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A Risk-Based Classification Scheme for Genetically Modified Foods: Establishing Levels of Concern to Guide Testing Requirements

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A Risk-Based Classification Scheme for Genetically Modified Foods: Establishing Levels of Concern to Guide Testing Requirements

VOLUME I

Eunice Chao

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List of Abbreviations

ADME	Absorption, distribution, metabolism and elimination
AFLP	Amplified fragment length polymorphism
cDNA	Complementary deoxyribonucleic acid
DBS	Double strand break
FIE-MS	Flow injection electrospray ionization- mass spectrometry
FISH	Fluorescence in situ hybridization
FT-ICR-MS	Fourier transform-ion cyclotron- mass spectrometry
FTIR	Fourier transform infrared (spectroscopy)
GC-MS	Gas chromatography-mass spectrometry
GISH	Genomic in situ hybridization
HMWC	High molecular weight chemical
IgE	Immunoglobulin E
LC-MS	Liquid chromatography-mass spectrometry
LMWC	Low molecular weight chemical
NHEJ	Non-homologous end joining
NMR	Nuclear magnetic resonance (spectroscopy)
PBPK	Physiologically based pharmacokinetic (model)
PCR	Polymerase chain reaction
PMM	Postmarket monitoring
PMS	Postmarket surveillance
ppb	Parts per billion
ppm	Parts per million
RBCS	Risk-based classification scheme
rDNA	Recombinant deoxyribonucleic acid
RNA	Ribonucleic acid
SCLPs	Straight-chained lepidopteran pheromones
SPT	Skin prick test
TILLING	Targeting induced local lesions in genomes
TTC	Threshold of toxicological concern

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ABSTRACT

Public discourse on genetically modified (GM) foods tends to focus on those aspects of recombinant DNA (rDNA) technology that heighten concerns about the potential risks of GM foods. Despite this focus of concern, a wide range of GM foods exist, including those that are modified by conventional approaches, the safety of which has not been assessed in detail. Also in contention is the likelihood of unintended effects with adverse health consequences in foods derived from crops genetically modified by more modern techniques such as rDNA, compared to those derived from crops modified by conventional techniques. Divergent views exist regarding the methods by which unintended effects should be detected and monitored. With advances in genomic technology, boundaries between conventional and modern approaches to genetic modification may become blurred. There is the need for a reliable method to efficiently differentiate GM foods that require more extensive testing and regulatory oversight from those that do not, regardless of the method of modification.

I propose a risk-based classification scheme (RBCS) to differentiate foods derived from GM crops based on levels of concern reflecting their potential to produce human health risks. Two categories of health effects are included in the scheme: (1) the potential for toxic/antinutritional effects, and (2) the potential for allergenic effects. To differentiate the sources of uncertainty about GM food risks, intended and unintended changes are classified separately with respect to potential toxic/antinutritional effects. Factors that may affect the level of risk are identified for each category of adverse health effects. Criteria for differentiating potential health risks are developed for each factor. The extent to which each factor satisfies the applicable criteria is rated separately. A concern level for each category of health effects is then determined by aggregating the ratings for the respective factors.

This thesis describes the factors, criteria, and aggregation rules used in determining concern levels in the proposed RBCS and the process used in its development. An exploratory evaluation of selected functional aspects of the scheme is provided. The implications of the classification scheme for the graded testing of GM foods are also discussed.

CHAPTER 1 - INTRODUCTION

1.1. Background

Foods are complex mixtures containing substances that may be shown to be either harmful or beneficial to health when subjected to conventional testing methods. While whole foods are not typically assessed for their potential to cause adverse health outcomes, they are generally presumed to be safe [Essers et al., 1998]. At the same time, there exist known cases in humans and animals of adverse effects from ingesting inherent substances contained in foods [Essers et al., 1998; Mattsson, 2000]. In contrast, man-made modifications to foods, such as the use of food additives, food processing, food irradiation, and genetic engineering, are viewed with caution and concern. An important population health goal is to optimize the balance between potential benefits and risks in the food supply, whether they are naturally occurring or introduced by technology.

The current paradigm for the premarket assessment of genetically modified (GM) foods tends to be process-based, with a focus on foods derived from crops modified by recombinant deoxyribonucleic acid (rDNA) techniques. This paradigm has wide acceptance despite recognition by the scientific community that the process of genetic modification is not a useful criterion for determining whether a GM product requires less or more oversight. A range of methods of genetic modification exist, including conventional approaches, such as mutation and wide-cross hybridization, the safety of which has not been assessed in detail. This focus on the process of genetic modification as a trigger for assessment also lags behind advances in modification techniques, that increasingly blur the boundaries between conventional and modern methods of genetic modification [Keenan and Stemmer, 2002; Madsen et al., 2002].

Also in contention is whether unintended effects with adverse health consequences in foods derived from crops genetically modified by more modern techniques are more likely to occur, as compared to those derived from crops modified by conventional techniques. As well, divergent views exist regarding the methods by which unintended effects should be detected and monitored. Many current guidelines also lack transparency in the criteria used in determining the level and extent of testing for GM foods that are modified by more complex techniques expected in the future. Therefore, there is the need for a systematic approach to efficiently

differentiate among GM foods that require more extensive testing and regulatory oversight from those that do not, regardless of the method of genetic modification. It is hypothesized that the concern level for a specific GM food and the nature and extent of its testing could be determined using risk-based principles and criteria. By identifying the sources of potential hazards in GM foods, the factors that influence their occurrence, and the level of human dietary exposure, and by applying appropriate criteria and integration rules, GM foods could be classified into different concern levels to which a graded level of testing could be applied. Graded testing based on the level of concern offers the potential for more efficient use of testing resources by focussing less on lower concern GM foods, and more on higher concern foods.

1.2. Research questions

This thesis was motivated by four research questions.

- What scientifically based factors and criteria should be considered in assessing the potential health risks of GM foods?
- How should the factors and criteria be integrated into a classification scheme that can provide guidance for testing GM foods based on their potential to produce human health risks?
- What toxicological and other testing methods can be applied to GM foods in a graded approach in proportion to their potential for adverse health effects?
- How should the technical and operational performance of the proposed classification scheme be evaluated?

1.3. Research objectives and scope

The primary objective of this research is to develop and evaluate a risk-based classification scheme (RBCS) to guide the testing of foods derived from crops that are genetically modified by a range of methods, including conventional and modern techniques. The proposed scheme aims to (1) differentiate and classify GM foods based on levels of concern, and (2) guide the application of testing methods for GM foods in proportion to their potential to produce human

health risks. The ultimate goal of this research is the continued strengthening of the health risk assessment and management framework for GM foods.

The focus of the proposed scheme is on potential human health risks from dietary exposure to *foods* derived from GM crops. GM crops are expected to remain an important component of overall agri-food production in the foreseeable future. Food uses of GM microorganisms and feed uses of GM crops, as well as the assessment of ecological risks, are outside the scope of the scheme. Also excluded are crops modified for therapeutic, environmental, or other industrial applications.

1.4. Research plan

Development and evaluation of the RBCS and proposed testing options for GM foods consisted of three phases (**Figure 1-1**). The following is a synopsis of the three phases, which are described in detail in subsequent chapters.

In **Phase 1 (conceptual development)**, factors that may affect or are associated with the production of health hazards along the pathway between the introduction of modified genetic material in a food crop and their entry into the food supply were identified through literature review. Based on well-established concepts and principles of risk analysis, criteria were developed for differentiating foods derived from modified food crops into different health concern levels for each factor identified. The scientific judgement of a multidisciplinary group of experts elicited through a self-administered questionnaire (expert consultation 1) was used to confirm the importance of the factors and the appropriateness of the criteria identified, and to guide the development of the scheme. A draft RBCS was developed by aggregating the factors and criteria supported by the results of consultation 1 into a manageable number (2-3) of concern levels for each major category of health effects of concern. This draft RBCS was reviewed by a different group of experts as well as by a few participants from consultation 1 (expert consultation 2) and refined prior to initiating Phase 2 of the project.

In **Phase 2 (testing options development)**, toxicological and other testing approaches that are currently used in evaluating the potential risks of GM foods, as well as of other classes of products, including defined food-related chemical substances (e.g., food additives, pesticide

residues) and chemical mixtures (e.g., plant-derived flavouring complexes, novel foods), were identified and appraised. The scientific literature and testing guidelines developed by regulatory and expert groups were included in this review. The applicability of emerging testing methods (with a focus on high throughput methods) identified through literature review was also assessed for their potential in reducing uncertainties in the safety assessment of GM foods. Two draft versions of a graded approach to testing based on levels of concerns in the proposed RBCS were proposed and reviewed by a multidisciplinary group of experts and two different groups of toxicologists using structured questionnaires, group and individual interviews in two separate consultations (expert consultations 3 and 4).

In **Phase 3 (evaluation)**, profiles were developed for a panel of reference GM food products that span a range of existing and possible future products with different characteristics. Public information sources were used in the development of the reference panel. The clarity of the proposed RBCS and of the descriptions of selected products in the reference panel was pilot-tested by experts with relevant expertise and experience in the evaluation of GM food products. Results of the pilot test guided the revision of product profiles and the development of a combined RBCS-product rating form. An evaluation of the performance aspects of the proposed scheme was conducted using two experts in toxicology, who independently assigned concern levels to the same panel of reference products and provided qualitative and quantitative feedback on the scheme (concordance study). The application of the RBCS is illustrated using a detailed example based on one reference product which received complete agreement in concern levels in the concordance study. This example serves to illustrate how the proposed RBCS and the corresponding graded testing approach may be applied.

1.5. Organization of the thesis

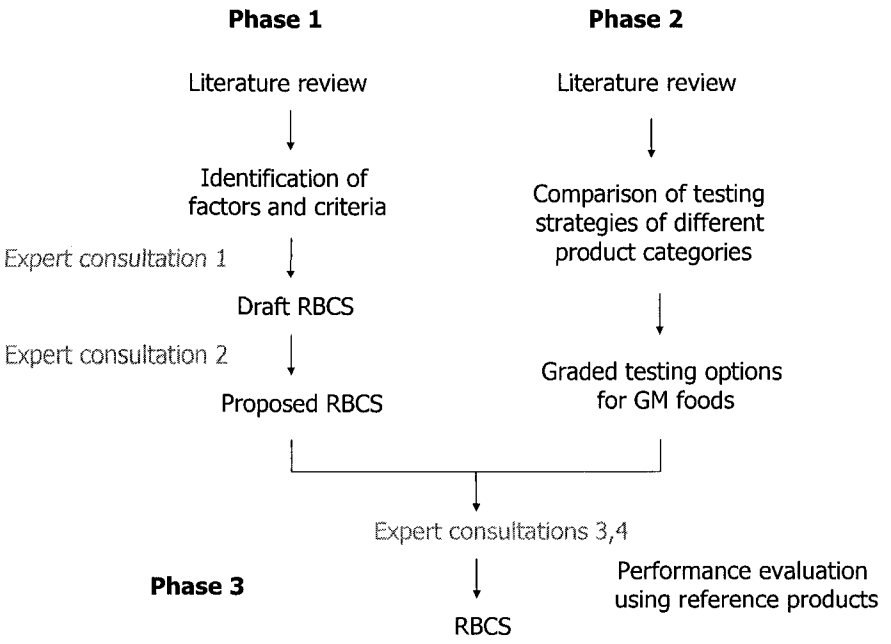
The thesis comprises two separate volumes. Volume I describes the scientific rationale for the proposed RBCS and testing strategies related to the levels of concern for a GM food as determined by the scheme. Volume I also contains a detailed report of the evaluation of the proposed scheme. Volume II (Appendices A-F) provides detailed supplementary information on the collection and analyses of data that support the development of the scheme and testing options in Phases 1 and 2, and the pilot test and research instruments used in Phase 3 for inter-rater comparison.

In Volume I, literature reviews of relevant topics (Chapters 2, 3, 6) precede descriptions of the proposed RBCS (Chapters 4 and 5) and graded testing (Chapter 7). Specifically, Chapter 2 provides an overview of the methods of genetic modification that are within the scope of the proposed RBCS. Both current and emerging technologies are discussed. The overview in Chapter 3 provides the scientific background for assessing the potential of genetic modification methods in producing genomic and metabolic effects in modified crops. Chapter 4 discusses the concept of health risk and its application to the development of a risk-based classification scheme. The development of the proposed RBCS is described in detail, including the development process, types of information used in classifying a GM food, the factors and criteria underlying the scheme, and their aggregation in determining concern levels. The conceptual framework of the proposed RBCS is presented in Chapter 5. Product characteristics represented by each concern level in the RBCS are included in this chapter. Chapter 6 contains an extensive review of available and emerging methods for testing relevant food-related substances and mixtures, as well as different toxicological endpoints that are generally evaluated in food safety determination. Their limitations and uses are also discussed. A proposed approach to graded testing of GM foods is presented in Chapter 7. Application of the proposed classification and testing schemes is illustrated in Chapter 8 using a product example worked through in detail. Evaluation of the proposed RBCS in a concordance study is described in Chapter 9, including the process used, the selection of products to be included in the reference panel, development of the product profiles, as well as the development of the evaluation tools. A pilot test of the data collection tools used in the concordance study is described briefly. Results of the concordance study are then discussed in detail with respect to the coverage of concern levels, the ability of the scheme to discriminate between foods of different levels of concern, its ease of use, and the degree of agreement between two raters in the assignment of concern levels for the reference products. Chapter 10 summarizes findings in the preceding chapters, and evaluates the strengths and limitations of this research. The contribution of the research to population health is indicated. The thesis concludes with recommendations for future research.

Volume II contains six appendices that describe the research instruments used in data collection and supplementary results not covered in Volume I. Ethics related documents (such as ethics approval certificates and consent forms) are also exhibited in Volume II. The objectives, methods, results, and analyses of the data collected are described in detail for Phases 1, 2 and 3 (pilot test) of the research project, comprising a total of five sets of data obtained through

structured questionnaires, as well as semi-structured group and individual interviews. Information provided to participants in the concordance study in Phase 3 is also described, including the data collection tools (combined RBCS-product rating form and a form for overall assessment), resources materials for raters, and complete descriptions of the 20 reference products used.

Figure 1-1. Research plan.



CHAPTER 2 - PRODUCTION OF GM FOOD CROPS

In order to differentiate and classify GM foods based on levels of concern about potential health risks, it is necessary to briefly describe the major methods of genetic modification used in crop improvement. This chapter provides an introduction to the discussion in the next chapter that reviews the molecular and metabolic basis of unintended changes in GM plants that may give rise to concerns about their potential for adverse health effects. This chapter begins with a brief explanation of the term "genetic modification" as its usage has conveyed different meanings.

2.1. Genetic modification - definitions

The term "genetic modification" associated with the RBCS may have meanings that are different from typical usage. This brief glossary clarifies the terms that are used in the context of the RBCS.

Genetic modification. Considerable variation exists in the definition of "genetic modification". Definitions in the science/regulatory policy literature include both broad and more specific definitions. The broad definition refers to the modification of the genotype of an organism by the application of biotechnology, such as gene transfer or chromosome set manipulation [NRC, 2002a]. More specific definition refers to the modification via transgenesis (inserting individual genes from an individual in the same or different species into another individual), or the targeting of specific changes in individual genes or chromosomes within a single species [The Royal Society, 2001].

A broad definition of genetic modification is adopted for the development of the RBCS. It encompasses all forms of *intentional manipulation* in changing the heritable traits of an organism, including conventional methods (such as induced mutation and wide-cross hybridization of sexually incompatible plants) and more modern methods (such as recombinant nucleic acid technologies) used in plant breeding. This usage is consistent with the Canadian Novel Food Regulations [Government of Canada, 1999].

Methods of genetic modification. In this document, "conventional" methods of genetic modification refer to intentional manipulation of the genetic make-up of crop plants by wide-

cross hybridization (crosses between different species or genera) and induced mutation (including mutagenesis by chemical agents, irradiation, and tissue culture). These methods have evolved over the past several decades. Some authors distinguish between traditional and non-traditional approaches to wide hybridization and mutagenesis [IFBC, 1990]. Non-traditional approaches include clonal propagation, somaclonal variation, and protoplast fusion. Other authors consider sexually compatible wide hybridization, including the use of a bridge cross[1], or physical or biochemical intervention[2], as conventional methods, while sexually incompatible hybridization is outside the scope of conventional breeding methods [EPA, 2001]. More "modern" methods refer to the exchange of genetic material through *in vitro* nucleic acid techniques [Codex Alimentarius, 2003]. These methods are also widely known as genetic engineering or bioengineering. Their application in the production of food crops may be referred to as agricultural biotechnology.

2.2. Conventional and modern methods of genetic modification

Modern agri-food production seeks continuous improvement in agronomic and other quality traits through a process of widening genetic variability and selection. Techniques for inducing genetic variability include methods such as hybridization and mutagenesis at the organism level, to tissue cultures at the cellular level (including somaclonal and gametoclonal variation and protoplast fusion), and deoxyribonucleic acid (DNA) recombination at the molecular level [IFBC, 1990]. Methods of genetic modification may also be distinguished on the basis of the physical methods of gene transfer used in the introduction of new genetic elements, ranging from sexual crosses, protoplast fusion, to recombination of known DNA sequences.

From the point of view of introducing novelty, these methods fall into two broad categories: (1) methods that introduce new allelic combinations or completely new genes from a donor organism into the modified plant (e.g., intraspecific, interspecific, or intergeneric hybridization within the plant kingdom or DNA recombination using a gene source outside the plant

[1] A bridge cross is the mixing of the first and the third plant through the formation of an intermediate zygote, in the event that a viable zygote would not be formed from the fusion of the gametes between the first and the third plants.
[2] Physical or biochemical intervention in a wide cross is the formation of viable zygotes through the use of any of the following means: surgical alteration of the plant pistil, bud pollination, mentor pollen, immunosuppression, *in vitro* fertilization, pre- and post-pollination hormone treatments, manipulation of chromosome numbers, embryo culture, or ovary and ovule cultures.

kingdom), and (2) methods that modify endogenous[3] genes by creating allelic differences in the modified plant without a donor organism (e.g., mutation induced by chemical mutagens, ionizing radiation, or tissue culture). In addition, induced polyploidy (increase in the number of chromosomes) can be achieved using chemicals such as colchicine, with or without cross-breeding, depending on the purpose of the intervention.

For illustrative purposes, this review will focus on selected examples from each of the major categories of genetic modification of crop plants. *Somatic hybridization* by protoplast fusion will illustrate wide crosses between plant species. *Nucleic acid techniques* allow recombination of DNA using a gene source outside the plant kingdom. *Induced mutation* includes modification by physical and chemical mutagenesis, as well as somaclonal variation produced by tissue culture. For a detailed description of these methods, several reviews and texts are available [Potrykus, 1991; Birch, 1997; Jain et al., 1998; van Harten, 1998; Mohan Jain, 2001; Predieri, 2001; Zhong, 2001; Chahal and Gosal, 2002; Davey et al., 2005a; Liu et al., 2005].

2.2.1. Somatic hybridization

Somatic hybridization typically involves the fusion of protoplasts (wall-less cells) in the development of hybrid plants that are impossible to achieve via traditional sexual hybridization. This approach enables nuclear and cytoplasmic genomes to be combined, fully or partially, across species and genera. Complex interactions between genomes and plasmones can lead to the formation of symmetric, asymmetric or cytoplasmic recombinants [Orczyk et al., 2003; Davey et al., 2005a; Liu et al., 2005]. Cybrids can be formed when the nucleus is derived from one parent and cytoplasmic components, including mitochondria and chloroplasts, are derived from the other.

Frequently in asymmetric hybridization, the donor organism is subjected to treatment (such as irradiation) to reduce the input of exotic genetic material from the wild relative. In some cases, genomic input of the recipient plant may also be limited. Prior to hybridization, the recipient protoplasts are always treated with some metabolic inhibitors to make them physiologically impaired for cell division. But when the recipient protoplasts are fused with the donor protoplasts, the heterokaryons can grow due to metabolic complementation [Liu et al., 2005].

[3] The word "endogenous" is used in this thesis to describe endogenous genes, proteins, enzymes, metabolites, allergens, food constituents, toxic substances and the like. In this context, "endogenous" refers to their inherent presence in the plant being modified.

Complete loss of donor chromosomes can also occur in asymmetric fusions where exposure of the donor organism to irradiation has resulted in the creation of cybrids.

2.2.2. Nucleic acid techniques

The most commonly used rDNA techniques for developing GM crops are biological Agrobacterium-mediated transformation and a biolisitic method based on direct microparticle bombardment. In the biological method, microbial plasmids are used to carry foreign genes into cells. Agrobacterium, a soil bacterium, is favoured because of its natural ability to transform cells by transferring and stably inserting its DNA into the plant DNA, causing the plant to read and express the transferred genes as if they were its own. By substituting the DNA of interest for the DNA of Agrobacterium that causes crown gall disease in plant, the bacterium can be modified to function as a vector for DNA recombination. Biolistics is a physical method of DNA delivery using microprojectiles, usually of tungsten or gold, coated with DNA that are propelled into the target cells by acceleration, powered by gun powder, gases, or an electric discharge. This method has the advantage of introducing DNA into virtually any tissue from any cultivar, whereas some crop plants (e.g., some monocots) are recalcitrant to Agrobacterium transformation without additional intervention [Hansen and Wright, 1999].

To a much lesser extent, electroporation and microinjection have also been used for gene transfer. Virus-mediated and plastid-based expression systems provide an attractive alternative to nuclear transformation in achieving readily obtainable high protein levels. Modified traits produced by virus-mediated gene transfer is not heritable, but can be maintained in subsequent nonsexually generated populations by vegetative means (such as grafting or from cuttings). Technically these plants are not genetically engineered, but they do carry new DNA and new traits [NRC, 2004a]. Chloroplast-based transformation may provide a biological means for transgene confinement.

2.2.3. Induced mutation

Two common methods of induced mutation in plant breeding are somaclonal variation from tissue culture and creating mutations using mutagenic agents. Somaclonal variation is the name given to spontaneous mutations that occur when plant cells are grown *in vitro*. Successful use of somaclonal variation depends on its genetic stability in subsequent generations [Mohan Jain, 2001]. Mutation breeding involves exposing plants or seeds to mutagenic agents (e.g., ionizing

radiation) or chemical mutagens (e.g., ethyl methanesulfonate) to induce random changes in the DNA sequence. There is no means to control the effects of the mutagen or to target particular genes or traits, except to adjust for the dose of the mutagen so that it is sufficient to result in some mutations without being lethal.

2.3. Extent of use

The importance of these methods to agricultural crop production varies geographically. Since the first report of successful application of rDNA technology to the modification of plant cells in 1983 and the first field trial of transgenic plants in 1986, transgenic food crops have been grown in about 21 countries, including 11 developing and 10 industrial countries. In 2005, the global area of approved bioengineered crops was 900 million hectares (222 million acres) [James, 2005]. Between 90 - 100 crop-trait events from over 20 developers have been approved for food use worldwide. The U.S.A., followed by Argentina, Brazil, Canada, and China, continue to be the principal adopters of bioengineered crops globally. It is expected that rDNA applications in agriculture will continue to be focused on economically important crops.

In contrast, relatively few somatic hybrids are capable of regenerating a whole organism, and fewer still are capable of sexual reproduction. This non-genetic engineering technique is not common in plant breeding as the resulting range of successful fertile hybrids has been limited. However, somatic hybrids can be used as bridging materials for breeding as a valuable source of novel germplasms for commercial exploitation. Somatic hybridization is a practical means of transferring traits present in species that are not sexually compatible with the crop species to be modified [Carputo et al., 1997; NRC, 2004a]. It is particularly suitable for transferring polygenic traits and genes of interest not physically identified, but which could be selected or screened for among hybrid cells and/or regenerated plants [Glimelius et al, 1991]. There appears to be renewed interest in the application of somatic hybridization in crop improvement targeted in specific genera, particularly, Citrus, Brassica, and members of the Solanaceae family [Glimelius et al., 1991; Orczyk et al., 2003; Davey et al., 2005b; Grosser and Gmitter, 2005; Liu et al., 2005].

The potential of somaclonal variation has also yet to be fully exploited by breeders. Relatively few cultivars have been developed in crops, such as Brassica, rice and some fruits and vegetables [Mohan Jain, 2001; Predieri, 2001]. Mutation induction by radiation was the most frequently

used method to develop direct mutant varieties (89%). The use of chemical mutagens was relatively infrequent. Worldwide, more than 2,250 different crop varieties have been released that have been derived from induced mutagenesis either as direct mutants or from their progenies. About 60% of these have been developed during the past 20 years. Many mutants have had a transnational impact on increasing yield and quality of several seed propagated crops [Ahloowalia et al., 2004]. In the United States, crop varieties ranging from wheat to grapefruit have been modified by mutation techniques since the techniques were first used in the 1920s. However, there are no records to retrace their subsequent use [NRC, 2004a]. In Canada, among food crops with novel traits that have been reviewed by Health Canada, five different crop species were modified by induced mutation, with chemical mutagenesis being the primary method of mutation. Somaclonal variants were also included. All the mutagenized crops reviewed were modified to confer herbicide tolerance, with the acetolactate synthase enzyme being the primary target of modification.

2.4. Emerging technologies

Projections on future developments in agricultural biotechnology have been reviewed in a number of reports and scientific papers [Kleter et al., 2000; PEW Initiative on Food and Biotechnology, 2001; NRC, 2002b; AEBC, 2003; König, 2003; Lheureux et al., 2003; Cockburn, 2004; ILSI, 2004; Dunwell, 2005]. Only broad future trends will be outlined here.

2.4.1. Complex metabolic engineering

It is expected that future improvements will continue to address four broad agronomic and social needs: improved agricultural characteristics, greater adaptation to post-harvest processing practices, improved food and nutritional quality, and mitigation of environmental pollution. The current generation of transgenic plants primarily involves improvement of traits through modification of single genes. The next logical step for expansion is the integration of single-gene traits, which has already occurred to some extent at the present [NRC, 2002b]. In the future, there is expected to be a proliferation of novel transgenic traits engineered into crop cultivars involving multiple genes [NRC, 2002b]. Complex changes in composition, as well as metabolic and gene interactions, may be expected with multi-gene modifications and the application of metabolic engineering [Verpoorte et al., 2000; DellaPenna, 2001; Momma et al., 2002]. Transcription factors have emerged as powerful tools for the manipulation of complex

metabolic pathways in plants. As opposed to most structural genes, transcription factors tend to control multiple pathway steps and have been found to impact flux through metabolic pathways [Broun, 2004]. These developments are expected to increase the complexity of health risk assessment of GM crops and derived foods [Kuiper et al., 2001; GAO, 2002; SOT, 2003].

2.4.2. Confinement of horizontal gene transfer

To date, the ecological risks of transgenic crops have garnered much attention. In food crops, transgenes that code for novel pharmaceutical or industrial compounds also need to be confined if dispersal from their site of production is possible. This has lead to concerted effort in the development of alternate technologies that are intended to reduce the impact of horizontal gene transfer. The Committee on Biological Confinement of Genetically Engineered Organisms reviewed technologies at different stages of development for their strengths and weaknesses as tools of bioconfinement [NRC, 2004b]. A few of the methods are based on existing agronomic or horticultural practices that have been tested already, while others are newly developed, or are only working hypothesis with untested efficacy. The implications of these technologies for human health risk assessment would require a separate review of the complexity they may create in evaluation. In this review, only marker elimination methods will be discussed to illustrate the type of issues that need to be considered in health risk assessment.

Promising methods that are being developed for the removal of marker genes from transgenic crops include cotransformation, homologous recombination, and recombinase-mediated excision [König, 2003; Miki and McHugh, 2004]. Cotransformation introduces changes into routinely used Agrobacterium-mediated DNA delivery protocols to obtain plant cells in which the gene of interest and the marker gene integrate into separate genomic locations, preferably on separate chromosomes, to allow segregation of the two insertions by breeding. Few additional safety concerns are likely to arise from this approach. However, this approach does require additional resources in development, as a considerably higher number of transformed cell lines would have to be handled, depending on the success rate of generating and selecting transformants with single copies of both the marker gene and the target gene. In the most successful experiments, up to 25% of the cotransformed cell lines contained single copies of both marker and trait-conferring genes that could be segregated through breeding.

Homologous recombination relies on the occurrence of base pairing between identical sequences that are in close proximity during the DNA replication process. This can lead to the excision of DNA sequences that are located between the two repeated DNA sequences. At present, recombination frequencies are very low in plants [Iida and Terada, 2004].

In recombinase-mediated marker excision, a recombinase enzyme specifically cuts DNA at the two short parallel DNA recognition sites and then reseals the two DNA strands after intervening DNA between the two sites has been removed. In crops in which markers are to be removed using this approach, the marker gene is flanked by the specific recombination target sequences for subsequent introduction of the recombinase protein. The most widely used among the available site-specific recombinases originate from the bacteriophage P1-derived Cre/*lox* system and the FLP/*FRT* system of the yeast 2μ plasmid [Baszczynski et al., 2003]. Three strategies are being investigated for introducing the recombinase protein: autoexcision, micro-injection or transformation, and sexual cross between a plant line containing the recombinase gene and a plant line that contains the trait-conferring gene.

2.4.3. Targeted gene insertion and expression

While site-directed transgene insertion and tissue-specific expression have the potential to reduce concerns about random gene insertion in GM crops and to limit the release of transgene expression products, the specificity of these technologies remains to be verified. At present, targeted transgene insertion in plants is primarily based on site-directed recombination and homologous recombination (gene targeting). As noted above, site-directed recombination requires specificity of the DNA-targeting enzyme and the efficiency of homologous recombination in plants has been very low. Recently, higher efficiency and "user-targetable" systems for site-directed genome modification are being developed by designing custom DNA-binding proteins or nucleic acid homology to confer specificity [Kolb et al., 2005]. For example, higher efficiency was achieved by using specifically designed zinc-finger nucleases to generate double-strand breaks (DSBs) at specific genomic sites. This approach is based on non-homologous end-joining that is favoured by plants for the repair of DSBs [Lloyd et al., 2005; Tzfira and White, 2005]. Another high-frequency gene targeting approach in plants uses a yeast DNA repair protein, Rad54, in the model plant Arabidopsis [Shaked et al., 2005; Tzfira and White, 2005]. Overexpression of the protein is apparently required for efficient gene targeting in plant cells. Questions remain regarding the mechanism by which the yeast protein is able to

dramatically stimulate homologous recombination in the recipient plant, which has an ortholog of the protein.

In theory, regulation of the expression of a transgene in GM crops can be advantageous in limiting the release and exposure of its gene products and in limiting deleterious effects on plant metabolism. Implications for risk assessment would depend on the system used in gene regulation. One such system is based on promoters responding to chemical inducers that differ in their ability to regulate the temporal, spatial, and quantitative expression patterns in concert with the transfer of compatible regulatory systems from non-plant sources [Potenza et al., 2004; Tang et al., 2004]. In addition, a variety of tissue-,organelle-, and cell-type-specific promoters, as well as synthetic ones, are available [Potenza et al., 2004]. Spatial control of transgene expression can also be achieved by plastid engineering where DNA is integrated in plant plastids (typically the chloroplasts) in a targeted manner via homologous recombination following DNA delivery [Heifetz and Tuttle, 2001; Maliga, 2002].

2.4.4. Non-food applications of GM food crops

There has been a growing interest in using plants as bioreactors in generating biopharmaceutical and industrial products (molecular farming) [Daniell., 2000; Fischer and Emans, 2000]. The production of target substances requires high expression level in these plants. While food crops modified for molecular farming are outside the scope of the proposed RBCS, these plants present risk management concerns with the potential to contaminate the food supply, particularly when food crops are used for molecular farming.

2.5. Blurred boundaries between conventional and modern methods

In practice, the boundaries between conventional and modern methods of genetic modification are not distinct. For example, both tissue culture and backcrosses can be an integral part of the plant breeding process. It is well known that tissue culture can induce pleiotropic effects, but backcrossing to an established cultivar can reduce such effects. *In planta* transformation is being developed for gene transfer that excludes the tissue culture step [Hansen and Wright, 1999; Pelletier and Bechtold, 2003].

Further, the techniques in both genetic engineering and conventional breeding are continually evolving and in some cases converging. It is expected that at some point in the future, the distinction between them may no longer be clear-cut [Keenan and Stemmer, 2002; Madsen et al., 2002]. For example, rapid developments are taking place in the technologies for genetic engineering where all-native plant gene sequences, including promoters and selection genes, are replacing the bacterial genes of the first generation transgenics [Rommens, 2004]. Also noted previously, advances in gene targeting technology in plants makes it possible to replace a gene, or to alter a minimum number of specific DNA sequences in an endogenous gene at its original locus in the plant genome [Madsen et al., 2002; Tzfira and White, 2005]. This will permit coding for the needed trait using the plant's own genetic regulatory systems.

Growing knowledge of genomes, more efficient gene transfer, and advances in DNA marker techniques will permit more precise transfer of desirable genes within species (intraspecific transfer), now accomplished by traditional breeding techniques. Directed molecular evolution based on DNA shuffling has the potential to accelerate the speed of selective breeding [Minshull and Stemmer, 1999; Lassner and Bedbrook, 2001; Stemmer, 2002; Snow, 2003]. Activation of site-specific recombination and targeted mutagenesis also offer new possibilities for chromosomal engineering in plants [Baszczynski et al., 2003]. Chromosome engineering is the term given to non-rDNA cytogenetic manipulations, in which portions of chromosomes from near or distant species are recombined through a natural process called chromosomal translocation [NRC, 2004a]. Recent refinements allow plant breeders to restrict the transferred genetic material. As a result, chromosome engineering is becoming more competitive with rDNA technology in its ability to transfer relatively small pieces of DNA.

As well, genes transferred by rDNA technique can be recombined or stacked using conventional breeding methods. In addition, site-specific gene deletion technology developed to remove marker genes for bioconfinement purposes may also be advanced to generate non-transgenic plant tissues that otherwise retain all of the benefits of transgenic plants. The basic design of a general gene deletion cassette has been proposed [Keenan and Stemmer, 2002]. Advances in genetic sequencing of crop plants also mean that it is now possible to effectively select induced mutations in specific plant genes. For example, a high throughput screening approach (targeting induced local lesions in genomes or TILLING) is now available to identify point mutations in chemically mutagenized crops [Colbert et al., 2001; Slade and Knauf, 2005].

2.6. Summary

In this chapter, selected examples of methods of genetic modification are presented to represent a range of approaches in crop improvement, some of which are used to a greater or lesser extent in producing GM crops. The lines separating these methods are not always distinct, and they are expected to converge further as technologies advance.

Current methods of genetically modifying crop plants differ in the specificity of the genetic material transferred or modified. These methods also vary in their complexity and the extent to which the genetic makeup and the metabolic activity of the modified organism can be altered. While emerging technologies offer the potential for reducing concerns about gene transfer and random gene insertion in the plant genome, the efficacy and stability of the intended effects of these technologies remain to be verified. These technologies may also increase the complexity of risk assessment of GM crops. Both current and emerging methods can give rise to unintended effects, which are discussed in the next chapter.

CHAPTER 3 - TYPES AND SOURCES OF POTENTIAL HAZARDS IN GM FOODS

Potential hazards in GM foods are those substances in foods that can produce an adverse health effect by virtue of their inherent toxic or antinutritional properties. Food substances in GM foods can be broadly divided into two categories, based on the types of adverse health effects they may produce: protein and non-protein substances. This division of potentially hazardous substances in GM foods is also related to how they are introduced into GM foods. Protein substances are generally the direct products of introduced or modified genes. Non-protein substances in GM foods are produced in metabolic pathways catalyzed by the introduced or modified enzymatic proteins. In GM crops, potential adverse health effects can also result due to intentional and unintentional changes in the nature or level of these substances.

This chapter will introduce the topic of potentially hazardous constituents of GM foods with an explanation of the terms "intended" and "unintended" effects. The types of adverse health effects associated with protein and non-protein substances will be described briefly. The molecular and biochemical basis of unintentional changes will be the focus of this chapter. A comparison of the types of unintended effects resulting from different methods of genetic modification will be made, as well as a summary of safety-related issues specific to genetic engineering.

3.1. Intended and unintended effects

Intended effects include both the direct and indirect effects of primary and secondary gene products resulting from genetic modification. A primary gene product is the protein expressed by the introduced/modified gene. An example of a direct effect of a primary gene product is the insecticidal effect of the protein produced by the *cry* gene of *Bacillus thuringiensis*. A secondary gene product refers to the principal metabolite(s) of interest of the target/modified gene. An example of an indirect effect is tolerance to herbicide bromoxynil. This indirect effect is mediated through an enzyme (nitrilase) produced by the gene of interest (the *bxn* gene of *Klebsiella ozaenae*). The enzyme derived from the bacterium converts the herbicide into a metabolite that is not phytotoxic. In this case, the secondary gene product is a metabolite of the nitrilase enzyme, which is non-protein in nature.

Unintended changes can be predictable or unpredictable. Predictable effects are unintended changes that go beyond the primary expected effect(s) of introducing the target gene(s), but that may be explicable in terms of our current knowledge of plant biology and how metabolic pathways and physiological processes interact and integrate. Unpredictable effects are those changes that fall outside our present level of understanding of gene regulation and gene interactions [Kuiper et al., 2001; Cellini et al., 2004].

3.2. Types of potential health hazards

The primary products of gene expression are predominantly protein in nature. In contrast, secondary gene products that are metabolites of biosynthetic pathways reflect a greater diversity of chemical classes. The expression products of transgenes introduced or modified via genetic engineering are generally known and sensitive assays for their detection are usually available. In comparison, the biochemical basis for the phenotype of interest produced using conventional breeding methods is generally untargeted and undetermined in most instances before transformation.

Three types of adverse health effects in GM foods are of particular concern: toxic, antinutritional, and allergenic effects. Toxic effects are the results of chemical disruption of the normal biological processes of living organisms [IFBC, 1990]. Antinutritional effects refer to the effects of substances that inhibit the normal utilization of nutrients with deleterious nutritional effects. Allergenic effects refer primarily to immunoglobulin E (IgE)-mediated reactions in genetically susceptible individuals. Toxic and antinutritional effects can be produced by protein and non-protein substances, while allergenic effects are attributable primarily to protein substances.

3.2.1. Protein substances

Food-borne toxic protein substances typically exhibit acute toxicity. With the exception of specific classes of protein, such as certain plant protease inhibitors, hemagglutinins, lectins, and some proteins induced in plant defense [Pariza and Foster, 1983; Sjoblad et al., 1992; Franck-Oberaspach and Keller, 1997; FIFRA SAP, 2000; Pariza and Johnson, 2001], there is generally little concern about chronic toxicity. When used as pesticides, these classes of proteins generally have a specific range of target organisms. Enzymes, a major category of gene expression

products in edible food crops, are generally not known to have toxic properties [Pariza and Johnson, 2001]. Many hydrolytic enzymes ubiquitous in plants are common constituents of foods. Their transfer between edible plants is unlikely to cause a problem unless they are expressed at relatively high levels, or are derived from non-edible plants [NRC, 2000a]. Knowledge of the function of an enzyme can help focus assessment on those metabolic pathways involved in the production of toxic secondary metabolites.

Proteins, including enzymes, the primary target gene expression products, are the focus of risk assessment with respect to allergenicity. Two phases of immunologically mediated allergic responses are important: sensitization and elicitation of response. Generally, there is no clear definition of a threshold level for the induction of sensitization, which appears to be highly dependent on host susceptibility [FIFRA SAP, 2000; Taylor et al., 2002]. However, it may be possible to use statistical modelling to determine minimum threshold levels of allergenic proteins required for allergenic reactions in individuals who have already been sensitized [Bindslev-Jensen et al., 2002]. Immunoglobulin E (IgE)-mediated food allergenicity, typically associated with acute and potentially severe reactions, is related to several physical, chemical and biological properties (e.g., heat stability, digestibility, amino acid sequence) of a protein substance [Taylor and Lehrer, 1996; Taylor and Hefle, 2001; Bannon et al., 2002]. While general characteristics of allergenic proteins have been reported, they are not shared by all common major protein allergens. Food processing or preparation methods, as well as food matrix and composition, can affect heat stability, digestibility and the immunogenicity of some allergenic proteins [Taylor and Lehrer, 1996]. Immunogenic effects other than allergenicity have not received as much attention in GM food risk assessment. They include cell-mediated reactions or hypersensitivity reactions elicited by food substances without the involvement of IgE antibodies [Goodman and Hefle, 2005].

3.2.2. Non-protein substances

Non-protein substances in plants linked to adverse health effects are typically secondary metabolites. In contrast to proteins, non-protein substances, many of which have a defense function in plants, can exhibit a range of toxicities and adverse health effects. Low molecular weight chemicals may be involved in an immune response by covalently binding to proteins forming a hapten-carrier complex [Kroes et al., 2004]. Non-protein amino acids in plants are also potentially toxic. They occur in unconjugated forms in a wide range of plants, particularly in

leguminous species. Some have been implicated in diseases of humans or animals [Cheeke, 1998; FIFRA SAP, 2000].

3.3. Molecular and biochemical basis of potential unintended health hazards

Unintended changes in GM crops of health concern include the generation of new toxic compounds, or modified expression of endogenous toxic substances or metabolites in different tissues or at different concentrations. These changes can arise at the genomic, transcriptional, or metabolic levels. Arguably, these distinctions are somewhat arbitrary, as these levels are integrated within an organism. These distinctions are only made for the purposes of systematically reviewing the issues relevant to assessing unintended effects in GM crops.

Specific issues considered in this section are: (1) disruption of genes at the genomic level, (2) gene interactions and epigenetic effects at the transcription level, and (3) altered biochemical pathways and flux at the metabolic level.

3.3.1. Genomic level

The potential for gene disruption is an aspect of rDNA techniques that has received much attention because of the randomness in the manner in which genes are inserted in the recipient organism. Random gene insertion can potentially disrupt or silence active genes and modify the expression of active genes or their interactions [Conner and Jacobs, 1999; Kuiper et al., 2001; Codex Alimentarius, 2003; SOT, 2003].

Activation of silent genes is theoretically possible, arising from read-through from highly expressed promoter regions of the transferred gene into coding regions of the flanking host DNA [Conner and Jacobs, 1999]. This possibility is of most concern if the previously silent gene encodes enzymes in biochemical pathways involved in the production of toxic secondary metabolites. These toxic metabolites may not be known to be present in the host plant, but their genetic code may have originated from its wild relatives. The wild relatives of many crop plants are known to contain various secondary metabolites with varying levels of toxicity. In addition, fusion proteins can also be produced from open reading frames formed by insertions of donor DNA with contiguous host genomic DNA. (An open reading frame is a nucleotide

sequence with an initiation codon at one end, a series of triplet codons, and a termination codon at the other end, potentially capable of coding for an as yet unidentified peptide or protein.)

Disruptions can also occur at the chromosome level. Four types of structural mutations within chromosomes are commonly distinguished: deletions, duplications, inversions, and translocations. The consequences of deletions depend on the role of the genes concerned. Effects of duplications are largely locus-specific, depending on whether one or more complete genes are involved, which may result in a double dose of the gene products. In most cases, inversions have little impact on phenotype. Effects of translocations will depend on which chromosome is affected and where on the chromosome the mutation occurs [van Harten, 1998].

To provide the context for the relevance of insertional mutagenesis produced by DNA recombination, this review will compare the type and extent of genetic disruptions of the three major methods of genetic modification reviewed in Chapter 3. Among the major methods of genetic recombination, comparison is made between wide hybridization and two types of nucleic acid techniques – Agrobacterium-mediated and biolistic approaches. Comparison is also made among three approaches to induced mutation: chemical mutagenesis, mutations induced by ionizing radiation, and somaclonal variation.

a) Recombination

There are two major mechanisms for genetic recombination in eukaryotes based on the most widely accepted double-strand break (DSB) model of DNA repair: homologous recombination (HR) and illegitimate recombination, a form of more general non-homologous end joining (NHEJ) process [Britt, 1996; Schnable et al., 1998; Vonarx et al., 1998; Haber, 2000; Cellini et al., 2004]. In plants, NHEJ is the predominate mechanism of genetic recombination, which is more error prone and which changes the original sequence with very high frequency. It is usually associated with deletions ranging from 1bp up to > 1kb. In both HR and NHEJ, recombination could theoretically occur randomly along the length of the chromosomes. However, in plants, as in other organisms, there are hot spots for recombination, including gene-rich regions, which, from an evolutionary perspective, has allowed the emergence of new alleles with novel characteristics within plant populations [Schnable et al., 1998; Cellini et al., 2004].

(i) Hybridization. Hybridization within species and wide crossing between species or genera in plant breeding is known to involve the transfer of large regions of uncharacterized DNA. Studies on the evolution of disease resistance genes and genes influencing quality traits suggest that genes arose from the shuffling of sequence domains between members of the gene family [Richter et al., 1995; Fridman et al., 2000; Cellini et al., 2004]. The majority of the transferred DNA is expected to contain linked genes of unknown function with only a small proportion carrying the desired character. The extent to which transfer of large chromosome segments in hybridization can be visualized in a range of crops is aided by recently developed techniques of GISH (genomic *in situ* hybridization) and FISH (fluorescence *in situ* hybridization) [Kato et al., 2005].

In wide crossing, parts or entire chromosomes in one species can be replaced by parts or entire chromosomes from another species. Where the genomes of the species are closely related, novel recombinant chromosome and introgression of new traits into a cultivated species can result. Gene introgression from wild species into crop plants, especially for genes conferring disease resistance, often involves homeologous recombination or induced translocation between chromosomes [Conner and Jacobs, 1999]. Where the fusion parents are phylogenetically distant species, complex interactions between nuclear and cytoplasmic genomes also occurs, from which diverse and *de novo* nuclear and cytoplasmic genomes and organelle combinations could arise with varied inheritance patterns [Davey et al., 2005b; Liu et al., 2005]. When somatic hybridization is used in tandem with polyploidy induction and chromosome duplication, the entire genome of both species could be retained in the hybrid.

Possible outcomes of somatic hybridization cannot be predicted. Only within a limited range can one influence chromosome elimination/recombination or organelle segregation/recombination [Orczyk et al., 2003]. While nuclear DNA from both fusion parents coexists in some hybrids with translocation and rearrangement of chromosomes, spontaneous elimination of chromosomes from either or both parents is also frequent in some hybrids [Orczyk et al., 2003; Liu et al., 2005]. Aberrations in chromosomal behaviour often accompany chromosomal elimination. In asymmetric hybrids where the donor species was irradiated, the pattern of chromosome elimination may not be random. For example, a non-random pattern of chromosome elimination was observed between cultivated potato (*Solanum tuberosum*) and the

donor *S. brevidens* that was irradiated [Orczyk et al., 2003]. The rate of chromosome elimination is also dependent on the radiation dose.

Several mechanisms could result in high expression of toxins in wide crosses [Mahon, 1989]: (1) homologous recombination, or chromosomal exchange of more efficient promoter elements or more active elements from the wild parent into the cultivated variety, (2) an active gene in the wild parent replacing an inactive allele in the cultivated variety, and (3) transfer into the cultivated variety of inactive trans-acting elements (on another chromosome) which regulate the expression of the toxin negatively, thereby allowing expression of the toxin.

(ii) rDNA techniques. Despite the ability to precisely characterize the genetic element to be transferred at the molecular level, the mechanisms of integration of foreign DNA into host plant modified by rDNA techniques are not well understood and are the subject of active research [Kohli et al., 2003]. The DNA fragment intended for transfer may become integrated as a single discrete copy or as repeated regions at one or more insertion sites, in a complete, truncated, or rearranged manner [Conner and Jacobs, 1999]. It is generally recognized that transgenes can cause insertion mutations at different locations along individual chromosomes [Conner and Jacobs, 1999; Svitashev et al., 2000]. The predominant mechanism of transgene integration in higher plants is believed to be based on illegitimate recombination, which involves little homology between recombining DNA strands, as is the predominant mechanism for hybridization used in conventional breeding [Kumar and Fladung, 2001; Svitashev and Somers, 2001; Hanin and Paszkowski, 2003; Cellini et al., 2004]. There are possibilities that some endogenous genes are interrupted as a result of gene integration during the transformation process. Preferential integration into an active transcription region, as shown in some cases of Agrobacterium-mediated transformation, can lead to disruption of normal gene function. In practice, however, insertion of transferred DNA, or T-DNA, into crucial genes is a relatively rare event in crop species as a consequence of crop improvement efforts [NRC, 2004a, p. 60].

DNA rearrangements have also been reported, including point mutations, small deletions, insertions and duplications in both the foreign DNA itself and in host DNA flanking the target site, resulting in linked arrays of transgenes [Maqbool and Christou, 1999; Hanin and Paszkowski, 2003]. This phenomenon of linked multicopy transgene insertion is supported by molecular analysis of transgene loci that reveals variable number of transgene copies and variable

number and length of interspersing genomic DNA sequences within the locus. These observations are believed to be consistent with chromosome rearrangements (translocation) [Maqbool and Christou, 1999; Svitashev et al., 2000; Svitashev and Somers, 2001; Kohli et al., 2003]. Not all transgene integrations result in linked multicopy loci that are prone to epigenetic and genetic instability. A certain proportion of transgenic DNA integration results in a single-copy locus or several unlinked loci that contain single copies of foreign DNA that can be separated by genetic segregation [Hanin and Paszkowski, 2003; Altpeter et al., 2005].

Similar models for transgene integration have been proposed for both direct (biolistic) and indirect (Agrobacterium-mediated) methods of gene transfer, the two major methods used in genetic engineering [Kohli et al., 2003]. Both methods appear to generate a similar range of integration events, including truncations, rearrangements and various copy numbers and insertion sites, with transformation parameters and host plant biology being important factors influencing integration [Birch, 1997; Kohli et al., 2003]. There is also little evidence for major differences in the levels of transgene instability and silencing when these transformation methods are compared in agriculturally important cereals and legumes, and other non-model systems [Altpeter et al., 2005]. However, differences between the two methods have been reported for earlier application of these methods of gene transfer, including a lower transgene copy number, lower frequency of transgene rearrangement, and smaller genomic DNA interspersions in Agrobacterium-mediated transformation compared to biolistic transformation [Conner and Jacobs, 1999; Kohli et al., 2003].

Preferential integration sites have been shown in Agrobacterium-mediated transformation (transcription units), as well as in biolistic transformation (distal ends of chromosomes), although random integration is also supported in both, as well as reasonably even distribution of T-DNA throughout the Arabidopsis genome [Zhong, 2001; Ecker, 2003; Kohli et al., 2003]. It is suggested that the integration of foreign DNA largely depends on the chromatin structure and host proteins involved in DNA replication, repair and recombination. How these proteins interact with exogenous DNA and the plant's own genome is unpredictable. Thus, plant genome factors, rather than how or in what form the DNA is delivered to the nucleus, appear to determine variations in transgene locus structures [Kohli et al., 2003; Somers and Makarevitch, 2004].

b) Induced mutation

Mutations in genetic elements in an organism can occur spontaneously without an exogenous stimulus or human intervention. Spontaneous mutation can be the result of spontaneous deletions, or errors in DNA replication, recombination, and repair. Mutation in plants can also be caused by cosmic rays or UV radiation, temperature shocks, geomagnetism, radioactivity (e.g., from radioactive isotopes in the soil), and chemical changes in plants due to ageing or other types of stress, as well as the action of transposons or mutator genes. Errors in DNA replication are probably the most important source of mutations [van Harten, 1998, p. 88]. In general, most mutations result in a loss of gene function. This type of mutation would have a health-related consequence if the active gene encodes enzymes in the removal of toxic, antinutritional, or allergenic constituent in the edible part of the mutant plant [NRC, 2000a, p. 61].

Compared with spontaneous mutation, induced mutations share certain common mechanisms. Although some differences in the mechanisms of initiation and repair may exist, there is little reason to assume that their mutation spectra should be fundamentally different [van Harten, 1998]. Genetic alterations associated with induced mutation employed in plant breeding include: alterations in DNA methylation patterns, variability in the copy number of DNA sequences, random point mutations (single DNA base-pair substitutions), position effects (i.e., changes in the phenotypic effect of one or more genes as a result of a change in their position with respect to other genes), transposition effects (destabilization of naturally occurring mobile genetic elements or transposons that may cause genes to be controlled in novel and unpredictable ways), chromosome mutations (duplication, translocation, inversion), and chromosome rearrangements (including random breaks in chromosomes, loss of chromosome fragments, or rejoining of chromosomes in different combinations) [Lee and Phillips, 1988; Phillips et al., 1994; Brar and Jain, 1998; Gupta, 1998; Kaeppler et al., 1998; Mohan Jain, 2001; The Royal Society, 2003]. Many factors ultimately determine whether an induced mutation will become expressed in a plant, including the rate of DNA damage and repair. Despite the generally non-directional nature of their damaging effects, a certain level of mutagen specificity and locus specificity can be expected from some mutagenic treatments.

(i) Chemical mutagens. Chemical mutagens produce primarily gene mutations whereas radiation-induced mutations are likely to occur at random positions in any chromosomal region. Chemical treatments generally give more non-random distribution of chromosome breaks than radiation

because of the preferential occurrences of breaks in specific chromosomes, as well as in definite regions within chromosomes [van Harten, 1998]. Some chemical mutagens such as sodium azide may induce mutations involving a relatively high frequency of point mutations with almost no accompanying chromosome aberrations. Nitroso-compounds are also very effective in inducing point mutations [van Harten, 1998]. Among alkylating chemical agents, ethyl-methane sulfonate (EMS) is the most commonly applied. EMS induces a higher proportion of chromosome aberrations than other chemical mutagens and high frequencies of base-pair substitutions, almost exclusively of the transition type at the G-C site, and little lethality.

(ii) Ionizing radiation. Ionizing radiation, including UV radiation, induces a broad spectrum of physical and chemical events in the DNA and RNA of prokaryotes and eukaryotes. Doses of less than 1 Gy are considered not to cause observable effects of any significance in plants. The phase within the cell cycle in which the cells are subject to irradiation is one factor determining the type of aberrations induced. Single hits are believed to result in small deletions and single-strand breaks, and are directly related to the dose and are not affected by dose rate in higher organisms. Chromosomal aberrations often require two or more radiation-induced breaks and are related to the frequency of their occurrence. Gamma rays and X-rays are the two most important sources for the production of mutant cultivars and are likely to induce small deletions and point mutations. UV radiation induces mutations that closely resemble the spontaneous ones and has a certain degree of specificity, compared to neutrons that may cause gross alterations like the induction of many chromosome breaks [van Harten, 1998].

(iii) Somaclonal variation. Somaclonal variation is common in plants that have been regenerated following an *in vitro* culture phase. Somaclonal variation arising from mutations of the nuclear genome cannot be transmitted to the next generation through germ cell mediated sexual reproduction. An exception would be if the tissue culture-induced mutations affected the genomes of organelles, such as mitochondria and/or chloroplasts, instead of the nuclear genome. Somaclonal variation arising from mutations of the nuclear genome can be transmitted to the next generation through asexual vegetative propagation, in which chimaeric plants are regenerated from multicellular tissues containing somaclonal variations. However, this can also be avoided by asexual propagation from single-cell lines (i.e., separation of single protoplasts).

As with chemical or radiation-induced mutation, the effects of somaclonal variation cannot be directed. The cellular and molecular mechanisms behind somaclonal variation are not well understood. Among the proposed mechanisms are: chromosomal instability from mitotic irregularities; gene amplification or deletion; gene inactivation or reactivation of silent genes; transposition and somatic crossing over; DNA methylation; and ploidy level variation [Lee and Phillips, 1988; Brar and Jain, 1998; Kaeppler et al., 1998; van Harten, 1998]. Somaclonal variation is associated with changes in restriction fragment length patterns at the DNA level, and a predominance of single-base changes at the protein level, excluding gross chromosome abnormalities [Kaeppler et al., 1998]. It has been suggested that chromosomal changes or breakpoints in somaclonal variation are not random but favour regions of chromosomes carrying heterochromatin blocks [Gupta, 1998]. It is well known that somaclonal variation induced by tissue culture can lead to biochemical changes in crop transformation using rDNA techniques.

3.3.2. Transcriptional level

a) Gene interactions

Unintended effects can result from epistasis which involves new unpredictable interactions between genes at different loci. For example, one gene can suppress the effect of another gene that is situated at a different locus. Rushlow et al. [1984, cited in Caplan, 1998] reported that chromosomal rearrangements in loci lying 15 kb away from a structural gene can greatly reduce levels of the RNA expressed by the structural gene. This study demonstrated that, in this case, the mutation has not altered the magnitude or specificity of expression, but rather the frequency with which the programme for expression is followed. In conventional breeding programs involving crossing the complete genomes of two organisms, there is the potential for epistasis and complementation between genetic elements from each parental species. Gene complementation from different parental lines may specify enzymes with different activities along biochemical pathways with alterations in metabolic effects. This can lead to unintended increases in secondary compounds, especially when parental lines include wild species in their pedigrees [Conner and Jacobs, 1999].

In transgenic breeding involving the insertion of specific foreign gene(s), position effects are also important as they relate to unintended effects. "Position effects" refer to the genomic context in which a gene is expressed. This means that the expression of a gene is influenced by its position

in the genome after integration [Conner and Jacobs, 1999; Zhong, 2001; The Royal Society, 2003, p. 55; Cellini et al., 2004]. Certain position effects can be mitigated by flanking transgenes with specific matrix-associated regions which are thought to insulate transcriptional regulation from the effects of surrounding chromatin, or by developing site-specific transgene integration systems [Conner and Jacobs, 1999; Zhong, 2001]. The application of these approaches in practical transgenic breeding remains to be fully evaluated [Zhong, 2001].

b) Epigenetic effects

Epigenetic effects refer to changes in gene expression without a change in DNA sequence and have been attributed to DNA methylation. Variations due to epigenetic effects are generally considered non-hereditary and reversible. Epigenetic effects are now well-recognized in genetically engineered plants as they are related to the highly unpredictable variability in transgene expression and silencing [Conner and Jacobs, 1999; Mazur et al., 1999; Wolffe and Matzke, 1999; Zhong, 2001; Qin et al., 2003]. It has been suggested that epigenetic factors may play a role in somaclonal variation induced by tissue culture [Kaeppler et al., 2000]. They are also expected to be important in gene expression in plants modified by conventional breeding methods, as has been widely observed in eukaryotic organisms [Strohman, 1994, 2002; Wolffe and Matzke, 1999].

3.3.3. Metabolic level

Secondary and pleiotropic effects of expression of the gene of interest introduced or modified in the recipient plant can arise from several sources: (1) alteration in flow-through of metabolites in biochemical pathways from, for example, over-expression of an enzyme which is the target gene product; (2) expression of new enzyme activity in the host plant, diverting metabolites from one secondary pathway to another; (3) expression of transcription-related factors involved in regulatory and signalling pathways; (4) increased concentration of catabolic products resulting from an increase in the concentration of an intended compound [Conner and Jacobs, 1999]. The likelihood of secondary effects of gene expression will in part depend on how the branch points and rate limiting steps in biochemical pathways are regulated. This type of effects can be partially predictable and testable, depending on the extent of prior knowledge of plant metabolism, which varies between crops as well as within the same crop species [NRC, 2000a; ILSI, 2004; NRC, 2004a, p. 78].

Future novel transgenic traits engineered into crop cultivars are expected to require multigene modification and the application of metabolic engineering [NRC, 2002b; Wolfenbarger, 2002]. At present, effects of metabolic engineering in modified crops cannot be assessed with confidence. Although the enzymological sequences and intermediates of many metabolic pathways in a small number of well-studied organisms are established, little is known in quantitative terms about the genetic and catalytic controls and integration of these pathways, including transport processes. An understanding of the signal sequences or transit peptides coding sequences attached to introduced genes is not sufficient to ensure proper targeting. Physico-chemical properties of transport protein such as charge and size may affect the efficiency of transportation into plastids. Redundancy of pathways and the adaptability of plants in using more than one enzyme in catalyzing a similar reaction pose other challenges [ILSI, 2004]. The manipulation of transcription factors that control networks of metabolism may help counter some of these problems in metabolic engineering of pathways [Kinney, 1998; Bruce et al., 2000].

Concerns have been raised about whether novel interactions in genetically engineered crops, to which one or a few genes from what might be radically different organisms from different kingdoms are transferred, would be different from gene recombination from genetically related organisms [The Royal Society, 2003, p. 57]. Novel regulatory elements are often required for transgene expression. Unexpected pleiotropic effects on the physiology of the host may occur at the metabolic level because the expression of a transgene could be outside the control of normal homeostatic feedback [Hallerman, 2003]. On the other hand, up or down regulation of endogenous or homologous genes can also result in interactions with a greater number of other genes/enzymes in the host, because the introduced gene shares a high degree of functional and sequence similarity with the corresponding gene in the host [Kuzma, 2002]. For example, a novel steroidal alkaloid demissine, not present in either parental line, was found in the somatic hybrids between *Solanum brevidens* and *S. tuberosum* [Laurila et al., 1996]. The authors hypothesized that demissine could be produced by hydrogenation of solanidine, a compound found in *S. tuberosum* but not in *S. brevidens*, by the hydrogenase enzyme of *S. brevidens*. Unknown compounds, likely derivatives of endogenous compounds, were also identified in somatic hybrids of Solanum and in wheat to which enzymes from grapevine was introduced by rDNA techniques [Serazetdinova et al., 2005; Väänänen et al., 2005]. Similarly, novel proteins can also be found in conventional crops, but they have not been subject to safety assessment [Kleter and

Peijnenburg, 2003]. Therefore, while genetic relatedness between host and donor organisms may influence the likelihood and range of unintended effects produced in the modified organism, it is the final product of a given modification, rather than the modification method or process, which determines whether the unintended changes would have an adverse effect with health relevance [NRC, 2004a].

3.4. Safety-related issues specific to genetic engineering of crop plants

Specific issues that have been associated with genetic engineering include: (1) novel nucleic acids, (2) gene transfer, (3) the use of viral DNA in plants and the potential for viral recombination, and (4) accelerated and wider scope of novelty. The safety of novel DNA and of gene transfer between plants, animals and microorganisms, including viruses, has been the subject of several scientific reviews and will not be repeated here [see Custers, 2001; Jonas et al., 2001; Tepfer, 2002; The Royal Society, 2002; van den Eede et al., 2004]. Only an overview of salient points relevant to the consideration of hazard factors for inclusion in a RBCS will be presented.

3.4.1. Novel nucleic acids

There is no indication to suggest that the toxicological or immunological properties of the DNA of inserted genes or their RNA transcripts from non-plant organisms will be different from those of existing food organisms. Neither does the present use of recombinant techniques in the production of GM crops introduce changes in the chemical characteristics of the nucleic acids, or have an impact on their digestibility or stability, or on the level of additional DNA ingestion [Jonas et al., 2001; The Royal Society, 2002].

3.4.2. Horizontal gene transfer from microorganisms

The potential of gene transfer between transgenes in GM crops and gastrointestinal microorganisms for health risks from the ingestion of GM foods has been assessed. Several conclusions can be made. (1) The likelihood of genetic transformation of ingested DNA of health significance is generally expected to be very low; for this to occur, a series of sequential and rare events will have to take place. (2) The likelihood of transfer of DNA to gut microflora from the ingestion of food and feed with health significance may be related to the stringency of processing of GM plant tissues and its effect on DNA digestibility and the levels of foreign DNA material [Chiter et al., 2000; Einspanier et al., 2001]. In addition, the consequences of

uptake of DNA by somatic mammalian cells differ from that of uptake of DNA by microorganisms, as DNA may be transmitted to subsequent generations in microbes but not in mammalian somatic cells. DNA transfer from plants to microorganisms has been demonstrated under laboratory conditions only if homologous recombination was possible. No other evidence exists for the transfer to and expression of plant or animal genes in microorganisms under natural conditions [NRC, 2004a, pp. 132-133]. Horizontal gene transfer from GM plants to bacteria is considered to occur with extremely low probability if no homologous sequences are present.

Specific concerns of horizontal gene transfer are related to the use of antibiotic resistance genes (as selectable markers) and of viral DNA (as gene expression promoters and as a source of protection against plant viruses) in plants. The risks associated with the use of antibiotic resistance genes in GM plants were reviewed by Bennett et al. [2004] and Goldstein et al. [2005]. The acceptability of antibiotic resistance marker genes can be judged based on their prevalence and their therapeutic relevance in human and veterinary medicine. From the perspective of food safety, the use of antibiotic resistance genes relevant for human therapy should be avoided [FDA, 1998; Bennett et al., 2004; Goodyear, 2004; van den Eede et al., 2004; Goldstein et al., 2005]. Removal of antibiotic resistance marker genes after transformation is also encouraged. However, the efficacy and limitations of different marker excision systems vary, as reviewed in sections 2.4.2 and 2.4.3.

Given that the potential consequences of gene transfer to microorganisms could be significant, a case-by-case assessment of the genes, constructs and organisms in question for possible enhancement of gene transfer potential in terms of probability, pathways and consequences has been recommended [EFSA, 2004].

3.4.3. Viral recombination

Evidence is lacking that plant viral genes can be transferred to the genomes of microorganisms, plants, or animals [Amabile-Cuevas and Chicurel, 1993]. It is also recognized that presently available methodologies limit a comprehensive assessment of potential health risks because less than ten percent of bacteria naturally occurring in the gastrointestinal tract can be cultured and analyzed. However, in the context of the use of viral DNA sequences that encode coat proteins of viruses in the production of viral resistance in transgenic plants, the likelihood of adverse

health effects in humans is considered to be very low. Plant viruses are highly divergent from bacteriophages and most animal viruses. Any potential risks associated with plant virus-derived coat protein encoding genes, promoter, and viral transformation vectors would be expected to be of a plant health nature, and not applicable to human health. The risk of recombination between viral DNA that may affect disease resistance in plants cannot be ignored because of the existence of replication-competent viruses. Therefore, it remains prudent to avoid the use of complete viral genes to confer viral resistance in plants. In those cases where the use of viral vector or viral sequences in plant bioengineering is justified, a case-by-case assessment of plant health risk will be required [The Royal Society, 2002].

3.4.4. Targeted gene integration, excision, and expression

Emerging technologies for targeted gene integration, excision, and expression can increase the complexity of risk assessment. In transgenic crops in which targeted gene insertion is achieved by homologous recombination, careful investigation of the stability of the remaining insert after recombination will be required, especially if recombination frequency is indicative of transgene insertion in recombination hotspots, or other less stable areas of chromosomes [König, 2003]. In recombinase-mediated marker removal, the likelihood of occurrence of unintended recombination events will need to be assessed through comprehensive study of the binding site specificity of the recombinase used. Despite their expected low frequency of occurrence compared with natural mechanisms for recombination in plant genomes, the potential for the presence of recombinase-recognition sites in plant genomes cannot be dismissed [König, 2003]. Questions of specificity and mode of action also apply to newer methods that offer more efficient recombination using nucleases or DNA repair proteins.

Concerns regarding targeted expression will depend on the nature or mode of action of the inducible expression system and how it interacts with the metabolism of the modified plant. Limitations also exist in tissue-specific expression, as many of the promoters should more accurately be described as tissue-enhanced because their expression may not be completely confined to a single tissue or plant part [Potenza et al., 2004]. A thorough knowledge of the functionality of both homologous and heterologous promoters in target host plants is essential in order to improve the efficiency and stability of transgene expression.

3.4.5. Accelerated and wider scope of novelty

The ability to transfer specific and well-characterized genetic material across species barrier allows rDNA techniques to overcome major limitations of conventional methods: reproductive incompatibility, difficulty in selecting desirable phenotypes, slow generation time, and tight linkages with undesirable traits. However, the advantages of *in vitro* nucleic acid techniques also vastly enlarge the novelty that can be introduced in the organism being modified, as well as the level of exposure to these substances. It is possible to engineer a metabolic pathway that is completely new to the host plant and that significantly alters cellular metabolism without any apparent phenotypic change [Tattersall et al., 2001; Kristensen et al., 2005]. The application of the same genes and constructs across all species may also accelerate the undesirable propagation of effects if they are potentially harmful [Mayer, 2002]. The speed of modification and selection, including multigene transformation, can be accelerated through techniques such as DNA shuffling and chromosome design [Lassner and Bedbrook, 2001; Snow, 2003].

In addition, the gene or genes of interest in genetic engineering are typically transferred with a marker gene (for selecting transformants) and appropriate control sequences (promoter and terminator for facilitating expression of transgene(s) in the recipient plant). The transfer of border DNA sequences (sequences from the plasmid or vector used for the transformation, but are not intended to be inserted into the host organism) has been shown to be a common event [Cluster et al., 1996, cited in Kohli et al., 2003]. The identity of the border sequences is more predictable for Agrobacterium-mediated than biolistic transformation. Generally, the characteristics of the donors of auxiliary sequences are not considered critical from a risk perspective if the donor organisms are merely used as a source of well-characterized DNA [ACGM, 2000]. The adoption of transgene constructs that are void of superfluous vector backbone is a positive development in minimizing this problem [Fu et al., 2000; Smith et al., 2001; Altpeter et al., 2005]

3.5. Summary and conclusions

Observations on the three approaches to genetic modification reviewed relevant to risk assessment are summarized below.

Genetic hybridization (recombination)

Given that DSB and NHEJ are the preferred mechanisms for DNA repair and recombination in plants, the process of transgene integration is expected to be similar to the natural recombination processes that occur in plants. Data also suggest that hot spots involving gene-rich regions are preferred by both mechanisms of natural recombination and of transgene integration. Intergeneric hybrids, including those obtained by cell fusion (that generally have a low commercialization rate) are expected to retain larger linkage blocks, compared to intra- or inter-specific hybrids that have similar genes and share a common ancestry [NRC, 2000a, p. 61]. Therefore, current evidence does not support the contention that transgene integration in plant chromosomes will result in DNA disruptions that are qualitatively different from those arising from natural recombination mechanisms.

Induced mutation

It is difficult to generalize the effects of chemical and physical mutagens in terms of size or number of structural changes, rearrangements, or duplications. In general, most mutations result in gene products being converted from an active to a less active form (loss-of-function mutation). These types of changes would pose a problem only when the active form is needed to remove an antinutritional, allergenic, or toxic constituent [NRC, 2000a, p. 61]. It is also difficult to maintain chromosomal changes that remain viable after the mutation process [van Harten, 1998]. It is not possible to determine with confidence whether there are major material differences between somaclonal variation and mutation induced by chemical or radiation [Larkin, 1998]. However, the potential for unfamiliar health effects using somaclonal variation or mutagenesis is expected to be similar [NRC, 2000a, p. 61]. Compared to nucleic acid technologies, induced mutation is unlikely to result in complex metabolic changes achievable by metabolic engineering using rDNA techniques. It would be difficult, by induced mutation alone, to change continuous or quantitative trait encoded by many independent genes (each with small effects on the plant phenotype).

Genetic engineering

With respect to genetic engineering, molecular analytic techniques have the potential to mitigate unintended effects of gene integration by permitting the selection of single-copy transformants [Zhong, 2001; Hanin and Paszkowski, 2003; The Royal Society, 2003, p. 54], and by improving the control of transgene insertion (gene targeting) by homologous recombination [Kumar and

Fladung, 2001; Hanin and Paszkowski, 2003; Iida and Terada, 2004]. *In planta* transformation holds promise as an alternative to tissue culture, thereby reducing unintended effects of induced mutation in somaclonal variation [Pelletier and Bechtold, 2003]. It is also possible to limit transgene expression by using inducible or tissue-enhanced promoters [NRC, 2004b; Potenza et al., 2004]. At present, however, major challenges have to be overcome before these genetic engineering technologies can be widely applied to major crops.

Role of selection and backcrossing in plant breeding

Equally important among considerations of GM crop production in relation to safety assessment is the recognition that selection is an integral part of crop improvement programs, regardless of the method of genetic modification. This includes positive selection for the target trait, as well as negative selection by which abnormal regenerating plants are discarded. In modifications involving tissue culture, the use of an appropriate growth medium can facilitate selection. In rDNA-based modification, selection can also be facilitated by expressing a marker gene that has been inserted in target cells. In all breeding programs, selection is an iterative process that continues in successive stages of plant development with varying degrees of technical stringency and power in identifying unintended changes [Hansen and Wright, 1999; Cellini et al., 2004; NRC, 2004a]. Selection is based on a combination of morphological, biochemical, cytological, and molecular markers. Selection can only be made for those traits observable phenotypically, or that can be measured by appropriate analytic methods. Other traits or modified traits that are not assayed will not be apparent. The objective of selection is to select for a minimal change in the phenotype while retaining all of the commercial agronomic characters in an established commercial variety. In conventional breeding, this objective would, to a point, limit the number and degree of non-phenotypically detectable changes that would be expected. In wide hybridization, including transgenic modification, with less genotypic homology and bypassing the natural restriction on genetic mixing, the extent of non-detectable changes that may be missed is unknown.

From a historical perspective, it is believed that during domestication, selection against toxic compounds took place and that many crops have either lost these compounds or contain significantly reduced amounts of them. Therefore, despite the limited testing that has been conducted in comparison to that used for synthetic compounds, classical plant breeding has introduced very few deleterious genes from wild relatives into crops.

The practice of backcrossing and testing that normally takes place during plant breeding is expected to eliminate deleterious mutations or underperforming genotypes. This practice is common regardless of the method of genetic modification. However, uncertainty remains regarding the effectiveness of this practice in eliminating unintended effects that may have health implications [Gepts, 2002; Cellini et al., 2004]. Documented reports on unintended effects in conventional breeding are rare, due in part to the high discard rate through the selection process that relies on observable phenotype in the early stages of selection [The Royal Society, 2003, p. 56; Cellini et al., 2004]. In conventional breeding, the effectiveness of the backcrossing process will depend on the size of the linkage block associated with the gene of interest, as a function of the number of backcrosses and the rate and type of recombination between the wild relative's chromosomes as well as those of the cultivated parent [NRC, 2000a]. Further, some types of unintended effects with biological relevance have only been detected after growing plants under specific environmental conditions [Cellini et al., 2004].

Conclusions

In general, there are no major differences in the type of random effects on genomic and chromosomal structure of the modified plant among the major methods of genetic modification reviewed. The extent of modification of genomic structure and the frequency of occurrence of a specific type of effect may vary among methods. Such effects are also influenced by epigenetic factors. However, the different methods of genetic modification pose different challenges in terms of how their effects can be identified and assessed. In this regard, molecular approaches provided by genetic engineering offer an advantage in being able to direct the needed analysis to obtain essential information on what genetic elements have been inserted into the transgenic plant, the regions into which they have been inserted, and any changes to the transferred elements that may have occurred after the transformation [NRC, 2004a, p. 60]. The specificity of molecular characterization of the target gene in genetic engineering permits its position in the modified host genome and its function to be assessed with greater precision. In contrast, there have been few applications of molecular analytic techniques for risk assessment of crops modified by conventional methods. Genetic changes at the molecular level in plants modified by non-rDNA methods are generally undirected and unpredictable. Molecular characterization of genomic changes in induced mutation or hybridization using conventional techniques for risk assessment is not widely practiced. In fact, food safety aspects of crops genetically modified by conventional methods have not received much attention to date. This may be partly attributable

to acceptance of potential health risks that may be associated with conventional genetic modification methods. However, this acceptance is not universal [Wilson A. et al., 2004].

Prior to the commercialization of GM food crops, new plant varieties are typically subject only to regulations related to plant variety protection and associated seed legislation, or their equivalent, that varies in stringency in different jurisdictions [Welsh, 1981]. Few health-related standards on food composition exist in registering a new variety of food crop. Exceptions include limits for erucic acid in oilseed rape and total glycoalkaloids in potatoes. Further, plant breeding for many species is now a commercial enterprise and the genetic heritage of cultivars may not be fully disclosed [Wilson A. et al., 2004]. As a growing number of non-transgenic methods become incorporated into commercial breeding programs, the current practice of basing premarket assessment of the potential risks of foods derived from GM crops exclusively or primarily on the method of genetic modification would require reexamination.

In conclusion, the preceding survey of three major approaches to genetic modification of food crops identify comparable mechanisms by which unintended effects could be produced in plants. The extent of modification of genomic and chromosomal structure and the frequency of occurrence of the specific types of changes may very among different methods and are difficult to quantify. However, it may be concluded that the types of unintended changes would be qualitatively similar, given the similar underlying molecular and biochemical basis of the changes. This in turn suggests that the potential health hazards from unintended changes may be expected to be similar for the major approaches to genetic modification of food crops. On the other hand, the method of genetic modification can have a significant impact on the scope of novel substances that can be introduced in the modified plant, as well as the types of food that contribute to new sources or altered levels of dietary substances in the food supply. In this regard, the safety of the donor organism and the recipient plant as sources of substances that are potentially harmful is a key consideration. The method of genetic modification can also affect the level of complexity in metabolic changes in the modified plant, which, in turn, also determines the complexity of risk assessment.

In the next chapter, the types and sources of potential hazards in GM foods reviewed in this chapter will be organized into an analytic framework based on the concept of risk. The concept of risk takes into account not only potential hazards, but also their likelihood of occurrence,

levels of exposure, and health consequence at the population level. The proposed risk-based framework aims to structure the assessment of concerns about the potential for adverse health impacts of GM foods and to guide the testing of GM foods in proportion to their likelihood of conferring human health risks in populations consuming such foods.

CHAPTER 4 - CLASSIFYING POTENTIAL HEALTH RISKS OF GM FOODS

This chapter begins with an introduction to the concept of risk, classification systems, and their application to the categorization of potential health risks of GM foods. The process used to develop the risk-based classification scheme (RBCS) for testing GM foods is then explained in detail. The focus of this chapter is to describe the scientific rationale for the factors and criteria that underlie the scheme, as well as the principles that guide the aggregation of these factors in determining concern levels for each category of health effects considered in the scheme.

4.1. Concept of risk

4.1.1. Definition of risk

There is currently no uniform definition of risk, although the term is generally used to refer to some probability of occurrence of an undesirable outcome [Covello and Merkhofer, 1993]. Accordingly, "risk" is a function of: (1) the possibility of an adverse outcome, and (2) uncertainty over the occurrence, timing, or magnitude of that adverse outcome. If either attribute is absent, then there is no risk. The probability of an adverse health outcome occurring depends on the potency of and the level of exposure to the agent, as well as the host's susceptibility. Factors influencing the seriousness of a health consequence include its nature, severity, and degree of reversibility [Krewski et al., 2002].

4.1.2. Health risk assessment

Health risk assessment can be conceptualized as a process that attempts to determine whether exposure to an agent in the environment exceeds the level at which an adverse health effect may or will occur [Chapman, 1996]. The model described by the United States National Research Council (NRC) of the National Academy of Sciences has been widely used by government agencies throughout the world. According to this model, risk assessment consists of hazard identification, dose-response assessment, exposure assessment, and risk characterization [NRC, 1983]. Depending on the application, the approaches to conducting risk assessment may vary in the emphasis and nomenclature used [Covello and Merkhofer, 1993; Williams and Paustenbach, 2002; Jardine et al., 2003]. With respect to food-related health risks, the Codex Alimentarius Commission, the international organization for food standards, recognizes four steps of risk assessment: hazard identification, hazard characterization, exposure assessment, and risk

characterization [Codex Alimentarius Commission, 2002]. The general approach used in assessing food-borne risk is similar to that embodied in the NRC model.

Hazard is a term closely related to risk. Specifically, hazard is an intrinsic property or the potential of a biological, chemical or physical agent to cause harm [HACCP Working Group, 1992; Silva et al., 1995; ANZFA, 1996; Brooke-Taylor, 2001]. In the NRC model, hazard identification is the determination of whether a particular agent or process is or is not causally linked to particular adverse effects. Alternatively, a hazard can be viewed as a (potential) source of risk or as an event [Covello and Merkhofer, 1993; van Gerwen et al., 1997]. According to this view, a hazard becomes a risk agent only if an exposure pathway exists and if exposures create the possibility of adverse consequences.

4.2. Classification systems

Classification is an information management technique that simplifies and orders complex and varied observations in scientific and applied research [Cvetkovich and Earle, 1985]. The classification of substances in terms of their hazardous properties or potential represents an essential first step in risk assessment and management [Basketter et al., 1999]. A range of classification systems have been developed along the hazard-risk continuum. Classification systems may be divided into two broad types based on the function or purpose of the classification system and the information required: hazard-based classification and risk-based classification.

4.2.1. Types of classification system

a) Hazard-based classification

Known or well-characterized hazards have been classified for the purposes of risk management in order to limit exposure based on toxic potency and to facilitate risk communication through product labelling. Examples include classification systems developed for pesticides and other hazardous chemicals [Copplestone, 1988; O'Bryan and Ross, 1988; Ignatowski et al., 1995; Pest Management Regulatory Agency, 2002; Pratt, 2002].

b) Risk-based classification

Classification systems have also been developed when the nature of a hazard has not been precisely identified or characterized in order to provide a preliminary assessment of *potential* or *possible risk*. Focusing on the major components of risk assessment, these systems classify potential risks based on identifying the types or sources of hazards or potential hazards, the extent of human exposure, and relevant factors influencing their occurrence. The type and severity of health effects may also be considered. For example, risk-based classification systems have been developed to help prioritize detailed assessment of contaminated sites [CCME, 1992] and medical devices [Health Canada 1998a, b].

As a qualitative risk assessment tool, a risk-based classification system can serve a screening function as part of a tiered or stepwise approach before investing resources in typically expensive and labour intensive comprehensive risk characterization [Silva et al., 1995; van Gerwen et al., 1997; Moutou et al., 2001; Ross and Sumner, 2002]. This approach facilitates the conduct of additional toxicological testing for the right reasons [Bristol, 1995]. Despite its qualitative nature, a risk-based classification can be used to inform structured problem-solving and to focus attention on factors that are most likely to affect risk.

4.2.2. Organization of classification systems

a) Structure and components

Common structure and components of classification systems identified in this literature review include:

(1) *categories* that reflect major types or sources of hazards or potential hazards,

(2) *factors* or attributes that describe key characteristics of the categories as sources of hazards or potential hazards,

(3) *criteria* that differentiate each factor into two or more levels of concern, and

(4) *classes or levels of concern* with the simplest type of classification consisting of binary classes (e.g., yes/no, or positive/negative). Indication of the level of confidence associated with a particular classification can also be described (e.g., likely, possible). Some ternary classifications add a class for equivocal results, to indicate the lack of certainty needed to provide an unequivocal assessment [Bristol, 1995].

b) Information required

Both hazard-based and risk-based classification systems aim to use minimal or easily accessible information on which to make a meaningful first evaluation [CCME, 1992; Lundgren, 1992; Finizio et al., 2001]. The use of expert-based scientific judgement or knowledge rules is common in both systems [O'Bryan and Ross, 1988; Jelovsek et al., 1990; van Gerwen et al., 1997; Chou and Williams-Johnson, 1998; Pohl et al., 1998; Bisaillon et al., 2001; Pratt, 2002; Sumner and Ross, 2002]. Where empirical evidence is available, the principle of weight of evidence is stressed in evaluating data quality [O'Bryan and Ross, 1988; Jelovsek et al., 1999; Pratt, 2002; Weed, 2005] Where empirical evidence is absent or inconclusive, expert knowledge can help assess theoretical hazard in context [O'Bryan and Ross, 1988; van Gerwen et al., 1997].

c) Aggregation

Methods of combining multi-attribute information in classification systems include (1) scoring, (2) matrix, (3) decision tree, and (4) combined approaches.

(1) *Scoring* is an additive numeric approach that can be applied with or without weighting [O'Bryan and Ross, 1988; CCME, 1992]. Alternatively, the highest score for any one component of a category may determine the score for the category or the score for all categories.

(2) A *matrix* is a qualitative scale that uses ordered categorical labels. In simple rating scales, two categories are combined at a time. For example, when one category is rated "high" and the other category is rated "low" separately, the combined rating of two categories would be "moderate" [Moutou et al., 2001].

(3) In a *decision tree*[4] or *flow chart* approach, a series of questions with binary response (yes/no) can be structured to lead to one final classification [c.f.., Cramer et al., 1978]. Alternatively, questions can be structured to rule out potential hazards from an exhaustive inventory [Bisaillon et al., 2001]. A variation of this scheme includes a progressively comprehensive

[4] In this thesis, the usage of the term "decision tree" follows that applied to flow diagrams used in the assessment of hazard potential of food-related substances [e.g., Cramer et al., 1978; FAO/WHO; 2001; Schilter et al., 2003; Kroes et al., 2004;]. It is noted that in other disciplines (e.g., decision analysis), a decision tree has several components, including the decisions to be made, chance events or possible outcomes and their probability of occurrence, as well as estimated values of the outcomes.

tiered approach to identifying and selecting potential hazards for subsequent targeted analysis [van Gerwen et al., 1997]. Decision trees can also be developed using quantitative methods (e.g., classification and regression tree analysis) [Eisenberg and Mckone, 1998; Qian et al., 2001].

(4) A combined approach using both numeric and descriptive information can be used to convert qualitative responses to questions into a numeric scale through a series of mathematical and logical steps [Ross and Sumner, 2002].

4.3. Development of risk-based classification scheme for testing GM foods

In developing a risk-based classification scheme as guidance for testing GM foods, *potential* health risk is conceptualized as the likelihood of known or potential adverse health effects arising from human exposure to substances that are intentionally introduced or modified in food crops, and that may be produced unexpectedly as a result of the genetic modification process. The classification of potential health risks of GM foods in the proposed scheme is based on identifying the sources of potential hazards in GM foods for each category of adverse health effects (i.e., toxic, antinutritional, and allergenic effects from intended and unintended changes resulting from genetic modification, as discussed in Chapter 3), the factors that influence their occurrence, and the level of human dietary exposure. For each factor identified, criteria for differentiating health risk potential are developed. The extent to which each factor satisfies applicable criteria is rated separately using qualitative measures (yes, no, or uncertain/questionable). A concern level for each category of health effects is then determined by aggregating the ratings for the factors using predetermined aggregation rules. An introduced or modified gene product in a GM food, as well as the GM food itself, is classified separately within the applicable category of health effects.

4.3.1. Development process

Development of the proposed scheme (Phase 1) started with a review of the scientific literature and national/international guidelines on risk assessment of GM foods and other food-related products. The objective of the literature review was to identify the relevant factors that can influence the introduction of potentially hazardous substances in GM foods and their exposure (as described in Chapters 2 and 3). Four expert consultations with a total of 45 experts from 10

countries were conducted on versions of the proposed scheme at different stages of development using questionnaires mailed electronically to participants. The first consultation aimed at verifying the relative importance of the factors and the appropriateness of the criteria for rating the factors based on a synthesis of the literature review. Ten participants with diverse expertise relevant to health risk assessment of GM foods participated in the first consultation. The second consultation tested the feasibility of differentiating GM foods into a manageable number of concern levels using defined factors and criteria. In the draft RBCS used in consultation 2, likely patterns of aggregation of the factors and criteria were considered in establishing concern levels. The second consultation involved a different multidisciplinary group of 14 experts, seven of whom also participated in consultation 1. In the third consultation, the revised RBCS included explicit rules for aggregating the factors and criteria into concern levels. The revised RBCS and a proposed graded approach to the testing of GM foods that corresponded to the concern levels in the RBCS were reviewed by another multidisciplinary group of 19 experts in consultation 3. Following consultation 3, the RBCS was simplified and was further reviewed by a separate group of nine toxicologists (consultation 4). The responses received from the four consultations were taken into account throughout the development phase of the classification scheme. Results of the consultations suggest that aggregation rules for determining concern levels should be separate from the application of criteria to individual factors within each category of health effects. This suggestion was incorporated before an exploratory study of the utility of the RBCS was conducted. Reports of consultations 1 to 4 are included in Appendices A to D in Volume II of the thesis. The reports detail participant recruitment, qualitative and quantitative data analysis, and how the results of the consultations were incorporated in successive revisions of the draft scheme. Early versions of the scheme and the questionnaires used in the consultations, as well as other supplementary information, are included in the consultation reports.

The following sections describe the scientific rationale for and organization of factors and criteria in the proposed RBCS, and how they are aggregated in determining concern levels for the three categories of health effects of relevance in the safety assessment of GM foods.

4.3.2. Information used in classification

Two types of information generally used in the premarket assessment of GM foods are distinguished in developing the RBCS: prerequisite information and core information.

Prerequisite information is required for all GM crops and will not provide a basis for differentiation. This includes information on genetic and phenotypic stability, quality of the data, and the intended purpose and performance of the modified trait. This type of information is currently required in the assessment of GM crops and derived foods in many jurisdictions.

Core information, in contrast, should have discriminating value for classifying GM foods. Core information is a subset of all the information that is typically required in premarket assessment. It provides the basis for the factors and criteria underlying the classification scheme. Core information is based on prior knowledge and baseline testing of the modified crop and derived foods.

Core information includes prior knowledge related to (a) biology of the host plant, donor organism (and/or genetically related organism), including associated toxicity and allergenicity, (b) prior history of food use, and (c) function and safety of the introduced/modified gene products and where applicable, their principal metabolites and breakdown products in plant and mammalian metabolism, as well as effects of processing.

Core information relevant to food safety assessment can also be generated from baseline testing or assessment at a screening level, including: (a) level of expression of gene products and principal metabolites of interest in the edible parts of the modified crops, (b) expected dietary exposure to the food derived from the modified crop and to the gene products of interest, (c) structure-activity characterization of the gene products of interest, and (d) basic compositional analysis of the edible parts.

Assessment based on prior knowledge and baseline testing provides the first step of an iterative process that continues throughout the GM crop development process. For the classification scheme to be useful as guidance in discriminating among GM foods that require more scrutiny and testing from those that do not, the factors and criteria underlying the classification scheme emphasize information normally compiled or generated as part of developing the desired crop. Ideally, this baseline information should also be easily assessed.

The nature and extent of testing may vary with the stages of product development and can result in a decision to terminate or to continue with the development process. Ultimately, sufficient information needs to be generated to support premarket risk assessment.

4.3.3. Factors relevant to health risk assessment of GM foods

There are two areas of focus in assessing the potential for health risks of a food derived from a GM crop: (1) the potential for toxic/antinutritional effects, and (2) the potential for allergenic effects.

To differentiate the sources of uncertainty about the potential risks of GM foods, intended and unintended changes are classified separately for potential toxic/antinutritional effects in the proposed RBCS. As explained in section 3.1, intended changes refer to the direct and indirect effects of primary and secondary gene products. Unintended changes refer to the predictable and unpredictable effects of genetic modification in the food derived from the modified crop. Predictable effects are unintended changes that go beyond the primary expected effect(s) of introducing the target gene(s), but that may be explicable in terms of our current knowledge of plant biology and metabolic pathway integration and interactions. Unpredictable effects are those changes that fall outside our present level of understanding of gene regulation and gene-interactions [Kuiper et al., 2001; Cellini et al., 2004].

Consistent with current international approaches to the assessment of the allergenic potential of GM foods, the classification of the potential for allergenic effects in the RBCS will focus on protein substances that mediate IgE-linked immediate hypersensitivity reactions. Given the influence of food matrix on allergenic effects, no distinction is made in classifying intended and unintended changes for this category of health effects.

An overview of the rationale underlying the factors used in determining concern levels for each of these three categories of health effects, which correspond to the three parts (Parts A, B, and C) in the proposed RBCS, is given below. Both the scientific justification for and the limitations of the factors are discussed. A detailed description of the factors is provided in **Table 4-1, Parts A, B, and C** (Tables 4-1A, 4-1B, and 4-1C).

Part A - Potential for toxic and antinutritional effects – intended changes

In the context of the RBCS, the risks posed by a dietary substance of interest are determined by its level of intake (dietary exposure) and hazard characteristics (the nature of the substance). Eight factors related to exposure and the nature of the substance that is intentionally modified in a GM crop can be grouped as follows.

Exposure to gene products

(1) Expression of gene product(s) in edible part(s) of the modified crop

(2) Prior history of consumption from foods

(3) Expected change in total dietary exposure

Nature of gene products

(4) Prior knowledge of safety/mode of action

(5) Structure-activity characterization

(6) Digestion and degradation products

(7) Synergism and interactions

Other factors

(8) Prior experience with gene products introduced/modified

Exposure to gene products

It is expected that many substances intentionally introduced or modified in GM foods will be similar to those already present in the food supply. Substances that have a prior history of consumption from foods will require less scrutiny than those that do not. Genetic modification may alter the food sources of these substances and their exposure levels. Further, depending on the strategy of the genetic modification, the extent of dietary exposure may be limited by the tissue of expression in the modified crop. Therefore, expected change in total dietary exposure is a critical factor in the RBCS.

For many food-derived substances, the discriminating value of information on the duration and frequency of exposure at the level of the food being consumed, as well as the size of the population being exposed, can be expected to be small, relative to a much wider variability in

intakes that can be expected at the level of food constituents. Therefore, these factors are not included as part of the core factors in the RBCS. However, consumption at the food level is considered in predicting the expected change in dietary exposure to the gene product of interest in the food. Susceptible or vulnerable population subgroups are not overlooked: they are included as part of considerations for testing (see section 7.3.2 and Table 7-1A, Level I, suggested further data acquisition/testing). Indirect or non-dietary routes of exposure via geobiochemical pathways or dermal and respiratory routes are also not included as part of the core factors in the RBCS, given their comparatively minor contribution to total exposure among the general population. However, respiratory routes of exposure to potential pollen-borne allergens are considered in Part C (see section 7.3.2 and Table 7-1C, Level I, suggested further data acquisition/testing).

Nature of gene products

The ability of a substance to induce adverse health outcomes is determined by its inherent properties, which depend on its structural and functional characteristics. For some substances with a previous dietary history, an understanding of their inherent properties can be based on prior knowledge accrued during the course of past consumption of that substance. The demonstration of structural similarity and functional equivalence with substances with a prior history of known or presumed safe food use is also a key factor in risk assessment. For a gene product with prior evidence of safety, structure-activity characterization aims to assess whether a heterologously expressed protein is structurally similar and functionally identical to the comparator/source protein for which prior safety information is available. In the context of GM foods, a novel gene product may be a protein that is encoded by DNA sequences that have been substantially modified, or is engineered for specific functional properties. In both cases, structure-activity characterization provides screening level information that may signal the need for further testing.

Consideration will also be given to the metabolic fate in the modified plant of a gene product that is new to the modified plant, or an endogenous gene product that is modified to express at substantially higher levels. Given that proteins in foods are normally broken down into amino acids for absorption, the proteolytic profile or metabolic fate of a protein gene product following food ingestion would only be relevant if a protein substance is resistant to digestion. A non-protein substance may be of concern if it is in a structural class for which there is less

knowledge of the metabolism, pharmacology and toxicology of that substance. These considerations are particularly relevant if the intake of these types of substances from the GM food exceeds normal dietary variations from the same or other foods or prior knowledge on their safety suggests the need for more information on potential human health risks.

The potential for adverse health effects from synergistic and interactive effects among intentionally altered substances and endogenous food constituents is another factor in the RBCS. In the context of foods as complex mixtures, the effects of food matrix, bioavailability, and processing are also relevant in considering the safety of a substance present in a new food source.

Factors related to gene transfer and viral recombination (see sections 3.4.2 and 3.4.3) are excluded from general consideration as core factors in the RBCS. One major concern about gene transfer is related to the use of antibiotic resistance genes as selectable markers. This type of concern can be addressed by employing best practices in the selection of marker genes encoding traits that pose little relevance in human and veterinary medicine. Similarly, concerns about viral recombination in the generation of new viral organisms (with respect to plant health) are also best addressed by sound practices such as avoiding the use of complete viral genes (without the presence of replication origins or gene/gene sequences that might enhance recombination). However, transgenes from less commonly used microbial sources may warrant a case-by-case evaluation as part of considerations for testing (see section 7.3.2 and Table 7-1A, Level I, suggested further data acquisition/testing).

Other factors

Prior experience with the gene products introduced/modified and the transformation protocol may reduce the level of scrutiny required if the necessary criteria are met. Additional factors may also be included in the scheme in the future as new genetic modification technologies evolve and new factors are identified, or as new tests are developed. The structure of the scheme is flexible enough to incorporate future developments.

Part B - Potential for toxic and antinutritional effects – unintended changes

Factors affecting the likelihood of occurrence of unintended changes with the potential for adverse health effects are grouped into four categories in the RBCS: genetic sources of toxic or antinutritional substances, the factors affecting their expression, indicators of their expression, and the health consequences of instability of the modified trait. Among the six factors in the RBCS listed below, factors marked with an asterisk may be considered as secondary factors. Failure to meet the criteria for secondary factors will trigger the need to justify the adequacy of the scope of other metabolites/parameters to be analyzed. For these products, an expanded scope of metabolites in addition to current standard practice may be triggered (see section 7.3.2, Part B).

Genetic sources

(1) Safety of donor organism

(2) Safety of host plant*

Factors affecting the expression of unexpected substances

(3) Potential for novel/altered patterns of metabolites*

- Knowledge of gene product function
- Impact of gene product function in the modified plant
- Substrate specificity of gene product

(4) Potential for disrupting coding or regulatory DNA sequences in the modified plant

- In the inserted/modified DNA
- In DNA sequences bordering the site of insertion/modification*

Indicators of unexpected expression of known or novel substances

(5) Chemical analysis of food derived from the modified plant

- Key nutrients
- Known toxicants/antinutrients
- Other metabolites/parameters

(6) Health consequences of instability of the modified trait

Genetic sources

The two main aspects of concern of unintended changes in GM foods with the potential for adverse health effects are: (1) modified expression of endogenous toxic substances (or metabolites) in different tissues or at different levels, and (2) generation of new toxic compounds. Therefore, the safety of the host plant and that of the donor organism (in the case of poorly characterized gene modification, e.g., wide-cross hybridization) are major considerations, as they represent the genetic sources of substances that may have adverse health effects at certain level of expression.

Available information tends to support the premise that unintended effects of genetic modification of food crops that have a history of traditional breeding, when they occur, would most likely result in a change in the level of expression of already recognized toxicants in the plant. This is deduced from observations that (1) the ability to synthesize particular classes of secondary metabolites is generally restricted to selected plant groups [Cheeke, 1998, p. 87; Osbourn et al., 2003]; (2) certain classes of plant chemicals with potent toxic effects (such as alkaloids) require highly substrate-specific enzymes and are highly compartmentalized in their biosynthesis [Croteau et al., 2000; Jørgensen et al., 2005]; (3) as genes encoding enzymes of specific pathways in eukaryotes are not organized and regulated as a unit as is the case for prokaryotes, there is the requirement for a higher level of coordination of multiple metabolic alterations in effecting the synthesis of potentially toxic metabolites [Hadacek, 2002]; (4) the production of secondary metabolites with potent biological activity is likely a rare property of any one molecule despite the vast diversity and metabolic flexibility of plant metabolism [Jones and Firn, 1991; Firn and Jones, 2003]; (5) only 21 food-borne toxic substances have been identified to be of plant origin (representing less than 0.1% of the total number of food constituents identified), and only 9 out of the 21 toxicants are biosynthesized by plants, with the remaining being pass-through contaminants in honey and milk [IFBC, 1990].

Therefore, it is less likely that critical toxicants inherent in the plant but hitherto unexpressed will be unintentionally induced by genetic modification [WHO, 1995]. At present however, there is insufficient evidence to conclude that the likelihood of its occurrence can be dismissed [Facchini et al., 2004]. It is also recognized that the genetic potential of secondary metabolism is never fully expressed [Hadacek, 2002]. The fact that the introduction of new pathways may not cause

metabolic problems or identifiable phenotypic changes also warrants consideration [Tattersall et al., 2001; Kristensen et al., 2005]. Therefore, the likelihood of occurrence of novel or naturally occurring constituents in the plant with previously unknown toxicity should be taken into account in the RBCS.

Factors affecting unexpected expression of known or novel substances

As explained in Chapter 3, unintended changes in GM crops can theoretically result from alterations (1) at the genomic level from structural changes to DNA sequences or to chromosomal organization; (2) at the transcription level from changes to gene expression resulting from position effects, the disruption, modification or silencing of active genes, or the activation of silent genes; and (3) at the metabolic level from interactions between novel gene products and endogenous enzymes and metabolites, as well as alterations in flow through of metabolites in biochemical pathways [Conner and Jacobs, 1999; Kuiper, 2001; Cellini et al., 2004] (see Chapter 3 for a detailed discussion).

Divergent views exist regarding the likelihood of unintended effects with adverse health consequences in foods derived from GM crops and the methods by which these effects should be detected. There is a lack of empirical data to estimate the relative magnitude of the potential for, or the probability of, health hazards associated with unintended changes in GM foods. While no generally accepted predictive factor(s) that may affect the expression of unexpected substances in GM crops have been identified, certain characteristics at the metabolic and genomic levels are suggestive of a higher level of uncertainty and the need for more scrutiny.

At the metabolic level, these characteristics relate to the function of the gene product, the likelihood for systemic effects in the modified plant, and the substrate specificity of an enzymatic protein. For example, based on current knowledge, it is generally assumed that recombinant pesticidal proteins that are introduced into food crops to date probably have minimal interactions with the plant's metabolic pathways [Ellis, 2001; ILSI, 2004, p. 51]. These types of protein are unlikely to induce a broad range of unexpected metabolic effects. Limited publicly available data appear to support this view in that the extent of metabolic interconnectivity may be predicted in part by gene function [Le Gall et al., 2003; Defernez et al., 2004; El Ouakfaoui and Miki, 2005].

At the genomic level, concerns about unintended changes relate to the likelihood of interrupting coding or regulatory sequences of the modified plant. At present, these concerns are addressed by identifying the presence and plausible expression of unknown DNA sequences (open reading frames) in the inserted gene and flanking regions. As concluded in Chapter 3, compared to poorly characterized gene modification using conventional methods, rDNA techniques offer an advantage in being able to direct the needed molecular characterization in this regard. The issue of the relative degree of genetic disruption for different methods of genetic modification was reviewed by the Committee on Identifying and Assessing Unintended Effects of Genetically Engineered Foods on Human Health convened by the U.S. National Research Council's Institute of Medicine [NRC, 2004a]. The committee concluded, based on expert judgement, that it is the final product of a given modification, rather than the modification method or process, that is more likely to result in an unintended adverse effect. With the exception of mutation breeding, the greater the genetic distance between the donor and host organisms, the higher will be the likelihood and the extent of unintended effects within a given method of genetic modification. On the other hand, due to the common practice in breeding programs of selecting favourable lines and discarding those exhibiting unwanted phenotypic characteristics, the extent of unintended effects in commercialized GM lines is expected to be reduced.

Similarly, as previously discussed (section 3.3.3), no generalization can be made regarding whether the introduction of a novel gene or a homologous gene will have a greater effect on the production of novel/altered patterns of metabolites. The expression of a novel gene (e.g., a gene for a biosynthetic enzyme not found in the host plant species) being controlled by novel regulatory elements may be outside the control of normal homeostatic feedbacks [Hallerman, 2003]. On the other hand, up or down regulation of endogenous or homologous genes can result in interactions with a greater number of other genes/enzymes in the host, because the introduced gene shares a high degree of functional and sequence similarity with the corresponding gene in the host plant [Kuzma, 2002].

Indicators of unexpected expression of known or novel substances

Past experience with the process used in commercializing crops modified by conventional breeding methods has generally resulted in very few documented cases of unacceptably high levels of known toxicants in modified foods. It has been suggested that foods derived from conventionally bred crops could provide a reference point by which the safety of crops modified

by rDNA techniques can be compared. Comparisons can be made at the molecular, metabolic, and phenotypic/morphologic levels. The level on which emphasis is placed can vary, depending on the method of genetic modification. For example, molecular characterization methods typically used in bioengineered crops are generally not amenable to assessing changes in plants modified by non-rDNA conventional methods. Therefore, identifying differences in expressed proteins and/or metabolites assumes greater weight in crops modified using conventional methods than characterizing changes at the molecular level.

Ideally, appropriate controls for comparison should include an unmodified counterpart (of isogenic or comparable genetic background) grown and harvested under the same environmental and agronomic conditions. Challenges exist in applying the comparative approach [NRC, 2004a; Wilson A. et al., 2004]. While its limitations are recognized, the comparative approach has remained the primary paradigm in assessing the potential health risks of genetically engineered crops [Kuiper et al., 2002; Kok and Kuiper, 2003; EFSA, 2004; NRC, 2004a]. Moreover, the debate continues whether the current targeted approach to comparative assessment should be replaced by a broad comparative approach. While a targeted approach has the advantage of focusing on the most likely impacts and of using established analytical methodologies, proponents of a broad approach emphasize the need to recognize that genetic alterations can induce nonlinear responses. Therefore, integrated systemic changes in the modified plant cannot be predicted, and can only be addressed using a global, untargeted analytical strategy. The RBCS proposes a tiered approach to determining the scope of analytes in the assessment of unintended effects (see detailed discussion in section 7.3.2, Part B).

As noted in section 3.5, the assessment of phenotypic alterations is an important part of screening in a plant breeding programs regardless of the genetic modification method. Phenotypic stability can be regarded as part of prerequisite information. While phenotypic screening is not a factor in the RBCS, it can be included as part of the justification for the scope of the metabolites to be analyzed. Major unintended effects that affect agronomic performance are routinely eliminated through a process of backcrossing and selection. While this process does not aim at eliminating unintended effects of human health relevance, it is a useful step in reducing the chances that unintended effects with adverse health consequences will be retained in the modified crop developed for commercialization.

Health consequences of instability of the modified trait

Although data to support genetic stability of the modified trait are generally required as part of premarket assessment, the duration of maintenance of genetic stability is uncertain [FIFRA SAP, 2004]. For food safety assessment and risk management, priority will be given to those health-related traits that are intended to reduce toxic or allergenic substances in crops for human consumption. It is also reasonable to expect that an identity preservation system will be in place for GM foods developed for their health benefits. Given commercial incentives, such a system can be operational even in jurisdictions where GM food tracing is not mandatory.

Part C - Potential for allergenic effects

The factors underlying the proposed RBCS are largely based on the 2001 Joint FAO/WHO report and subsequent Codex guidelines. Judgement of the potential for allergenic activity from the protein substance introduced or modified in a GM crop is based on the following factors.

(1) Allergenicity of the gene source

Characteristics of the protein substance

(2) Potential to cross react with known allergens

(3) Stability in pepsin digestion

(4) Secondary factors

- Structural/functional characteristics
- Heat stability
- Abundance

(5) Allergenicity of the host plant

The following section provides an overview of the issues relevant to assessing the allergenic potential of GM foods, followed by a brief explanation of the factors included in the RBCS. See section 6.3.1.b for a detailed discussion of the methodological issues related to the assessment of potential allergenic effects of GM foods.

Issues relevant to assessing the allergenic potential of GM foods

An allergenic reaction is an interaction between an individual with a genetic (atopic) background and an allergen that is recognized by an immunoglobulin E (IgE). The specificity and affinity of IgE binding to a given allergen can vary widely due to the genetic heterogeneity of the population, as well as the nature, extent, and duration of exposure [Bannon et al., 2003]. Given the nature of an allergenic reaction, allergenic substances in foods are primarily protein in nature, although low molecular weight chemicals may be involved in an immune response by covalently binding to proteins to form a hapten-carrier complex [Kroes et al., 2004].

There are two stages in the development of allergic reactions: (1) sensitization, and (2) elicitation of a reaction. Correspondingly, the assessment of potential for allergenic effects in GM foods consists of two elements: (1) assessment of allergenic activity (the propensity of a substance to elicit allergic reactions), and (2) allergenicity (the likelihood of inducing *de novo* sensitization in a nonallergic individual) [Spök et al., 2005].

Alterations in the allergenic potential of GM foods can take several forms [Buchanan, 2001; Wal, 2001; Lehrer and Bannon, 2005], including:

- transfer of known allergens from existing food sources to new food sources,
- introduction of novel protein from sources without a history of exposure that may become allergenic in man,
- increase in the levels of endogenous allergens of an existing allergenic food source, and
- increase in allergenic activity or allergenicity of known allergens or other proteins (due to increased exposure, post-translational modification, changes affecting protein structure such as folding, unmasking previously unavailable epitopes, or changes in the cellular matrix in a new host).

Several studies and reviews have examined the relative importance of these sources of allergenic risks. The following conclusions may be drawn from this body of work.

1) At an aggregate level, the likelihood of selecting and transferring a gene encoding an allergen in transgenic modification can be considered small. This is inferred from the observation that despite the vast number of protein substances in nature, most proteins are not allergens [Haslberger, 2003; Lehrer, 2004; Goodman et al., 2005].

2) The proportion of allergenic proteins from foods is also relatively small. They cluster in few protein families, with four families accounting for more than 65% of plant food allergens [Jenkins et al., 2005]. Of particular significance is a general lack of IgE cross-reactivity observed both within and between the other subfamilies. However, it has not been determined whether this observation also applies to the proportion of protein gene products that are selected for food crop modification [Sathe et al., 2005].

3) Our understanding of how food matrix or adjuvant factors may affect the allergenicity of a protein is limited [Teuber, 2002], despite growing knowledge about the relationship between protein structure and allergenic potential.

4) The likelihood of increasing the levels and reactivity of endogenous allergens of an allergenic host plant may be considered as a function of general unintended effects.

5) With a growing interest in the use of biological agents in pest control and other applications in environmental biotechnology, increased exposure to potential allergens may represent a broader problem that is not limited to GM foods [de Jong et al., 2004]. Processing procedures can also affect food allergenicity in ways that are not entirely predictable [Besler et al., 2001; Wal, 2003].

Goodman and Hefle [2005] concluded that the primary consideration in assessing the allergenic potential of GM foods is to avoid the transfer of any allergen or protein likely to cause a cross-reaction in those already sensitized. A secondary consideration is to avoid introducing a protein with characteristics that are likely to sensitize individuals. A final consideration is whether the allergenic potency of a transformed crop that commonly causes serious food allergies is altered. (This possibility can be examined by comparing the relative allergenicity or abundance of endogenous allergens in the GM crop with the unmodified crop.) The factors included in the RBCS are consistent with these considerations.

Current methodological constraints necessitate that assumptions be made in the present approach to assessing the allergenic potential of novel proteins in GM foods. Two specific assumptions, stemming from our knowledge about two classes of known food allergens ("sensitizing elicitors" and "non-sensitizing elicitors"), are recognized. (1) A protein that has a low likelihood of having allergenic activity is also less likely to induce sensitization. (2) Conversely, a protein that can induce sensitization is likely to have allergenic activity. If it

demonstrates cross-reactivity with another known allergen, it could be predicted by *in silico* methods, even if it is a new protein without a history of prior exposure.

Allergenicity of the gene source

At the present, a protein from an allergenic source would be considered to be highly probable of being allergenic, unless proven otherwise [FAO/WHO, 2001].

Potential to cross-react with known allergens

A major challenge in assessing the allergenic potential of GM foods is the lack of validated methods for testing the allergenicity (the ability to sensitize individuals who have not been previously exposed) of novel proteins. Current approaches focus on assessing the allergenic activity (the propensity to elicit allergic reactions in sensitized individuals) of proteins in GM foods. Identifying the presence of individual epitopes (minimal peptide units within proteins that can be recognized by the immune system) on the protein of interest that shares sequence identity with known allergens remains a principal objective of the assessment of the allergenic potential of a novel protein.

In silico approaches to predicting cross-reactivity with known allergens based on amino acid sequence identity represent a principal tool currently being used in assessing allergenic potential of transgenic proteins. While the predictive value of this approach has not been verified, the methodology continues to evolve [Becker and Reese, 2001; Goodman et al., 2002; Kleter and Peijnenburg, 2002; Brusic et al., 2003; Stadler and Stadler, 2003; Björklund et al., 2005].

Stability in pepsin digestion

The property of digestive stability is not shared by all common major protein allergens. In addition, while the link between food allergen structure, stability to digestion, and IgE binding has been demonstrated, the correlation between protein stability in digestion and sensitization is less well understood [Dearman et al., 2002; Bannon et al., 2003].

While the correlation between resistance to proteolysis and allergenic activity is not absolute and cannot be used alone for defining a potential allergen, the *in vitro* digestibility test confirms that the protein is unstable in the presence of digestive fluids and that it is not unusually persistent in the digestive system. At present, protein stability appears to be the only reliable characteristic

being used to distinguish known food allergens from normal dietary proteins [Helm, 2001]. However, the pepsin digestibility assay is not meant to mimic precisely the fate of protein under *in vivo* conditions [Mendelsohn et al., 2003]. The rationale for using stability in digestion as an indicator of the potential for protein allergenic activity is summarized in section 6.3.1.b.

Secondary factors

Given the overlap of characteristics of allergenic and non-allergenic proteins and the methodological limitations in testing for allergenicity, no single factor or test can fully predict the allergenic potential of any new protein. Judgement based on a combination of factors reflecting a weight of evidence approach is increasingly favoured over a strict sequential decision tree application of testing strategies in the 2001 report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology [FAO/WHO, 2001; Codex Alimentarius, 2003; König et al., 2004; Poulsen, 2004]. Secondary factors are proposed in the scheme. They may add weight to the concern that a protein could be allergenic where the evidence on the above (primary) factors is equivocal, particularly if the uncertainty is related to pepsin digestibility.

Structural/functional characteristics. Post-translational glycosylation, and specifically carbohydrate structures containing an α(1,3)-fucosyl residue and/or β(1,2)-xylose, may act as a cross-reactive carbohydrate determinant capable of binding to IgE from individuals sensitized to unrelated sequences [van Ree et al., 2000]. The role of cross-reactive carbohydrate determinants in IgE recognition versus their ability to elicit allergic reactions may depend on binding affinity and the rest of the protein structure [Goodman et al., 2005; Spök et al., 2005].

There is some overlap between this secondary factor and a primary factor (the potential to cross react with known allergens) outlined previously, in that both factors are based on criteria related to sequence identity. However, there are limitations to identifying potential cross-reactivity of a protein with known allergens based on linear sequence alone. It has been shown that the level of sequence homology is not always correlated with the conservation of allergenicity [Mills et al, 2003]. Low levels of homology can exist with conservation of allergenicity and vice versa.

Heat stability. Many food allergens are heat stable. However, heat stability is not a prerequisite for allergenic proteins. Some allergens (e.g., lipid transfer proteins) are known to be heat labile, including: Bet v 1-related proteins and profilins.

Abundance. Highly allergenic proteins are often expressed at relatively high levels [FAO/WHO, 2001]. However, it is not possible to define a level of expression below which a protein can be considered safe from an allergenicity point of view. As a factor in assessing the allergenic activity of foods produced through agricultural biotechnology, abundance is likely related both to the probability of sensitization and elicitation, although threshold levels for sensitization would be difficult to determine [Taylor and Hefle, 2001; Haslberger, 2003; Goodman et al., 2005].

The criterion relating to the level of protein in a food as a predictor of its allergenic potential should not be confused with the threshold level for sensitization or elicitation. The threshold level is a function of the dose, which depends on the quantity of the food consumed. The duration of exposure or the number of repeated exposures may also play a role in sensitization. It has been suggested that the induction of allergic sensitization in a previously naïve individual almost invariably requires exposure to concentrations of the allergen that are greater than those necessary to elicit a response in a sensitized subject [IPCS, 1999].

Allergenicity of the host plant

For the host plant that is an allergenic source, unintended changes in the level or potency of endogenous allergenic proteins would increase concerns about health risk. Practical considerations for testing may restrict the application of this factor to common sources of allergen, as further testing, typically involving serum screening, requires the use of sera from patients known to be sensitized to the protein or the food source of a sufficient sample size [FAO/WHO, 2001]. The health impact of an unintended change in an allergenic protein in a known source of food allergen may be relatively small, as the offending food would likely be avoided by individuals who have been positively diagnosed. It is less clear if and to what extent these changes would sensitize or elicit reactions in previously clinically asymptomatic individuals. If this were to occur, there would be a higher likelihood that more people would be affected by common allergens than by less common allergens. Given the practical limitations in testing, higher priority should be given to assessing unintended changes in allergenic proteins in common allergenic foods.

4.3.4. Criteria for differentiating health risk potential of GM foods

In this section, common elements underlying the criteria used in differentiating GM foods in the RBCS are outlined. The specific criteria are listed in **Tables 4-1A, 4-1B, and 4-1C**. For detailed explanations and interpretation of the criteria that are useful for applying the scheme to specific GM foods, refer to the combined RBCS-product rating form used in the concordance study as part of the evaluation of the RBCS (discussed in Chapter 9). The rating form is included in Appendix F (Volume II).

a) Types of criteria

Three types of criteria may be distinguished in the RBCS on the basis of the level of evidence and judgement required or data available in deciding whether a criterion for a specific factor is met for a GM food product.

- Data-based criteria requiring little judgement (e.g., whether a gene product is expressed or not based on the objective of the genetic modification).
- Data-derived criteria requiring that the data be interpreted using a numeric cut-off value (e.g., above or below amino acid sequence identity of 35%).
- Interpretive criteria requiring considerable expert judgement (e.g., determining the adequacy of the scope of analytes).

Some criteria may be considered provisional and can be updated in the future as new evidence or new testing methods become available. In some cases, where quantitative criteria are expected to minimize subjective interpretation, such criteria were included by this researcher based on the best available information in the absence of generally accepted values (e.g., criteria for factor C.4.3 - abundance in Table 4-1C). The types and strength of evidence that can be used to determine whether criteria are met or not also vary. They range from (1) product-specific testing, (2) generally accepted knowledge, (3) theoretical knowledge or estimates, (4) data based on bioinformatics, to (5) expert judgement.

b) General concepts underlying the criteria

For factors related to both intended and unintended effects, the concepts of familiarity and novelty underlie several criteria for discriminating hazard characteristics or potential (e.g., prior knowledge based on the same or similar substance) and exposure (e.g., history of consumption

from food). The concept of familiarity is based on the fact that most GM crops are developed from plants the biology of which is well understood [European Commission, 2003]. Familiarity can be defined as indirect knowledge or experience obtained from similar gene products, plant varieties, or progenitor varieties grown under similar conditions and used for similar purposes [NRC, 2000a, p. 73]. Familiarity allows the risk assessor to draw on prior knowledge and experience with the introduction of similar crops or similar structural and functional attributes of substances of interest. In contrast, novelty represents the lack of or limited prior knowledge and will necessarily require that an assessment be made on a less certain basis. Consequently, the greater the novelty, the greater will be the likelihood that the level of testing will be increased [Madsen et al., 2002].

For some factors, the criteria vary depending on the nature of the substance being assessed (intended effects) or the method of genetic modification as a function of available analytical capability to characterize changes at the genomic level (unintended changes). This acknowledges the need to tailor the criteria to more appropriately match the characteristics of the substance or the process.

For factors related to unintended changes, it is expected that complexity of the metabolic pathway or network being modified may increase the potential for unexpected effects [Kuiper et al., 2001; SOT, 2003]. Consequently, the level of testing would likely be increased in proportion to the need to reduce uncertainty. However, the increased need for testing does not necessarily mean that a product is more risky.

c) Application of the criteria

The criteria developed are used to rate each factor in the RBCS. All criteria are stated in a manner such that a "yes" rating to a criterion means that the criterion has been met. A low concern level (Level I) for a category of potential health effects requires meeting the criteria for all applicable factors (see section 4.4 - aggregation). Other ratings are also possible: "no" (one or more criteria for a factor are not met), or "uncertain" (information is insufficient or equivocal for rating), except where only a "yes" or "no" rating is acceptable (e.g., whether a gene product is expressed or not). An uncertain rating implies that further testing or assessment is required. An option for "not applicable" is also provided.

4.3.5. Aggregation of factors

An aggregated concern level is assigned separately for each category of health effects based on the ratings for individual factors in each category. The rules for aggregation are summarized in **Table 4-2** (Tables 4-2A, 4-2B, and 4-2C) and the accompanying text. The format presented here is a revised version made in response to comments received in the concordance study (discussed in Chapter 9). Aggregations rules were presented in a different format in the combined RBCS-product rating form used in the concordance study (included in Appendix F, Volume II of the thesis). It should be emphasized that the aggregation rules are not based on likely combinations of factors. Rather, how the factors are aggregated in determining concern levels is determined primarily by scientific considerations, as explained below.

As already noted in previous sections, not all factors in the RBCS are of equal importance in their contribution to the potential for a specific health effect. Therefore, critical and secondary factors may be distinguished in how they are aggregated.

The approach to aggregation is developed with the following considerations in mind.

1) All GM foods should be classifiable.

2) The assignment of concern levels should be mutually exclusive. This means that a GM food should be assigned into only level for each category of health effects.

The extent to which the above two objectives were achieved are discussed in Chapter 9 in relation to the range of characteristics in a reference panel of GM foods, coverage of concern levels, and discriminating ability of the scheme (sections 9.3.1, 9.5.2, 9.5.3, and 9.6).

3) Not all factors are of equal importance or certainty in their contribution to the level of concern. Qualitative weighting may be applied without using arbitrary numeric weighting values.

One example of the application of this principle is the weight placed on the need to justify the adequacy of the scope of metabolites analyzed in assessing the potential for unintended changes (factor B.5.3) when the criteria for one or more secondary factors in Part B are not met. More weight is placed on assessing metabolic changes (factor B.5) compared to the secondary factors

(B.3) because of the less certain nature of the contribution of the secondary factors to the potential for unintended adverse health effects. More importantly, changes in the levels of metabolites directly reflect changes in components in foods that are eaten by consumers and thus reflect what really matters with respect to metabolic products [FIFRA SAP, 2004]. Another example is how secondary factors (C.4) are considered in assessing the potential for allergenic effects. In this case, secondary factors in Part C are triggered when the criteria for pepsin digestibility (C.3) is not met. Additional factors are considered in this case because of the limitations of the pepsin digestibility assay, as discussed in section 4.3.3, Part C.

4) In applying the aggregation rules, there should be flexibility in more appropriately matching the characteristics of the product and the availability of analytical methods for the factors for which specific criteria should be met.

This consideration can be illustrated by giving more weight to the need to justify the adequacy of the scope of metabolites/parameters analyzed than on the need to meet the criteria for molecular characterization in the case of foods derived from crops modified using non-nucleic acid based techniques. This recognizes differences in available methods for molecular characterization between characterized and poorly characterized gene modifications. There are also differences of opinion about the value of complete DNA sequencing, including junction regions, in different regulatory jurisdictions [FDA Food Advisory Committee, 2003; EFSA, 2004; ILSI, 2004].

5) The concern levels determined using the RBCS should reflect a gradation in the nature and extent of assessment and testing on GM foods. This means that assessment and testing of GM foods in the lower concern levels should generally be less than those in higher concern levels.

Briefly, there are three concern levels for Part A, and two concern levels for each of Parts B and C. Concern Level I in all three parts of the RBCS is assigned only when the criteria for all applicable critical factors are met (i.e., receiving a "yes" rating"). For Part A, failure to meet the criteria for only one critical factor would lead to Level II; failure to meet the criteria for more than one critical factor, or any of the "other" factors, would lead to Level III. This aggregation pattern takes into account the extent and nature of further assessment and testing associated with each concern level. For Part B, failure to meet the criteria for any one critical factor would

result in Level II. One critical factor (B.5.3 – other metabolites/parameters) is triggered if the criteria for any one of the secondary factors are not met. For Part C, failure to meet the criteria for one critical factor (C.3 – pepsin digestion) will trigger the need to meet the criteria for all secondary factors.

In the next chapter, an overview of the conceptual framework of the scheme and product characteristics represented by the different concern levels in the scheme are presented, along with a discussion of the strengths and limitations of the proposed scheme.

The implications of the proposed RBCS for testing GM foods are discussed in Chapter 7. A case example is presented in Chapter 8.

Table 4-1. Factors and criteria in the RBCS for determining concern levels.

Types of evidence
1: Product-specific testing
2: Generally accepted knowledge
3: Theoretical knowledge or estimates
4: Data based on bioinformatics
5: Expert judgement

Note: For a detailed explanation of the factors and criteria outlined in this table, please refer to the Combined RBCS-Product Rating Form included in Appendix F in Volume II of the thesis.

PART A - Potential for toxic and antinutritional effects from intended changes resulting from the primary and secondary gene products introduced/modified. Applicable to both protein and non-protein substances, unless stated otherwise.

Factors	Criteria	Type of Evidence
Dietary Exposure to Gene Products		
Factor A.1. Expression of gene product(s) in edible part(s) of the modified crop.	A.1.1. Substances with NO nutritional role *and* NOT expressed in the modified crop due to intentional gene silencing, *or*	1, 3
	Non-protein substances only A.1.2. Total dietary intake of gene product(s) is below the threshold of toxicological concern for the chemical class where applicable, *or*	3
	A.1.3. Gene products NOT expressed, detected or changed in the edible part(s) of the modified crop a/ tissue-specific expression NOT involving the edible part(s) of the modified crop, *or* b/ the edible part is used as a food ingredient in a limited form as a highly refined and well-defined chemical entity (e.g., refined oil from oilseed rape and cottonseed by hot solvent extraction).	1, 3 1, 2
Factor A.2. Prior history of consumption from foods.	a/ Naturally occurring food constituents, *or* b/ Other substances that have a history of consumption from foods without reported adverse health effects.	2
Factor A.3. Expected change in total dietary exposure (based on theoretical estimates).	***Substances with known toxicity concerns*** a/ Total dietary intake below established upper limit (ADI, tolerance level, or equivalent) with an adequate margin of exposure. ***Other substances*** b/ Negligible change or changes within normal variation in dietary intakes.	2 3
Nature of Gene Products		
Factor A.4. Prior knowledge of safety/mode of action.		
Factor A.4.1. Food constituents and substances with a prior dietary of consumption from foods (with dietary history).	***Substances with known toxicity concerns*** a/ Established upper limit (ADI, tolerance level, or equivalent).	2

Factors	Criteria	Type of Evidence
	Other substances b/ Well-established biological function or specificity of mode of action with NO potential for mammalian toxicity, antinutritional, and adverse metabolic effects under normal conditions of consumption, *or* c/ Limited empirically based toxicity profile *and* simple chemical structure or predictable metabolism suggestive of low order of oral toxicity.	2 2
Factor A.4.2. Substantially modified or novel substances (without dietary history).	***Pesticidal and enzymatic proteins*** a/ NO structural or functional similarity to known proteins with toxic or antinutritional properties relevant to mammals *and* at low expression level. ***Non-protein (low molecular weight) substances*** *b/* WITHOUT structural similarity to known mutagen or carcinogen *and* < 0.5 ppm in the total diet. ***Other protein and non-protein complex substances*** *c/* WITHOUT structural similarity to known mutagen or carcinogen *and* < 0.1% diet.	1, 4 1, 3
Factor A.5. Structure-activity characterization.	***Proteins*** *a/* NO amino acid sequence identity when compared with known mammalian toxins, rapid and complete pepsin digestion using standard testing conditions, *and* structurally similar and functionally identical to comparator/source protein. ***Non-proteins*** *b/* Structurally identical to natural food constituents.	1, 4 1, 3
Factor A.6. Digestion and degradation products.	In edible parts of modified crop. a/ NO potentially toxic metabolites expected from catabolism of an introduced/modified substance or of an exogenous chemical (e.g., detoxification of a herbicide or mycotoxin), *and* In mammalian metabolism from consumption of the gene product. b/ NO products of digestion or bioactivation at levels of immunological, pharmacological, or toxicological significance.	2, 3 2, 3
Factor A.7. Synergism and interactions.		
Factor A.7.1. Between gene products introduced/modified.	a/ NO synergistic toxic effects expected.	2, 3
Factor A.7.2. Between gene products introduced/modified and food constituents.	a/ NO negative effect on bioavailability of food constituents expected.	2, 3
Factor A.8. Prior experience with gene products introduced/modified.	a/ Comparable in expression levels, gene source, transfer method, and host plant, *and* b/ No expected increase in total dietary exposure and approved by at least one national competent authority.	1, 3

PART B - Potential for toxic and antinutritional effects from unintended changes in the modified food from genetic modification of the host plant. Applicable to the food derived from the modified crop.

Factors	Criteria	Type of Evidence
Factor B.1. Safety of donor organism (gene source).	a/ NOT a source of known toxicant, antinutrient, or pathogenicity/infectivity.	2
Factor B.1.1. Characterized gene modification.	a/ Absence of transfer of undesirable characteristics to the host plant is supported by experimental evidence.	1
Factor B.1.2. Poorly characterized gene modification.	a/ Genetically related organisms (in the same genus) are NOT sources of known toxicant or antinutrient, *or* b/ Extent of backcrossing is sufficient to remove large percentage of genetic material from the donor organism.	2 1, 3
Factor B.2. Safety of host plant.	a/ NOT a source of known/significant toxicant or antinutrient.	2
Factor B.3. Potential for novel/altered patterns of metabolites.		
Factor B.3.1. Knowledge of gene product function.	a/ Protein gene product(s) have well-characterized function in the modified plant.	1, 4
Factor B.3.2. Impact of gene product function in the modified plant.	a/ Expression product of the introduced/modified gene does NOT have a metabolic, regulatory, or signalling function in the modified plant, *or* b/ Expression of the introduced/modified gene is NOT expected to have a systemic effect on the metabolism of the modified plant.	2, 3 2, 3
Factor B.3.3. Substrate specificity of gene product.	a/ Narrow substrate specificity (i.e., unlikely to interact with endogenous substrates to form novel compounds).	1, 3
Factor B.4. Potential for disrupting coding or regulatory DNA sequences in the modified plant.		
Factor B.4.1. In the inserted/modified DNA.	a/ NO novel open reading frames (ORFs), disruptions of existing ORFs, or functional expression.	1, 4
Factor B.4.2. In genomic sequences bordering the site of insertion/modification.	a/ NO novel open reading frames (ORFs), disruptions of existing ORFs, or functional expression.	1, 4
Factor B.5. Chemical analysis of food derived from the modified plant.		
Factor B.5.1. Key nutrients.	a/ Within acceptable ranges of natural variation, *or* b/ NOT expected to have adverse nutritional, metabolic, or toxicological effects.	1, 5
Factor B.5.2. Known toxicants/ antinutrients.	a/ Within acceptable ranges of natural variation, *or* b/ NOT expected to have adverse nutritional, metabolic, or toxicological effects.	1, 5
Factor B.5.3. Other metabolites/ parameters	a/ Reasonably justified to be sufficient in scope, *and* b/ NO unknown compounds or significantly altered	1, 5

Factors	Criteria	Type of Evidence
	levels of endogenous metabolites outside the range of natural variation, *or* c/ If changes occur, the likelihood of negative health effects can reasonably be justified to be low.	
Factor B.6. Health consequences of instability of the modified trait.	Applicable if the modified trait is intended to provide nutrition or health benefits. a/ Adequate quality control should in place to ensure consistency in lot-to-lot expression of the targeted gene product.	3

PART C - Potential for allergenic (IgE-mediated) effects from intended and unintended changes. Applicable to protein substances.

Factors	Criteria	Type of Evidence
Factor C.1. Allergenicity of donor organism (gene source).	a/ NOT a known source of allergenicity or gluten-sensitive enteropathy.	2
Protein gene products (factors C.2-C.4).		
Factor C.2. Potential for protein gene product(s) to cross react with known allergens.	a/ Comparison of amino acid sequence identity with known allergens: NO sequence identity of ≥35% over 80 or more amino acids based on a sequential search	4
Factor C.3. Pepsin digestion (under standard testing conditions).	***Novel or substantially modified proteins*** (without prior dietary history) a/ Rapid and complete digestion with NO stable fragments (< 10 min*). ***Other proteins*** (with prior dietary history) b/ Rapid or moderate digestion with NO stable fragments (< 15 min*).	1, 5
Factor C.4. Secondary factors.		
Factor C.4.1. Structural/functional characteristics.	a/ NO post-translational modification involving glycosylation with primarily N-linked, specific xylose or fucose substitutions, *or* b/ NO structural or functional relationship to specific protein families or functional categories commonly associated with allergenicity.	1, 4
Factor C.4.2. Heat stability.	a/ Heat labile (NOT applicable to lipid transfer proteins).	1, 2
Factor C.4.3. Abundance.	***Pesticidal and enzymatic proteins*** a/ < 0.1% protein. ***Other proteins*** b/ < 1% total protein.	1, 5
Factor C.5. Allergenicity of host plant.	a/ NOT a common source of allergenicity or gluten-sensitive enteropathy, *or* ***Host plant is a common source of allergenicity or gluten-sensitive enteropathy*** b/ Rating for factors B.5.1, B.5.2, *and* B.5.3 is "Yes" or "Not applicable".	2 5

* Listed here are provisional cut-off values for concordance study only. Guidelines for interpreting digestion assay results remain to be established [Bannon et al., 2003].

Table 4-2. Aggregation of factors in determining concern levels.

PART A - Primary and secondary gene products with the potential for toxic or antinutritional effects from intended changes

Level I - Substances with prior dietary history, negligible exposure, or prior experience

Ia - Substances with a prior dietary history

Ib - Substances with negligible dietary exposure

Ic - Substances produced by well-characterized GM process based on prior experience

Level II - Substances with limited and defined concerns with respect to either expected exposure or toxicity profile

IIa - Substances with a prior dietary history

IIb - Substances with a questionable dietary history (dietary substances with modified structures)

IIc - Substances without a dietary history (novel substances with limited/defined structural concerns)

Level III - Substances not meeting criteria for Level I or II

Substances with or without a dietary history having concerns with both expected exposure, toxicity profile, or other factors related to metabolism or interactions, or any combination of more than one of the factors.

Factors	Aggregation of critical factors						
Levels	**Ia**	**Ib**	**Ic**	**IIa**	**IIb**	**IIc**	**III**
A.1. Expression of gene product(s) in edible part(s) of the modified crop		M					
A.2. Prior history of consumption from foods	M			M	M/?	NM	NM-1
A.3. Expected change in total dietary exposure (theoretical estimates)	M			?[a]	M		NM-1
A.4. Prior knowledge of safety/mode of action	M			?[a]	M	M	NM-1
A.5. Structure-activity characterization	M			?[b]	?/NM	?/NM	NM-1
A.6. Digestion and degradation products	M			M	M	M	NM
A.7. Synergism and interactions	M			M	M	M	NM
A.8. Prior experience with gene products introduced/modified			M				

M: Criteria for the factors indicated must be met, or not applicable.
?: Limited or equivocal information.
NM: Criteria are not met for any of the factors indicated.
?[a]: Limited or equivocal information on only one of the factors indicated (A.3, A.4, or A.5).
?[b]: For factor A.5, uncertainty is limited to status of pepsin digestion test only.
NM-1: Criteria are not met for more than one of the factors indicated.

PART B - GM foods with the potential for toxic or antinutritional effects from unintended changes

Level I - GM foods that meet baseline requirements for testing for unintended effects

Ia - GM foods derived from crop transformed by characterized gene modification (based on nucleic acid techniques)

Ib- GM foods derived from crop transformed by poorly characterized gene modification (based on undirected mutagenesis, somaclonal variation, or wide-cross hybridization)

Ic - 3 subgroups are included:
- GM foods derived from crops transformed using tissue-specific modification not involving the edible parts of the modified crop,
- GM foods with limited ingredient use of the edible part and the ingredient is highly refined,
- GM foods derived from crops transformed by genetic modification process based on prior experience

Level II - GM foods that require further assessment beyond baseline testing for unintended effects

Factors	**Aggregation of critical factors**			
Levels	**Ia**	**Ib**	**Ic**	**II**
B.1. Safety of donor organism *Applicable if donor organism and host plant are different.*	M	M		NM
B.1.1. Characterized gene modification	M†			
B.1.2. Poorly characterized gene modification	M†			
B.2. Safety of host plant				
B.3. Potential for novel/altered patterns of metabolites				
B.4. Potential for disrupting coding or regulatory DNA sequences in modified plant				
B.4.1. In the inserted/modified DNA	M			
B.4.2. In genomic sequences bordering site of insertion/modification				
B.5. Biochemical analysis of edible part of modified crop				
B.5.1. Key nutrients	M	M		NM
B.5.2. Known toxicants and antinutrients	M	M		NM
B.5.3. Other metabolites/parameters *Applicable if criteria for factors B.2, B.3, and B.4.2 are not met.*	M¶	M		NM
B.6. Health consequences of instability of the modified trait *Applicable if the modified trait is intended to provide nutrition or health benefits.*	M			NM
Criteria 1.3 of Part A*			M	

*Criteria 1.3 of Part A: Gene products not expressed, detected, or changed in the edible part(s) of the modified crop, including: (a) tissue-specific expression not involving the edible part(s), or (b) the edible part is used as a food ingredient in a limited form as a highly refined and well-defined chemical entity.
M: Criteria for the factors indicated must be met, or not applicable.
M†: Criteria must be met if information for factor B.1 is limited or equivocal.
M¶: Criteria must be met if information for factors B.2, B.3, and B.4.2 is limited or equivocal.
NM: Criteria are not met for any one of the factors indicated.

PART C - Protein gene products and GM foods with the potential for allergenic effects from intended and unintended changes

Level I - Intended protein gene products and GM foods that meet baseline requirements for testing for allergenic activity

Ia - Expression in edible parts

Ib - Negligible expression in edible parts and in derived food ingredient

Level II - Protein gene products and GM foods that require further assessment beyond baseline testing for allergenic activity

IIa – Characterized protein and gene modification

IIb – Uncharacterized protein and gene modification (e.g., somatic hybrid)

Factors	**Aggregation of critical factors**			
Levels	**Ia**	**Ib**	**IIa**	**IIb§**
C.1. Allergenicity of donor organism *Applicable where donor organism and host plant are different.*	M		NM	NM
C.2. Potential for protein gene product to cross-react with known allergens	M		NM	
C.3. Pepsin digestion	M			
C.4. Secondary factors *Applicable if criteria for factor C.3 are not met.*				
C.4.1. Structural/functional characterization	M†		NM-1	
C.4.2. Heat stability	M†		NM-1	
C.4.3. Abundance	M†		NM-1	
C.5. Allergenicity of host plant	M		NM	NM
Criteria 1.3 of Part A*		M		

*Criteria 1.3 of Part A: Gene products not expressed, detected, or changed in the edible part(s) of the modified crop, including: (a) tissue-specific expression not involving the edible part(s), or (b) the edible part is used as a food ingredient in a limited form as a highly refined and well-defined chemical entity.
§ Information on protein expression products may be limited in poorly characterized gene modification.
M: Criteria for the factors indicated must be met, or not applicable.
M†: Criteria must be met if information for factor C.3 is limited or equivocal.
NM: Criteria are not met for more than one of the factors indicated.
NM-1: Criteria are not met for any one of the factors listed if factor C.4 is applicable. Factor C.4 is applicable when information for factor C.3 is limited or equivocal.

CHAPTER 5 – PROPOSED RISK-BASED CLASSIFICATION SCHEME

The previous chapter explains in detail how the proposed RBCS was developed, including the scientific rationale for the factors and associated criteria, and their aggregation into concern levels. In this chapter, the proposed RBCS is presented at a broad conceptual level. A description of the characteristics of products that fall into the different concern levels will illustrate the practical implications of the concern levels in the RBCS. The chapter concludes with a comparison of this scheme with several risk-oriented classification systems and a discussion of their strengths and limitations.

It is recognized that more detailed explanation and interpretation of the factors and criteria is needed in a formal classification process. A version of such a guide was developed for the concordance study (see Combined RBCS-Product Rating Form included in Appendix F in Volume II of the thesis.

5.1. Overview of conceptual framework

The RBCS comprises a total of seven concern levels in three categories of potential health effects, with 2-3 levels in each of the three categories. The three categories of health effects and the respective concern levels are as follows:

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - intended changes resulting from the primary and secondary gene products introduced/modified (3 levels).

Level I. Dietary substances (protein and non-protein) that have a history of known or presumed safe consumption from foods and have insignificant changes with respect to expected exposure. They can also be gene products of well-characterized modification using an established transformation procedure.

Level II. Substances that have limited and defined concerns with respect to *either* their expected exposure *or* their toxicity profile.

Level III. Substances that do not meet the criteria for level I or II.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - unintended changes in the modified food from genetic modification of the host plant (2 levels).

Level I. GM foods that meet baseline requirements for testing for unintended effects.

Level II. GM foods that require further assessment beyond baseline testing for unintended effects.

PART C - POTENTIAL FOR ALLERGENIC EFFECTS (2 levels).

Level I. Intended protein gene products and GM foods that meet baseline requirements for testing for allergenic activity.

Level II. Intended protein gene products and GM foods that require further assessment beyond baseline testing for allergenic activity.

5.2. Product characteristics by concern levels in the proposed RBCS

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - intended changes resulting from the primary and secondary gene products introduced/modified.

The factors and criteria in Part A of the RBCS are applicable to both protein and non-protein substances, unless stated otherwise.

Level I. Dietary substances that have a history of known or presumed safe consumption from foods and have insignificant changes with respect to expected exposure.

Three subcategories of substances in Level I are distinguished based on dietary exposure and prior experience with the genetic modification protocol.

[Ia] A substance in Level I must meet all applicable criteria for the following six factors.

(1) The substance has a prior history of known or presumed safe consumption from foods.

(2) The change in total dietary exposure is expected to be within normal variation in dietary intakes. For a substance with known toxicity concern, expected total exposure will not exceed established upper limit of intake.

(3) There is either an established upper limit for intake (for a substance with known toxicity concern), or well-established biological function or specificity of mode of action with no potential for mammalian toxicity, antinutritional, and adverse metabolic effects under normal conditions of consumption (for other substances), taking into consideration comparability in the methods of food processing/preparation between the food derived from the modified crop and that from the gene source.

(4) For a substance with a prior history of known or presumed safe consumption from foods, the counterpart produced in the modified plant must be demonstrated to be structurally similar to the naturally occurring comparator/reference substance on which prior knowledge about safety is based. For a protein substance, functional equivalence should also be demonstrated.

(5) No unusual or novel metabolites are expected from proteolytic or catabolic degradation of the gene product(s) in the edible part(s) of the modified plant and from the digestion and metabolism of the substance following ingestion of the food.

(6) There are no synergistic toxic effects between gene products and no interactions between gene product(s) and food constituents that may negatively affect bioavailability.

Level I includes two additional special categories of foods derived from GM crops.

[Ib] Certain types of gene modification can result in negligible dietary exposure to the intended gene products. This can be the result of intentional gene silencing, tissue-specific expression not affecting the edible part of the modified crop, or the edible part is used as a food ingredient in a limited form as a highly refined and well-defined chemical entity. This special category does not apply to substances with a nutritional role. Analytical confirmation of the absence of gene

product(s) in the food is required. A substance at a level below the threshold of toxicological concern will also meet the criteria for Level I.

[Ic] The gene modification is well-characterized and is based on prior experience with respect to gene products introduced/modified, expression levels, gene source, transfer methods, and the host plant. Its use has been approved by at least one national competent authority. Replication of the same gene modification does not lead to appreciable increase in dietary exposure.

GM food risk assessment will focus on the edible part of a crop plant consumed by humans. It is recognized that some food crops have very limited food use for human consumption. If their human food use is restricted only to the production of highly refined ingredients of well-defined chemical composition (e.g., the only food use of oilseed rape is the production of oil by extraction using a highly refined process), verification of the absence of an intended primary gene product without a nutritional role in the extracted fractions should be sufficient testing for a crop with a limited food use. Currently available standards for the testing and assessment of food ingredients will apply. Similarly, in tissue-specific expression of introduced/modified genes that do not affect the edible parts of a food crop, verification of the absence of intended gene products in the relevant plant parts should be sufficient. In these two specific cases, the application of the RBCS in its entirety would not be necessary.

In the case of intentional silencing, unintended effects will need to be assessed, as well as intended changes in any secondary gene products associated with the modification.

Level II. Substances that have limited and defined concerns with respect to *either* expected exposure *or* toxicity profile.

Three subcategories of Level II substances may be distinguished based on dietary history.

[IIa] A substance with a dietary history is defined as a naturally occurring food constituent that has a history of consumption from foods without reported adverse health effects.

A naturally occurring food constituent with insufficient or equivocal information on *only one* of the following three categories of characteristics (without other concerns regarding digestion and degradation products or adverse synergistic and interactive effects with other gene products or food constituents) may be considered as Level II:

- expected change in total dietary exposure;
- prior knowledge of safety or mode of action; or
- pepsin digestibility (for protein substances only).

This means that a naturally occurring food constituent with only one of the following characteristics may be classified as Level II if the only uncertainty is with limited data on expected changes in dietary exposure.

a/ Low molecular weight chemicals with known toxicity profile and established upper limit for intake.

b/ Low molecular weight chemicals with limited empirically based toxicity data but no identifiable characteristic for concern.

c/ High molecular weight chemicals (complex substance consumed at macro-levels) with no potential health concerns related, but not limited to, alterations in digestibility and/or metabolic effects under typical conditions of consumption.

On the other hand, if the information on these characteristics of a naturally occurring food constituent is limited or uncertain, the information on expected changes in exposure should be considered sufficient in order to assign a concern Level II. In addition, a protein substance derived from a food source with equivocal information with respect to resistance to proteolysis without other apparent concerns about safety or expected changes in exposure can also be classified as Level II.

[IIb] A substance with a questionable dietary history is a food constituent that has been substantially modified. If the introduction of such a substance to a modified food results in negligible change in or very low expected dietary exposure, it may be classified as Level II if it has one of the following characteristics.

a/ For a protein substance: does not have structural-functional similarity to proteins that have known toxic or antinutritional properties.

b/ For a non-protein (low molecular weight) substance: has simple chemical structure suggestive of a low order of oral toxicity, or physical-chemical properties identical to the native/reference substance with low toxicity concerns.

c/ For other protein and non-protein complex (high molecular weight) substances: has no structural similarity to known mutagen or carcinogen.

[IIc] A novel substance without a dietary history may also be classified as Level II if it has exposure level and other characteristics similar to those for subcategory [IIb] described above.

Level III. Substances that do not meet the criteria for level I or II.

A substance that does not meet the criteria for Levels I or II will be classified as Level III for intended changes with a potential for toxic and antinutritional effects.

This means that a naturally occurring food constituent or a substantially modified or novel substance may have *one or more* of the following characteristics.

(1) The substance has more than one of the characteristics described for Level II.

(2) Ingestion exceeds an established upper intake level, if known, based on theoretical estimates (necessitating more detailed or probabilistic modelling to estimate expected intakes or discontinuation of product development).

(3) The substance does not meet one or more of the criteria regarding hazard characteristics: safety or mode of action, structure-activity characterization.

(4) The substance does not meet criteria regarding digestion and degradation products or adverse synergistic and interactive effects with other gene products or food constituents.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - unintended changes in the modified food from genetic modification of the host plant.

The factors and criteria in Part B of the RBCS are applicable to the food derived from the modified crop.

Level I. GM foods that meet baseline requirements for testing for unintended effects.

To be classified as Level I, a GM food will meet applicable criteria in four groupings comprising a total of six factors. The four groupings of criteria are:

(1) genetic sources (the safety of the donor organism, and the host plant);

(2) factors affecting the expression of unexpected substances: potential for novel/altered patterns of metabolites, and for disrupting coding or regulatory DNA sequences in the modified plant;

(3) indicators of unexpected expression of known or novel substances: key nutrients, known toxicants and antinutrients, and other metabolites and parameters; and

(4) health consequences of instability of the modified trait.

Applicable criteria vary between two subcategories of GM products:

(a) characterized gene modification based on nucleic acid techniques, and

(b) poorly characterized gene modification including undirected mutagenesis, somaclonal variation, or wide-cross hybridization.

(1) For both characterized and poorly characterized gene modifications, the safety of genetic sources must be demonstrated by prior knowledge or in combination with empirical data

- for crops modified by rDNA techniques: evidence of transfer of only the target gene
- for crops modified by conventional techniques: evidence of a greater level of genomic contribution from the host plant with an established history as a food crop in proportion to concerns about the safety of the donor organism

(2) With respect to the potential for novel/altered patterns of metabolites and for disruption of coding or regulatory DNA sequences in the host plant, a judgement of low likelihood can made

based on a combination of prior knowledge and experimental data on biochemical and molecular characterization.

a/ Prior knowledge and biochemical characterization will demonstrate

i/ the function of the protein gene product(s) in the modified plant;

ii/ that the gene product has no metabolic, regulatory, or signalling function in the host plant; and

iii/ low substrate specificity if the protein has an enzymatic function.

b/ Molecular characterization will demonstrate the absence of open reading frames (potentially active genes with an unknown function) and their functional expression

i/ in the inserted/modified DNA; and

ii/ preferably, also in the sequences bordering the site of insertion/modification.

The relative weight placed on molecular characterization can vary depending on the nature of the genetic modification. It is recognized that molecular characterization methods typically used in bioengineered crops are generally not amenable to assessing genomic changes in plants modified by conventional non rDNA techniques, for which alternative analyses at the level of metabolites are emphasized. Even for bioengineered crops, comparative sequence analysis may not always be possible due to limited genomic databases for the crop species in question at the present [EFSA, 2004].

(3) With respect to indicators of unexpected expression of known or novel substances, for both characterized and poorly characterized gene modifications, chemical analysis of the food derived from the modified plant must demonstrate that

a/ the levels of key nutrients and known toxicants/antinutrients are within acceptable ranges of natural variation, or are within limits that are unlikely to have adverse nutritional, metabolic, or toxicological effects.

b/ In addition, if the information on other factors described in (2) is limited or missing, the scope of nutrients, toxicants and antinutrients analyzed in (3a) and any additional metabolites/parameters analyzed should be reasonably justified to be adequate and should demonstrate that

- no unknown compounds or significantly altered levels of endogenous metabolites are outside the range of natural variation, or
- if changes occur, the likelihood of negative health effects can be justified to be low.

For characterized gene modification with limited information on the potential for interruption in genomic sequences bordering the site of insertion/modification, and for poorly characterized gene modifications, satisfying information requirement (3b) is also essential.

(4) Health consequences of instability of the modified trait.

Where the modified trait is intended to provide nutrition or health benefits for humans, adequate quality control is in place to ensure consistency in lot-to-lot expression of the targeted gene product. This requirement is applicable irrespective of the method of genetic modification.

Two special cases in Part A can also be classified as Level I for Part B. They are modifications where

a) gene products are *not* expressed, detected or changed in the edible part(s) of the modified crop achieved by either one of:

- tissue-specific expression not involving the edible part(s) of the modified crop, or
- the edible part being used as a food ingredient in a limited form as a highly refined and well-defined chemical entity; or

b) there is prior experience with the gene products introduced or modified with respect to expression levels, gene source, host plant, and gene transfer method. The lack of an appreciable change in total dietary exposure and prior approval by at least one national competent authority are demonstrated.

For GM foods in both of these cases, they should satisfy requirements discussed earlier in Part A.

Level II. GM foods that require further assessment beyond baseline testing for unintended effects.

A GM food that fails to meet any of the applicable criteria for Level I will be classified as Level II for further assessment/testing with respect to the potential for unintended changes with toxic and antinutritional effects.

This means that a food derived from a crop modified by rDNA technique in Level II may have *one or more* of the following characteristics.

(1) The transfer of uncharacterized genetic material from the gene source cannot be ruled out, where knowledge about its safety is limited.

(2) The presence or expression of open reading frames in the inserted/modified sequences cannot be ruled out.

(3) The levels of endogenous nutrients, toxic or antinutritional substances being outside the range of natural variation and their potential for adverse human health effects are uncertain.

(4) The adequacy of the scope of metabolites/parameters analyzed is not justified, or the health relevance of altered levels of analytes is uncertain, where one or more of the following characteristics are present.

a/ The host plant is a source of known or significant toxicant or antinutrient associated with adverse health effects in humans or animals, i.e., the substance of concern is present with narrow margins of exposure. (Margin of exposure is the ratio of the no-observed-adverse-effect-level to the estimated exposure dose.)

b/ There is uncertainty about the function of the gene product, its likely metabolic impact in the modified plant, or about substrate specificity if the protein has an enzymatic function.

c/ The potential exists for open reading frames (unknown DNA sequences) in the regions bordering the site of insertion/modification to be expressed or functional.

(5) For modified traits that are intentionally introduced to provide nutrition or health benefits for humans, quality control measures to ensure consistency in the level of compounds introduced/modified are limited. This is of particular concern where the intended health trait is related to the reduction of a toxic or allergenic substance.

The above characteristics (1, 3, 4, 5) for GM foods in Level II derived from crops transformed with well-characterized gene modification also apply to GM foods derived from crop modified by poorly characterized gene modification, achieved using conventional techniques. For the latter group of GM foods, information is likely to be limited for characteristic (2, 4c), and possibly also 4b. In addition, where the safety of the donor plant is uncertain, Level II is triggered when evidence may be considered equivocal whether sufficient genetic material from the donor organism has been removed by backcrossing.

PART C - POTENTIAL FOR ALLERGENIC EFFECTS

The factors and criteria in Part C are applicable to protein substances introduced or altered in GM foods, as well as GM foods containing common allergens.

Level I. Intended protein gene products and GM foods that meet baseline requirements for testing for allergenic activity.

A protein substance (including the GM food) is classified as Level I when the criteria for all of the following factors are met.

(1) The donor organism (gene source) is not a known source of allergenicity or gluten-sensitive enteropathy.

(2) The likelihood of the protein gene product to cross-react with known allergens is low, as determined by recommended criteria for amino acid sequence comparisons with known allergens.

(3) The protein is completely degraded within a specified duration in pepsin digestion under standard testing conditions.

Where information on pepsin digestibility is equivocal for eligibility for Level I, criteria for the following additional factors will be met.

a/ The protein does not have structural/functional characteristics that are similar to specific categories in certain protein families that have a high proportion of allergenic proteins, or are suggestive of allergenic property (e.g., specific post-translation glycosylation patterns for heterologously expressed proteins).

b/ The protein is not heat stable, except for lipid transfer proteins that are generally heat labile.

c/ The protein is present in the food at a low level (< 0.1% protein for pesticidal and enzymatic proteins; < 1% protein for other types of protein).

(4) The food is derived from a modified plant that is not a common source of allergenicity or gluten-sensitive enteropathy. If the food is derived from a common allergenic source, it should have characteristics corresponding to having a low potential for unintended changes (based on assessment in Part B).

Two special cases in Part A can also be classified as Level I for Part C (see description for Level I, Part A).

Level II. Intended protein gene products and GM foods that require further assessment beyond baseline testing for allergenic activity.

A GM food containing a protein that fails to meet any of the applicable criteria for Level I will be classified as Level II for further assessment/testing with respect to the potential for allergenic effects.

This means that a protein substance (including the GM food) in Level II may have *one or more* of the following characteristics.

(1) The donor organism (gene source) is a known source of allergenicity or gluten-sensitive enteropathy.

(2) The protein gene product is likely to cross-react with known allergens based on amino acid sequence identity with known allergens.

(3) If the protein is moderately to highly resistant to pepsin digestion, it also has one or more of the following structural-functional characteristics.

a/ The protein has structural/functional characteristics that are similar to specific categories in certain protein families that have a high proportion of allergenic proteins, or are suggestive of allergenic property (e.g., specific post-translation glycosylation patterns for heterologously expressed proteins).

b/ The protein is heat stable (excluding lipid transfer proteins that are generally allergenic and heat labile).

c/ The protein is present in the food at a relatively high level ($\geq$ 0.1% protein for pesticidal and enzymatic proteins; $\geq$ 1% protein for other types of protein).

(4) The food is derived from a modified plant that is a common source of allergenicity or gluten-sensitive enteropathy and has characteristics corresponding to having a high potential for unintended changes (based on assessment in Part B).

5.3. Discussion

The discussion here will cover the conceptual aspects of the proposed scheme. In Chapter 9, a detailed evaluation of several functional aspects of the proposed RBCS will be presented.

5.3.1. Characteristics of the proposed RBCS

Three components of the proposed RBCS provide the building blocks to determining concern levels as guidance for the testing of GM foods. The three building blocks are: factors, criteria, and aggregation. The building blocks were developed using basic concepts and fundamental

principles of health risk assessment, using current knowledge about the sources of potential hazards in GM foods and the factors influencing their occurrence and levels of exposure. Factors in the scheme are rated individually using explicit criteria. The ratings of individual factors are then integrated according to predetermined aggregation rules in assigning concern levels. Concern levels are determined separately for each of the three categories of potential adverse health effects of relevance to GM foods. Each component of the scheme can be modified independently of the other components. This flexible framework allows revisions to be readily made in the future. Refinements will likely be necessary as genetic modification technology evolves and new factors that may need to be considered emerge. Similarly, as new analytical and other testing methods are developed, the criteria used may need to be adjusted.

The explicit criteria and the types of evidence on which the criteria are based endow the rationale on which concern levels are determined with both clarity and tranparency. The conceptual framework of the RBCS (as described in this chapter) can also permit a rapid assessment of where a specific product may fall within the continuum of concerns relevant to GM foods.

The core factors in the proposed RBCS are generic in nature, based on basic concepts of health risk assessment, and are expected to be applicable to the majority of current and new GM foods, irrespective of uncommon applications or future developments. It is probably unreasonable to expect that a classification scheme such as the one proposed here will be able to anticipate all future possible approaches to genetic modification and their associated intended and unintended effects. In its present form, the proposed RBCS deals with exceptional cases under "other considerations" (further discussed as part of implications for testing in Chapter 7). Exceptional cases include foods derived from crops that are modified by uncommon techniques or by using uncommon source organisms, or that are intended for ingredient use in foods targeted for vulnerable population groups. "Other considerations" ensure that special cases that require more detailed case-by-case assessment are not overlooked, while permitting an efficient application of the scheme for the majority of cases that do not require additional considerations. Further, as noted already, the fundamental framework of the proposed scheme is designed to be of sufficient flexibility so that revisions can be readily made in response to new developments.

5.3.2. Comparison with other classification schemes

a) Aggregation

As noted in section 4.2.2, scoring, matrix, and decision tree approaches are among the common approaches to aggregating information in classification schemes.

The scoring approach has the advantage of simplicity of use and ease of comprehension. This approach can offer flexibility by separating known vs. potential effects and by allowing users to enter a numeric value that more accurately reflects the situation being modelled than that assigned by the classification system [CCME, 1992; Ross and Sumner, 2002]. However, the assignment of numeric values and cut-off scores for different classes may be arbitrary [Finizio et al., 2001]. This approach is suited to aggregating a large number of independent factors with none being a dominant factor.

The matrix approach is suited to a small number of independent factors. Combining two factors at a time, as described by Moutou et al. [2001], is intuitively attractive but the theoretical rationale is unclear. The scaling of qualitative information may be warranted when it is supported by quantitative data [Ross and Sumner, 2002]. However, qualitative systems for the rating of risks may not provide sufficient information to discriminate accurately between quantitatively small risks and quantitatively large risks [Cox et al., 2005].

The decision tree approach is appropriate where there are conditional dominant factors. While a decision tree or flow chart is structured to lead to one of the prescribed classes, it can be complex to follow [e.g., Cramer et al., 1978; The Edmond's Institute, 1998]. The quantitative decision tree method requires a substantial amount of well-defined and quantifiable response and predictor variables. It is generally not suitable for use as a tool for screening or preliminary assessment.

Combined numeric and descriptive approaches can be computerized to make them user-friendly. There will be the need to guard against the separation of the supporting logic and the outcome of the classification by making the documentation of the logic or knowledge rules underlying the classification easily accessible.

Compared to these common approaches to aggregating information in classification systems, the proposed RBCS has several tailored features that encompass some of the useful attributes of the common approaches while minimizing some of their disadvantages.

- The method of aggregation adopted in this proposed RBCS incorporates a qualitative approach to the weighting of factors that vary in their relative importance or level of certainty in their contribution to the potential for adverse health effects. This qualitative approach to weighting avoids the need to arbitrarily assign numeric weights that would be necessary in a scoring approach.
- The proposed scheme permits the aggregation of multiple factors into concern levels without combining ordered categorical labels common in a matrix approach that may be intuitively attractive, but with unclear theoretical basis.
- Given the number of factors to be considered in GM food safety assessment, a decision-tree or flow chart approach would be cumbersome to apply and detract from the objective of devising a transparent scheme that is more accessible to non-experts.

b) Applications of classification systems

Classification systems have broad applications in chemical and environmental risk assessment [Cramer et al., 1978; CCME, 1992; Schilter et al., 2003]. They have been applied to the classification of microorganisms with respect to safety practices in research activities, medical practice, and industrial production [e.g., Jank et al., 1996; Lelieveld et al., 1996; Doblhoff-Dier, 1999; risk group and biosafety level definitions issued by different organizations, including the European Economic Community, U.S. National Institutes of Health and the Center for Disease Control, and the Canadian Laboratory for Disease Control, available at http://www.absa.org/resriskdef.html]. Classification systems have also been developed for ecological risk assessment of GM crops [FAO, 2001; Traynor et al., 2001; Hancock, 2002; Koivisto et al., 2002; Strauss, 2003].

With respect to the application of classification systems for foods, microbiological and chemical hazards have been the focus of these systems [e.g., van Gerwen, 1997; Bisaillon et al., 2001; Ross and Sumner, 2002]. Among food applications using a matrix approach consisting of two parameters (exposure and chemical structure), the U.S. FDA classifies direct food additives into

three concern levels. The objective of the classification is to provide guidance to industry on the type of toxicity testing required in the premarket assessment of direct food additives in the U.S. [FDA, 2003]. An *ad hoc* expert panel [LSRO, 1999] proposed an expanded version of FDA's approach to food additive classification to cover novel food ingredients.

While classification systems have been developed to assess ecological risks of GM crops and chemical and microbiological risks of foods in general, to this researcher's knowledge, the risk-based classification scheme developed in this doctoral thesis is the first of its kind for assessing the potential health risks of foods derived from or containing GM organisms.

5.3.3. Comparisons with current guidelines for GM food safety assessment

Current guidelines for the safety assessment of GM foods are generally descriptive and generic in nature. Explicit criteria for case-by-case exceptions are limited. Application of assessment procedures may be guided by the use of decision trees or flow charts with a focus on specific aspects such as allergenic or unintended effects [e.g., Custers, 2001; FAO/WHO, 2001; NRC, 2004a]. The flow charts developed by the Edmunds Institute [1998] were based on strategies intended to provide different margins of safety. The notion of varying degree of scrutiny required for the assessment of GM organisms was expressed in the guidelines prepared by the Association of South East Asian Nations [ASEAN, 1999]. However, no criteria were included for food safety assessment. Similarly, while there is the recognition in recently published guidelines [e.g., EFSA, 2004] that not all GM foods or their components require the same level of testing, no systematic characterization or explicit criteria were developed to facilitate a common approach to more efficient assessment and testing.

In comparison, the proposed scheme has a general conceptual framework that is comprehensive in scope comprising separate categories of health effects and a range of factors related to the generation of intended and unintended effects in GM foods. The scheme is supported by a set of detailed criteria based on their ability to differentiate among GM foods their relative potential to produce adverse human health effects. Its implications for testing are discussed in Chapter 7.

5.3.4. Potential limitations

A number of limitations in the development of the proposed RBCS are recognized. Major limitations include: a paucity of empirical data for addressing several fundamental questions

related to unintended effects (Chapter 3); limitations in current methods for assessing certain potential health hazards on which several criteria are based (Chapter 6); and a focus on the scientific aspects of risk assessment without incorporating the broader considerations of health risk assessment using an integrated approach (Chapter 10). There could be bias in the selection of participants in the expert consultations in that participants by necessity were volunteers. However, this limitation is balanced by the broad-based and purposive recruitment. Over the course of the research project, multiple consultations were conducted with more than 40 experts recognized in their respective disciplines and expertise relevant to the assessment of agricultural biotechnology products and health risk assessment. Divergent views were received from the participants during the consultation process. This suggests that despite the voluntary nature of the participation process, a range of input was achieved, reflecting what can be expected based on the literature. Limitations in the underlying assumptions and areas for future research are discussed in more detail in Chapter 10.

5.3.5. Potential uses of the proposed RBCS

The clearly identifiable factors, explicit criteria and aggregation rules, as well as the conceptual framework could allow the RBCS to be used as part of a formal product assessment process, as well as in a less formal process where information generated and testing conducted for a specific product can be screened for their completeness as part of premarket assessment. It is also anticipated that the proposed scheme can be used iteratively during different stages of developing a GM crop to guide decision-making in whether or not further development and/or testing should continue. In addition, the conceptual framework could probably be adapted to communicate with interested parties, providing understandable information on how decisions about testing requirements are established in GM food risk assessment. This would be one step towards facilitating public understanding of how potential risks of a new technology in food production are assessed and managed. It should be emphasized, however, that the scheme is *not* intended to predict the level of inherent health risk of GM foods. Rather, it is intended to provide a basis for establishing levels of concern as guidance for testing.

In conclusion, the proposed RBCS presents a new, systematic approach to thinking about the basis of potential health risks of GM foods and how their testing should be prioritized in proportion to their potential risks. The implications for testing of the proposed scheme are

discussed in Chapter 7, following the literature review in the next chapter on testing approaches for food-related categories and different health endpoints.

CHAPTER 6 - TESTING OF FOOD-RELATED PRODUCT CATEGORIES

The objective of this chapter is to review current approaches to the testing of food-related product categories with a view to providing the scientific rationale for the proposed graded approach to testing GM foods presented in the next chapter. This chapter begins with an introduction to current approaches to the testing of GM foods. Strategies for testing other product categories relevant to the assessment of GM foods are reviewed, with a focus on toxicity testing. Limitations of current methods and prospects for emerging methods are discussed.

6.1. Current approaches to testing GM foods

Guidance for the safety assessment of foods derived from genetically engineered crops have been published by national and international government agencies in recent years [for example, Health Council of the Netherlands, 2002; Codex Alimentarius, 2003; Scientific Institute of Public Health, 2003; EFSA, 2004; FSANZ, 2004]. Non-governmental organizations have also recommended testing procedures [The Edmonds Institute, 1998; Custers, 2001]. With few exceptions [Health Canada, 2003], published guidelines focus primarily on crops modified by rDNA techniques. The list in **Table 6-1** represents the type of information generally required for premarket assessment. There are differences, however, among guidelines from different jurisdictions. For example, acute toxicity testing of expressed pesticidal proteins is a general requirement of the U.S. EPA, while under European guidelines, protein toxicity testing might not be required when both the plant and the new proteins have a history of safe consumption by humans and animals [Mendelsohn et al., 2003; EFSA, 2004]. Where protein toxicity testing is required in the European guidelines, a 28-day oral toxicity test is indicated. There are also differences of opinion about the value of complete DNA sequencing including junction regions of gene inserts in GM crops [FDA Food Advisory Committee, 2003; EFSA, 2004].

The current approach to the testing of genetically engineered foods is based on assessing functional and chemical changes that result from the genetic modification [Kuiper et al., 2001; Kuiper and Kleter, 2003; Cellini et al., 2004; König et al., 2004]. While government guidelines tend to emphasize a case-by-case approach, particularly with respect to the need for toxicological testing, critics generally advocate a comprehensive approach, including detailed molecular

characterization, multiple toxicity tests, and allergenicity testing that require a clinical component as well as the assessment of *de novo* sensitization using animal models [Freese and Schubert, 2004; Wilson A. et al., 2004; Spök et al., 2005]. The authors of one guidance document suggested that the "margin of safety" is a function of the completeness of assessment [The Edmonds Institute, 1998]. However, no criteria for "completeness" were provided. No explicit structured approach to testing has been proposed that integrates multiple factors that influence the health risk potential of a GM food with the extent and nature of testing. Factors that influence the health risk potential of GM foods, as elaborated in Chapter 4, include the nature of the trait being modified, the mode of action of gene products introduced or modified in the modified crop, and the characteristics and history of food use of both the donor organism and the modified crop.

6.2. Strategies for testing food-related product categories

To provide the basis for recommending a graded approach to testing GM foods, factors that influence the nature and extent of testing are illustrated by reviewing testing strategies of selected product categories that are relevant to the risk assessment of GM foods. Testing strategies that are currently used or have been proposed fit into one of four approaches or are combinations of more than one approach: battery, tiered, tailored, and multilevel [NRC, 2006]. The approach of choice often depends on the legislative or regulatory mandate, scientific developments, risk management objectives, and availability of resources.

A *battery* is a specific or minimum set of toxicity tests applied to all chemicals in a group. In *tiered* testing, the results of a specific or basic set of toxicity tests and risk management needs are used to guide decisions about the nature and extent of further testing. A substance may first be assigned to categories (e.g., based on structure or exposure) that guide testing sequences. Tests in lower tiers aim to provide an initial characterization of toxicity, followed by investigation of mode of action in higher tiers. Often, lower tier tests with a screening function use low-cost, high-throughput tests that may have a high false positive rate but low assurance of relevance to humans. Positive tests at a lower tier trigger more rigorous and relevant tests at successively higher tiers with higher associated costs [NRC, 2000b]. In *tailored* testing, prior information (e.g., exposure circumstances, suspected adverse effects, and knowledge of mechanism of action) is used to determine the scope of tests to be conducted on a given chemical or class of chemicals. Tailored testing strategies may also be flexible in that different tiers or types of testing may

evolve in an iterative manner based on scientific judgement [NRC, 2006]. The *multilevel* approach does not specify the direction in which tests move across levels. Testing can be done independently at any level or at several levels, depending on the particular compound, the risk assessment questions to be addressed, the anticipated pattern of human exposure, and the uses intended for the compound [NRC, 2000b]. Increasingly, the value of flexibility in testing within a structured framework that focuses on generating data that will be used in risk assessment is being recognized [Health Council of the Netherlands, 2001; Carmichael et al., 2006].

6.2.1. Simple substances

This section focuses on non-protein low molecular weight substances. Substances in this chemical class that are naturally occurring in foods are not generally subject to systematic premarket safety assessment. Instead, chemicals added to food will illustrate different approaches to the testing of food-related chemicals. Three categories will be examined: food additives, pesticide residues, and substances with low exposure levels. Determining upper levels of intake for nutrients and related substances in foods have been the subject of a recent extensive review [FAO/WHO, 2006]. This publication details the approaches that can be used to evaluate available data for hazard characterization and health risk of inherent food constituents. Therefore, this category of food substances will be excluded from this review.

a) Food additives

Premarket assessment of food additives in the U.S. follows a tiered approach that recommends specific batteries of test for each tier [FDA, 2003]. The tiered approach to food additive testing is based on two parameters that influence the health risks of a potentially hazardous substance: its chemical structure and exposure level. Two categories of food additives are distinguished: indirect food additives and direct food additives. For indirect food additives, the nature and extent of testing is determined by three exposure levels: virtually nil exposure (< 0.05 ppm), insignificant exposure (> 0.05 ppm), and significant exposure (> ~1ppm). For direct food additives, testing is determined by both exposure level and chemical structure that form the basis for three concern levels (**Table 6-2**). Structure category A includes compounds associated with low toxic potential or identified as normal cellular constituents, such as alkanes, complex carbohydrates, and fatty acids. Structure category B includes compounds associated with non-cancer adverse effects in animals or humans such as certain amino acids, carboxylic anhydrides,

peptides, and proteins. Structure category C includes compounds associated with mutagenicity or carcinogenicity, such as benzofurans, epoxides, and phenols.

Progression in stringency in the type and extent of testing is evident for each successive concern level (**Table 6-3**). Extensive toxicity testing is reserved for additives with high exposures and potentially reactive structures and for additives that induce adverse toxic effects at low doses or after short periods of exposure. Testing requirements are the same for the highest exposure level for indirect food additives and the highest concern level for direct food additives. The recommended tests for the highest concern level are intended to be sensitive enough to detect nearly all types of observable toxicity, including malignant and benign tumours, pre-neoplastic lesions, and most other signs of chronic toxicity. Some tests are contingent on other test findings (e.g., teratology studies), or are suggested (e.g., metabolism studies), but there is no formal guidance on proceeding from one test to another on the basis of negative or positive results [NRC, 2006]. Among specific endpoints, short term tests for carcinogenicity are required at all exposure and concern levels, except for indirect food additives considered to have virtually nil exposure. A multigenerational reproduction feeding study is required for direct food additives at concern levels II and III and for indirect food additives at the highest exposure level.

Tiered toxicity testing requirements were also introduced by the Scientific Committee on Food in Europe. For example, testing may be reduced for three categories of food additives (food enzymes, flavouring substances, and substances migrating into food from packaging materials) based on their known or likely human exposure [Barlow et al., 2002].

b) Pesticide residues

Three types of pesticides (chemical, microbial, biochemical) require different testing strategies according to guidelines of the U.S. Environmental Protection Agency (EPA) and the Organization for Economic Cooperation and Development (OECD). Chemical pesticides are generally synthetic materials that directly kill or inactivate the pest. Biochemical pesticides are certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals. Biochemical pesticides include substances such as insect sex pheromones, which interfere with mating, as well as various scented plant extracts that attract insect pests to traps. These two types of pesticides are discussed because of their relevance to plant-incorporated pesticides encountered in GM crops.

The testing of chemical pesticides that can end up as food residues currently requires a comprehensive standard battery of tests. To improve the efficiency and accuracy of the safety assessment process for agricultural chemicals, the International Life Sciences Institute (ILSI) has developed proposals for an alternate tiered approach [Barton et al., 2006; Cooper et al., 2006; Doe et al., 2006]. The proposals addressed three types of testing: systemic toxicity in young adult animals, testing in different life stages, and the acquisition and application of absorption, distribution, metabolism, and excretion (ADME) data. A strength of the proposal is the consideration of margin of exposure in guiding the selection and extent of testing [NRC, 2006].

As a class, biopesticides are usually inherently less toxic than conventional pesticides. Biopesticides generally affect only the target pest and closely related organisms, in contrast to broad spectrum, conventional pesticides that may affect a wide range of non-target species. They often are effective in very small quantities comparable to natural emissions and often decompose quickly, thereby resulting in lower exposures and largely avoiding the pollution problems caused by conventional pesticides.

Reduced data requirements for premarket approval may be justified for some types of biopesticides based on exposure and structure. For example, studies of teratogenicity and subchronic exposure can generally be waived if long-term exposure above ground levels can be excluded, or if a substance is a member of a well-characterized chemical group, such as straight-chained lepidopteran pheromones (SCLPs) consisting of simple, unbranched aliphatics and functional groups such as alcohol, acetate or aldehydes. Required data may also be waived if the toxicity of a substance has been described or published, or if surrogate data are available [OECD, 2002]. The EPA has a formalized three-tier approach to the testing of biopesticides. Biochemical pesticides used on food crops are subject to required testing for acute toxicity and screening for hypersensitivity, immune response, and genotoxicity. Tier II tests (mammalian mutagenicity tests, immunotoxicity) are conditional on positive findings from tier I tests, whereas tier III tests (chronic exposure and oncogenicity) are conditional based on pesticide use pattern, expected human exposure, and findings in lower tiers.

c) Substances with low exposure levels

Structure-based thresholds of toxicological concern (TTC) have been proposed to enhance the efficiency of testing of chemicals with low exposure levels. A threshold of toxicological concern

is a generic human exposure threshold value for all chemicals or groups of chemicals below which there would be no appreciable risk to human health. The TTC is derived from the distribution of toxicological potencies of relevant classes of chemicals for which good toxicity data exist. Assuming that the substance in question is no more toxic than the most potent chemical in the class of interest, a theoretical upper bound on the potency of this substance can be inferred [Krewski et al., 1990; Munro 1990]. It is a pragmatic risk assessment tool, the main advantage of which is that the risk of low exposures to a chemical with known structure can be evaluated without the need for chemical-specific animal toxicity data [Kroes et al., 2004; Renwick, 2005]. The TTC concept could be useful in setting priorities for testing among large functionally similar groups of chemicals to which exposure is generally very low, as is the case with flavourings and substances used in food contact materials. In fact the concept of TTC has been used for many years for screening the safety of packaging migrants by the U.S. FDA, and of flavouring substances by the JECFA (Joint FAO/WHO Expert Committee on Food Additives) [FDA, 1995; Renwick, 2004; Smith et al., 2004; 2005]. The TTC concept could be particularly useful, for example, for determining the need for detailed assessment of a newly discovered contaminant in food, for which there is no toxicological information.

A systematic decision tree approach to the evaluation of low levels of diverse chemicals in food and the scientific basis for the approach were elaborated by ILSI in Europe [Kroes et al., 2000; Kroes and Kozianowski, 2002; Renwick, 2005]. Only the practical implications of the approach are summarized here.

Applicability. The TTC concept is not applicable to several types of substances, including proteins, heavy metals, aflatoxin-like, azoxy-, nitroso-compounds, and polyhalogenated dibenzo-p-dioxins and its analogues, steroids, as well as substances that are likely to accumulate in the body. The use of the concept is also not appropriate for assessing endocrine-mediated effects and allergenicity for which threshold levels cannot be determined at present [Barlow, 2005].

Exposure considerations. A critical aspect of the appropriate application of the TTC principle is the necessity for reliable exposure data, including exposure from non-food sources. TTCs are expressed in terms of micrograms per person per day, but can be converted to an approximate equivalent dietary concentration by assuming that an adult person may consume 1.5 kg of food and 1.5 kg of beverages per day (an additional adjustment for children based on body weight is

suggested). Three structural classes for chemicals within the TTC concept were proposed. Class I consists of substances with simple chemical structures and for which efficient modes of metabolism exist. A low order of oral toxicity is suggested. Human exposure thresholds for this class of chemicals (1.8 mg/person/day, or a dietary concentration of 0.6 ppm) are 20 times higher than chemicals in Class III, consisting of substances with chemical structures that permit no strong initial presumption of safety or may even suggest significant toxicity or have reactive functional groups. Additional TTC levels were developed for organophosphates (< 6ppb) and compounds with structural alerts but with no concerns for potential genotoxicity (< 0.5 ppb). At these levels of exposure, the risk for potential genotoxicity is judged to be negligible (probability of life-time cancer risk of less than 1 in 10^6).

The application of assumed tolerable exposure levels has also been proposed for determining the need for testing in other cases where toxicity data are available, but a "no observed adverse effect" is not available. By comparing the assumed tolerable exposure level with the estimated exposure level, an assumed margin of safety can be calculated. A margin higher than one means that testing may be considered to be of low priority or deferred [Tischer, 2001].

Use of the TTC in regulatory context. The TTC concept has been applied to the regulation of chemicals, taking into consideration genotoxic and carcinogenic risks. For migrants from food contact materials, a level in food of 0.5 ppb was established as the threshold of regulation because most known carcinogens pose less than one in a million lifetime risk if present in the diet at 0.5 ppb or less, and because this represents a level 2000 times lower than the dietary concentration at which the vast majority of studied compounds are likely to cause non-carcinogenic toxic effect [Rulis, 1986; FDA, 1995; Renwick, 2004].

Several approaches have since been proposed to extend the application of the TTC concept originally developed for indirect food additives to a wider range of chemicals. Integrating hierarchically a combination of data derived from structure-activity relationships, genotoxicity, and short-term oral toxicity, Cheeseman et al. [1999] supported the extension of a single threshold of regulation to a range of dietary concentrations between 0.5 and 15 ppb (1.5 and 45 μg/day), depending on whether an untested substance is predicted to be a likely potent carcinogen, if in fact it is a carcinogen at all. Carcinogenic endpoints were determined to be the most conservative toxicity endpoint, based on comparison with the distribution of lowest effect

levels for reproductive toxins and other compounds with multidose toxicity data. The dietary range estimated by Cheeseman et al. is within the range for threshold of toxicological concern already discussed for compounds with structural alerts, but with no concerns about potential genotoxicity.

6.2.2. Complex substances

Food-related complex or high molecular weight substances may be represented by macronutrients such as lipids, carbohydrates, and proteins, which are digested into their basic units of fatty acids, simple sugars, and amino acids before absorption. While inherent macronutrient substances are generally not known to be toxic, they do have health effects, both beneficial and adverse. In fact, there are no known intake levels that are without risks for these food substances [FAO/WHO, 2006]. Genetic modification of crops can expand the scope of dietary sources of these substances (e.g., marine-derived fatty acids in food crops). Crops can also be modified to synthesize specialized macro-substances (e.g., cyclodextrins) for extraction as food ingredients. For pesticidal proteins introduced into GM crops, there are concerns that they could be structurally related to known toxins, although these proteins are typically expressed at very low levels. Therefore, testing approaches for complex substances may be distinguished based on their abundance in the food and in the diet.

Since the standard approach to assigning concern levels for food additives is not appropriate for macronutrient substances because of differences in their dietary levels, authors of the LSRO report [1999] recommended two separate categories of complex chemical entity. For complex compounds with exposure levels corresponding to less than 0.1% of the diet, animal testing using classical protocols was considered appropriate, with additional attention required for allergenic effects. However, for complex compounds at exposure level greater than 0.1% of the diet, an alternative approach was proposed. The need for an alternative approach to testing novel food-related complex substances that is different from the approach traditionally used in testing simple chemically defined substances is now well recognized. Discussed below is a generally accepted approach [IFBC, 1990; Howlett et al., 2003; Kruger and Mann, 2003; Schilter et al., 2003; Walker, 2004; Edwards, 2005].

a) Complex substances at macro levels

Complex substances such as carbohydrates, proteins, and lipids are generally not of toxicological concern unless they are resistant to digestion, or consist of unusual basic units rendering the complex substances structurally or functionally dissimilar to nutritive dietary substances. Therefore, the extent and type of testing depends largely on their digestion products. Where toxicological testing is indicated, hazard characterization may be complicated by the need to clearly distinguish between effects that are adverse versus physiological or adaptive homeostatic effects without adverse health outcome. Because of their relative mass in the diet and the limits of their incorporation in the diets of animals in toxicity testing, only a narrow margin of safety can be achieved through classical approaches to testing.

Given these characteristics of food-related complex substances, there will be an increased need to understand their biodisposition through comparative studies and their mode of action in order to guide the design and interpretation of toxicity testing [Hattan, 1994; LSRO, 1999, p. 95]. There will also be an increased need for testing in humans, including postmarket surveillance, to assess effects such as tolerance, composition and activity of gastrointestinal microflora, colonic fermentation, and allergenicity not easily detected or relevant in typical laboratory animal models. In addition, how complex substances at macro levels may replace other food components also need to be determined [Munro et al., 1996]. Nutritional and compositional characterization prior to toxicity testing is emphasized in the assessment of complex food-related substances.

Selectivity in the conduct of long-term studies of novel food ingredients is recommended in European guidelines [Poulsen, 1990]. Food ingredients that yield hydrolysis products identical to normal metabolites are not required to undergo long-term studies. Chronic toxicity and carcinogenicity may be unnecessary endpoints for assessing peptides, proteins, carbohydrates and fats, when these compounds can be shown to consist of well known sequences of amino acids, mono- and di-saccharides and fatty acids by chemical and biochemical (including metabolism) studies. These compounds may warrant long-term toxicological testing if they are to be used in large amounts or have widespread use. To address the concern of a lower safety factor in animal testing, more sensitive markers of effects and susceptibility will need to be developed. This may be possible with advances in toxicogenomics. In some cases, for these food ingredients with exposure estimates below 0.1% of the diet, prerequisite chemical-physical

and metabolic evaluation would provide sufficient evidence to render subsequent testing unnecessary.

b) Protein substances with low exposure levels

Recombinant proteins introduced or modified in GM plants have become a focus of risk assessment of foods derived from GM crops. Many pesticidal proteins and other enzymatic proteins are usually expressed at very low levels (ranging from less than 1 ppm up to 2% of total protein in the modified crop [Peumans and Van Damme, 1996; Betz et al., 2000; Heil and Bostock, 2002]. At these levels of expression, they contribute below 0.1% of total dietary protein typically consumed in a mixed diet. The primary factor determining whether there is a need for toxicity testing is structural and functional equivalence with the source protein. Where equivalence with a protein that has a history of consumption from food is established, further testing is generally not required. Where further testing is indicated, certain inherent properties of protein make extensive testing unnecessary, which is in contrast to the testing of many chemicals without a history of food use. These properties are summarized below [Pariza and Foster, 1983; FIFRA SAP, 2000; Pariza and Johnson, 2001].

- Protein substances, if toxic, generally have acute effects, with a few exceptions, such as protease inhibitors and lectins/hemagglutinins.

- Peptide fragments resulting from the breakdown of protein are less toxic than intact protein, with some exceptions:
  - inactive precursors activated by specific proteolysis (e.g., ribosome inhibiting protein);
  - biologically active peptides from specific plant proteins (e.g., exorphins from casein); and
  - non-dietary proteins (e.g., human protein pro-opiomelanocortin; toxic small peptides like snake neurotoxin).

- Protein substances are unlikely to be genotoxic as the probability that they or their digestion products would interact with DNA or other chromosomal material is low. Experience in testing enzymes for food application has failed to detect a single mutagen. *In vitro* mutagenicity is deemed unnecessary when following a systematic approach to ensure that relevant questions are addressed [Pariza and Johnson, 2001].

- Dietary proteins have not been associated with teratogenic, mutagenic, or carcinogenic effects in rodent models.

- Enzymes are generally not toxic [Ness et al., 2001]. Very few toxic agents have enzymatic properties. The few exceptions that exist are unrelated to food applications (e.g.,

diptheria toxin and some enzymes in snake venoms). The primary consideration in the genetic modification of enzyme is the safety of the source organism.

- For modified enzymes, it is unlikely that changes which improve upon desired enzyme function will result in the creation of a toxic protein. Data are lacking that a structure change will transform an otherwise non-toxic protein or enzyme into a toxin. Wide variations exist in enzyme structure and function. Despite changes in characteristics such as stability and substrate specificity, general tertiary structure and catalytic activities are usually maintained, with variation similar to that observed in nature.

Given these properties, novel pesticidal and enzymatic proteins that are expressed at very low levels do not require extensive testing. Only short-term toxicity tests of the purified protein (repeated dose oral toxicity study of 28 days) are generally required. Additional targeted tests (e.g. immunotoxicity) may be indicated by the 28-day test. Whole food 90-day toxicity may be required for membrane-bound proteins, or where the potential exists for interactions among multiple new proteins, or for interactions between the new protein and other new or endogenous food constituents [EFSA, 2004]. For proteins that are resistant to pepsin digestion in standard tests, stable breakdown products of the expressed protein would need to be characterized and assessed for hazards linked to their biological activity, including allergenicity [EFSA, 2004; Akkerdaas et al., 2005].

6.2.3. Complex mixtures

Compared to the testing of defined substances typical in classical food toxicology, the testing of unintended effects of GM foods with the potential for adverse health outcome presents additional challenges [Hattan, 1994]. Additional challenges stem from the nature of food, which is a complex mixture of many poorly characterized components that have both beneficial and adverse effects [Knudsen and Ovesen, 1995; Essers et al., 1998]. This section focuses on general strategies of toxicological testing of poorly characterized chemical mixtures. Approaches to the testing of three examples of food-related complex mixtures (plant-derived mixtures including flavouring complexes and food supplements, irradiated foods, and novel foods) are discussed briefly. In the next chapter (section 7.3.2, Part B), the relevance of these strategies to the testing of unintended changes in GM foods is examined and integrated with biochemical characterization of GM foods.

A mixture is characterized by simultaneous exposure to the constituents as a result of their joint occurrence and the presence of unidentified or only partially known components [Feron et al.,

2004]. Two categories of complex mixtures may be distinguished: simple mixture and complex mixture. A simple mixture is defined as a mixture that consists of a relatively small number of chemicals (say, 10 or less), the composition of which is qualitatively and quantitatively known. Examples are a cocktail of pesticides and some flavouring preparations. A complex mixture is defined as a mixture that consists of tens, hundreds or thousands of chemicals, the composition of which is qualitatively and quantitatively not fully known [Feron and Groten, 2002]. Examples include drinking water and food. For simple mixtures that can be readily characterized analytically, their safety evaluation will tend to follow along well established approaches for single chemically defined substances [IFBC, 1990] and will not be elaborated further.

a) General strategies

Testing strategies and options for complex mixtures are evolving and depend to a large extent on the questions and objectives of testing. If the objective is to assess the effects of the mixture, toxicologic evaluation of the whole mixture in its entirety would be the preferred strategy. If the objective is one of prediction, hypothesis generation, or identification of causative agents, then evaluation of the components or bioassay-directed fractionation is recommended [NRC, 1988].

The whole-mixture approach to toxicity testing requires no knowledge of mixture components and may be a preferred option if discontinuation of product development is the default decision in the event of positive findings. However, knowledge of the mixture components is critical in guiding the design of toxicity studies and in ensuring that study results can be interpreted with confidence. The lack of biochemical characterization of the GM food used in toxicity testing would also make comparison with other similarly modified crops of the same species difficult, if not impossible. This makes toxicity testing of biochemically uncharacterized GM foods less useful for the purposes of screening for unintended adverse effects in GM foods.

Component-based testing of a complex mixture is another strategy that can be considered when the mixture is available for testing in its entirety [Feron and Groten, 2002]. Two strategies have been proposed. An underlying assumption for both strategies is that selected chemicals in the mixture are representative of the hazard and risk of the entire mixture. Representative chemicals of the mixture can be selected using two approaches: "top n" and "pseudo top n". The "pseudo top n" approach is suggested for complex mixtures that consist of many widely varying chemicals with no obvious ranking of individual constituents according to potential health risk.

"Top n" approach means identification of the n (say the 10) most risky (not most toxic) chemicals in a complex mixture. "Pseudo top n" approach comprises first, identification of the n most risky classes of chemicals, followed by identification of a representative chemical or pseudo-chemical for each class, using a lumping technique. The selected "pseudo top n" chemicals are either representative chemicals of each class or pseudo-chemicals representing a fictional average of a certain class. The lumping technique is based on grouping chemicals with relevant similarity such as the same target organ and/or similar mode of action. Once the "top n" or "pseudo top n" chemicals have been identified, they will be approached as a simple (defined) mixture.

b) Plant-derived mixtures

In this subsection, two categories of plant-derived complex mixtures (flavouring complexes and food supplements) are discussed separately as they reflect different considerations in assessing complex mixtures.

(i) Flavouring complexes

Plant-derived flavouring complexes represent an enormous challenge in risk assessment because there are over 3,000 substances present in flavouring agents, with toxicity data available on only a fraction of these [Renwick, 2004]. This problem is similar to that encountered in evaluating the potential adverse health effects of unintended changes in plant metabolites in GM foods, the toxicity of which is largely unknown. This brief review will outline only those concepts and principles of risk assessment relevant to this group of compounds [Munro et al., 1998; Smith et al., 2004, 2005] that may also be applicable to addressing concerns about the potential health risks of unintended changes in GM foods.

One major principle used in prioritizing the need for toxicity testing of natural flavouring agents is the organization of constituents of the complexes into congeneric groups based on knowledge of structure-activity relationships and of the metabolic pathways by which flavouring agents are synthesized in plants. Available data for different representative members in each of the congeneric groups support the conclusion that the toxic, genotoxic, and carcinogenic potential of individual constituents adequately reflect these same characteristics for the corresponding congeneric group [Smith et al., 2005]. Following detailed chemical characterization, compounds with known chemical structures in the mixture are assigned into the respective congeneric

groups and to one of three classes of toxic potential, each with its own exposure threshold of toxicological concern (see TTC concept in section 6.2.1.c). The predicted metabolic fate of the compounds is another key criterion in assigning a compound into one of the classes of toxic potential, although other factors, such as probable exposure and availability of toxicity data on structurally closely related substances, are also important [Munro et al., 1998].

For constituents without identified chemical structures, the level of intake of the compound as a component of commonly consumed food is compared with the level of intentional addition to food. If the consumption ratio markedly exceeds one, then concerns about health risks would be considered low. If the consumption ratio is below one, the total potential intake of all unidentified compounds are considered together in comparison with the exposure threshold of toxicological concern for the structural class of highest concern (class III). Additional steps are taken if exposure estimate exceeds the threshold of toxicological concern. Additional steps include further chemical characterization, generation of new toxicity data, or assessment of existing toxicity data on a comparable complex. The decision to take one or more of these steps would depend on the rigour of the analytical methodology used in characterizing the complex, the extent of current and expected exposure to the complex, as well as its history of use.

(ii) Food supplements

Plant-derived food supplements, including botanical preparations, also vary widely in their complexity [Bast et al., 2002; Kruger and Mann, 2003; Schilter et al., 2003]. Approaches proposed for assessing this category of mixtures are similar to those for complex substances, with an emphasis on product and chemical characterization as the first phase in determining the extent and nature of further testing. Another important consideration in establishing testing requirements is the level and type of prior of use of the product in question. Four levels of prior use may be distinguished: history of food use at levels comparable to expected levels, history of food use at significantly higher levels, history of non-food uses (e.g., medicinal), and no history of use (completely novel) [Walker, 2004].

c) Irradiated foods

Limits on the level of irradiation that can be applied to different food categories have been established in relation to the intended purpose of irradiation and the level of the food customarily consumed in the diet. The type of toxicological testing depends on whether the

irradiation level is above or below the threshold. Risk assessment of irradiated foods focuses on the identification of radiolytic compounds which are enzymatically digested before conducting *in vitro* genotoxicity testing [Elias, 1989]. *In vivo* testing (specifically, a 90-day feeding study) is required for foods that exceed the threshold. Early toxicity studies of irradiation foods highlighted the importance of establishing nutrition standards for long-term animal studies. Because the radiolytic products formed are more determined by the chemical composition of the food than by the irradiation alone, foods that are sufficiently similar with respect to chemical composition and the condition of irradiation may be reviewed as a group for regulatory purposes [Hattan, 1994]. The identification of metabolic marker compounds in the modified plant may be a relevant concept in determining the type and extent of testing of foods derived from crops modified by processes with a prior history of use.

d) Novel foods

As noted in the introduction to this thesis, traditional foods are generally not subject to testing for adverse health effects. The safety of traditional foods and food ingredients is usually accepted on the basis of their history of use unless there are overt indications to the contrary [Edwards, 2005]. However, a growing interest in the development of novel foods facilitated by advances in food technology and nutritional science has prompted a need for regulatory scrutiny of their potential risks. As in the approach proposed for complex substances, product characterization as a first step will guide the testing of novel foods. Product characterization comprises the collation and in-depth appraisal of information on the origin, production, composition and analysis, nutritional characteristics, history of previous human exposure, and anticipated use of the material as a novel food. The use of a worldwide net of global, regional, local and ethnobotanical positive lists for food plants to guide the decision on novelty was proposed as an initial step in determining the "novelty" of the food in question [Knudsen et al., 2005].

Where a food is considered to be novel, pre-test characterization would constitute a formal primary evaluation. In some cases, this pre-test phase may be sufficient to permit a conclusion as to its safety. In other cases, it identifies knowledge gaps and areas of concern, and provides a basis for defining the extent and objectives of any additional studies that are relevant for risk assessment [Howlett et al., 2003; Edwards, 2005]. Where animal feeding studies are necessary, the minimum is likely to be a 90-day study in a rodent model, including the full range of

toxicological parameters normally considered in this type of study [Howlett et al., 2003; Edwards, 2005; Knudsen et al., 2005]. Additional parameters might be included in some cases, depending on the characteristics of and information reviewed in the pre-test characterization step.

Because of the complex composition and the nutritional role of foods that are typically consumed at levels much higher than chemically defined substances added to foods, conventional toxicological testing strategies are inadequate as the sole basis for establishing acceptable intakes of novel foods that do not have a prior dietary history. Human studies are necessary to confirm nutritional quality and the absence of adverse effects indicated by animal testing. Postmarket surveillance may be necessary in confirming anticipated usage patterns and exposure levels. No structured approach to tiered testing was proposed for the testing of novel foods. This is understandable, given the diversity of the nature and exposure levels of foods as complex mixtures. However, a stepwise approach to testing may be inferred from the pre-test characterization step that guides the type and extent of subsequent testing.

For complex mixtures such as food, the possibility of matrix effects also needs to be considered. Matrix effects include the effects on bioavailability, absorption and pharmacokinetic behaviour of substances in the food. However, toxicokinetic and genotoxicity studies will be possible only for defined chemicals or simple mixtures [Howlett et al., 2003; Edwards, 2005]. For complex foods, some assurance of the absence of a potential for genotoxicity may be gained by appropriate investigations of bone marrow or peripheral lymphocytes undertaken as additional elements of repeat feeding studies in rodents [Howlett et al., 2003; Edwards, 2005]. Indications from prior knowledge may suggest additional studies on isolated components of complex foods.

e) GM foods

European guidelines [EFSA, 2004] specify a 90-day toxicity study of the whole GM food in rodents as a minimum testing requirement whenever the composition of the GM plant is modified substantially, or where there are indications for the potential occurrence of unintended effects, based on molecular, compositional, or phenotypic analysis [EFSA, 2004]. The need for further toxicity tests would be considered on a case-by-case basis, taking into account the toxicological profile of the food revealed in the 90-day study [König et al., 2004]. Other guidelines [Health Canada, 2003; FSANZ, 2004] emphasize assessment of compositional

changes and associated nutritional impacts in addressing concerns related to unintended effects, without indicating the conditions under which animal testing may be required.

For practical reasons, whole food testing may be a more feasible approach for testing novel proteins because of the difficulty in extracting and purifying sufficient quantity of the protein without altering its structure or function. There are also concerns about the structural and functional equivalence between the plant-derived protein with the protein produced from an alternate microbial source. Therefore, whole food testing may have the dual objectives of assessing both the intended effects of the introduced or intentionally modified gene products and unintended changes resulting from genomic and metabolic perturbations.

6.3. Specific testing - indications, limitations, and advances

The preceding review of the testing of food-related product categories revealed that where toxicological evaluation is indicated, repeated dose (subchronic) toxicity studies for a period of one month or longer are usually considered to be core studies for risk assessment purposes. Repeated dose toxicity studies are designed to identify target tissues where toxic effects may be manifest and to reveal dose-response relationships for induced toxicological responses [Barlow et al., 2002]. While standardized testing guidelines promote the reliability and comparability of studies, they also restrict flexibility in tailoring testing strategies or study designs for the product being assessed.

The relevance of animal studies depends on prior knowledge of the similarities and differences of physiology and metabolism in the test species and humans relative to the test substance. In practice, however, the selection of animal species for risk assessment purposes is usually done more on the basis of availability of and background information on the species and strain than on any knowledge on metabolic similarities to humans. Other questions remain regarding toxicity testing of food-related chemicals, such as the optimum duration, dosing frequency, and the parameters or endpoints to be measured [Poulsen, 1990; Barlow et al., 2002]. With the exception of pharmaceuticals and selected pesticides in heavily exposed workers, few data exist on which to assess concordance in toxicity data between test animals and humans. Olson et al. [2000] reviewed data compiled from 150 pharmaceutical compounds with 221 reported human toxicity events. Where animal models, in one or more species, identified concordant human

toxicity, 94% of these cases were first observed in studies of one month or less in duration. An overall human toxicity concordance rate of 71% was reported for rodent and non-rodent species combined, with non-rodents alone being more predictive (63%) than rodents alone (43%). On the other hand, a no-effect level in a 90-day rat study on a compound without any other supporting data will only provide limited assurance regarding its safe widespread use by humans [Knudsen, 1999]. Knudsen recommended that the total data set for each compound includes different cell types during different sensitive stages during the lifetime of test organisms. However, as noted previously for complex food-related mixtures, a 90-day study in a rodent species is generally considered the minimum amount of toxicological data that can be used in evaluating safety or to determine the need for further studies.

In practice, toxicological endpoints identified in subchronic studies normally lead to further studies of longer duration and of specific endpoints of concern when considered in conjunction with other factors such as likely exposure to humans and the nature of the substance. In contrast to the testing of major food-related product categories for which guidance on the need for higher-order toxicity testing is generally expected, few specifics in this regard are provided in official guidelines on premarket testing of GM foods. Higher-order toxicity testing refers to chronic toxicity and testing characterized by specific endpoints, such as immunotoxicity, reproductive-developmental toxicity, genotoxicity and carcinogenicity [Schwetz and Morrissey, 1989]. Higher order toxicity testing warrants attention because of concerns about latent health effects feared by some segments of the general public and some critics of GM foods [Environics Research Group, 2000; Lambraki 2002; Pollara Research and Earncliffe Research and Communication, 2003; The Royal Society, 2003]. However, a detailed review of these types of testing is beyond the scope of this thesis and recent reviews are available [Barlow et al., 2002; IOM, 2004; NRC, 2006]. Instead, this section will focus on general concepts of tiered approaches to higher-order toxicity testing, limitations of current test methods, and emerging methods that are being developed.

The following discussion begins with an examination of testing characterized by specific endpoints, followed by metabolism and biokinetic studies, human studies, and integrated testing strategy incorporating non-animal models and emerging technologies. Approaches to dietary exposure assessment and postmarket monitoring as related to the assessment of complex

substances are described. Trends in biochemical and molecular characterization provide perspectives on the testing of unintended changes in GM foods.

6.3.1. Immunotoxicity and allergenicity

The immune system is comprised of a number of organs, tissues, and cells throughout the body. A successful immune response depends on cooperation between different cell types. Appropriate functional tests are important in detecting chemicals with an immunotoxic potential [Barlow et al., 2002]. Two main types of immunotoxic responses may result from xenobiotic exposure: immunosuppression (inhibition of immune function) and antigenicity, a type of immunostimulation commonly known as hypersensitivity or allergy. A third, less studied immunotoxic effect is autoimmunity [NRC, 2006]. The distinction between immunotoxicity and allergy is that immunotoxicity is characterized by a non-specific interaction between a toxic chemical and either cells within the immune system or with other cells necessary for the proper function of the immune system. An immunotoxic chemical would have the potential to affect any individual exposed at a sufficiently high level. In contrast, an allergic response is an antigen-specific response only in certain atopic individuals with a genetic predisposition to respond in this manner [Barlow et al., 2002]. Of particular relevance to food-related substances are allergy (acute responses mediated by immunoglobulin E) and late-phase or delayed reactions mediated by other classes of immunoglobulins. Food-related chemicals with the potential to induce direct immunosuppressing or autoimmune responses are expected to be rare, although several dietary factors have been implicated in autoimmune diseases [Embry, 2004; Mark and Carson, 2006]. A well-known food-related example is gluten-sensitive enteropathy, which may be considered an autoimmune disorder [Sollid and Jabri, 2005; Chand and Mihas, 2006].

The immune system is highly conserved among higher vertebrate species, with the immune components and their interactions being similar in mice, rats, and humans. Thus, it is reasonable to use laboratory rodents to test for potential immunotoxic effects of chemicals sharing similar toxicokinetic properties between humans and animals [Barlow et al., 2002]. However, for the reasons explained above, this generalization does not apply to the testing of allergenic effects. Separate discussions on immunotoxicity and allergenicity follow.

a) Immunotoxicity

There is general agreement that immunotoxicity testing should follow a tiered approach. Different suggestions for tiered testing of immunotoxic effects have been proposed [IPCS, 1996; Barlow et al., 2002]. Screening level detection of immunotoxic effects can be included as part of either 28- or 90-day repeat dose toxicity tests. Positive findings in screening tests can indicate the need for specific immunotoxicity tests. Triggers or causes for concern for immunotoxicity include structural alerts (e.g., belonging to a chemical class known to be associated with immunotoxicity), findings in preceding toxicity studies, findings in humans, and intended use or the probability of susceptible groups (e.g., the ability of the compound to cross the placenta and/or secreted in milk) [Holsapple et al., 2005]. Several immunotoxicity tests have been developed to date; the choice of a specific immunotoxicity test is dependent on expert evaluation of the results of routine toxicity tests. Functional assays are an important part of testing at higher level in a tiered approach.

The 2-tier approach adopted by the U.S. FDA to assess food additives consists of a minimum set of standard or core tests. Level I tests do not require perturbations of the test animal and provide flexibility for expanded tests within each level on a case-by-case basis. Level I serum chemistry screens include quantification of immunoglobulins. Depending on the type of immunoglobulin affected, level II screening may include cell-mediated immune response using delayed-type hypersensitivity assessment and evaluation of reversibility of the effects [Hinton, 2000]. Considerations are also given to the allergenic propensity of low-molecular-weight food constituents through structural-activity assessment and *in vitro* studies in determining the chemical's haptenic potential [Hinton, 2000]. Hinton emphasized that while the individual tests in the standard screen may not be specific for a given target, results are reviewed in the context of the total toxicologic profile and the dose dependence of the observed effects.

b) Allergenicity

Acute allergic reactions are mediated by Type 1 IgE-based mechanisms. A substantial number of IgE-mediated food allergies involve cross-reactions in which the primary route of sensitization is the respiratory tract. Delayed or chronic allergic responses are mediated through antigen-specific T-cell responses or a combination of IgE and T-cell responses [Goodman and Hefle, 2005]. Cell-mediated allergic reactions to food are less common and much less well understood. Non-IgE mediated immune responses have received little attention in the assessment of the

immunostimulating potential of novel proteins in GM foods, although assessment of genetically engineered microorganisms generally includes testing for a broader scope of immunogenic reactions.

While food allergens are mostly glycoproteins of high molecular weight, allergenic responses to low molecular weight chemicals (LMWCs) induced via the oral route can occur but are rare. In contrast to proteins, LMWCs are recognized by the immune system only by conjugation to a carrier protein (hapten-carrier mechanism), which can be any protein in the body. Some LMWCs (prohaptens) need to be metabolically converted to a reactive hapten [Eisenbrand et al., 2002].

As noted in Chapter 4 (section 4.3.4, Part C), there are two stages in the development of allergic reactions: sensitization and elicitation of a reaction. The assessment of potential for allergenic effects in GM foods should consist of two elements: (1) allergenicity (the likelihood of inducing *de novo* sensitization in a nonallergic individual, and (2) assessment of allergenic activity (the propensity of a substance to elicit allergic reactions in a sensitized individual) [Spök et al., 2005].

However, at present, the testing of allergenic effects of new protein substances in GM foods focuses on their potential to elicit IgE-mediated allergenic activity. Preliminary assessment is based on known properties shared by many, but not all, food allergens, including stability to pepsin digestion using an *in vitro* assay. Bioinformatics approaches predict cross-reactivity with known allergens based on comparisons of amino acid sequence identities. Information derived from these screening tools can be coupled with prior knowledge of the allergenic attributes of the gene source and of the modified crop to obtain a more complete profile of the allergenic potential of the protein. The current approaches to testing for allergenic effects are based on recommendations of an expert consultation sponsored by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) [FAO/WHO, 2001]. The recommendations were adopted in guidelines published by the Codex Alimentarius, with a weight of evidence approach (not defined) [Codex Alimentarius, 2003]. Limitations of current approaches and how these limitations should be addressed have been contested [Goodman and Hefle, 2005; Goodman et al., 2005; Spök et al., 2005]. Major contentions centre on the lack of validation of the predictive value of the recommended testing approaches.

Protein digestibility is an important element in the assessment of allergenic potential. The likelihood that a protein will be allergenic is higher if it is resistant to digestion than if it is completely digested. This relationship between protein digestibility and allergenicity is not absolute, however. It is based on the premise that a protein that is stable to proteolysis has an increased chance of reaching the intestinal mucosa and subsequently the immune system of the intestine. Further, protease resistant fragments less than 3 kDa (29 amino acids) are expected to lack the ability to degranulate mast cells. This is based on theoretical considerations and assumptions of sequence specificity of pepsin substrate and the minimum peptide size required for eliciting clinical symptoms of allergy [Bannon et al., 2002]. The link between food allergen structure, stability to digestion, and the immunodominant IgE-binding epitopes was demonstrated within a population of individuals allergic to an allergenic protein in peanuts [Stanley and Bannon, 1999, cited in Bannon et al., 2003]. However, the correlation between protein stability in digestion and sensitization is less well understood [Dearman et al., 2002]. Results of the protein digestibility assay are most useful when considered in conjunction with other data on the protein [Helm, 2001].

Since proteolysis reduces the availability of conformational epitopes more efficiently than linear epitopes, susceptibility to *in vitro* pepsin digestion may not be indicative of a lower potential for allergenic activity that is dependent on linear epitopes [Goodman et al., 2005]. Other limitations of the pepsin digestibility test include the lack of guidance for interpreting assay results, disregarding non-oral routes of sensitization, and the lack of predictive value for class 1 food allergens (allergens that both sensitize and elicit allergic reactions) among individuals with stomach insufficiency or elevated stomach pH (e.g., young children, elderly, individuals with chronic atrophic gastritis or taking anti-acidic medications) [Bannon et al., 2003; Spök et al., 2005; Jensen-Jarolim and Untersmayr, 2006].

The bioinformatics approach is based on the assumption that T-cell and B-cell epitopes are of minimal size [Becker, 2001]. However, non-contiguous or conformational epitopes are not identified by current algorithms. The *in silico* approach is also limited by the availability of sequence information of known allergens in public databases and in the scientific literature [Codex Alimentarius, 2003]. *In silico* methods are still evolving [Becker and Reese, 2001; Goodman et al., 2002; Kleter and Peijnenburg, 2002; Brusic et al., 2003; Stadler and Stadler, 2003; Li et al., 2004; Björklund et al., 2005]. Steps to be taken to ensure the quality of sequence

searches have been suggested [Goodman and Hefle, 2005]. Expansion of bioinformatics approaches to include computer-predicted epitope identification and comparison of 3D structures has been proposed [Scientific Institute of Public Health, 2003; EFSA, 2004]. However, while these methods may be useful in complementing current approaches in specific cases, their general utility in replacing or enhancing the predictability of linear sequence comparison has yet to be established [Goodman and Hefle, 2005].

Positive results from preliminary assessment based on the gene source of the protein, bioinformatic analysis, and pepsin stability may lead to the need for serum screening using an *in vitro* IgE binding assay. Two types of serum screening (specific and targeted) have been recommended [FAO/WHO, 2001]. Specific serum screen refers to the use of sera from subjects allergic to the source of the test protein. In a targeted serum screen, sera are tested from subjects allergic to food and inhalation allergens from broadly defined organisms related to the gene source of the test protein. For example, proteins from dicots would be tested with sera from those allergic to tree pollen, weed pollen, peanuts and tree nuts. Proteins from bacteria would not be required to undergo targeted serum testing, as few (if any) individuals are allergic to bacteria. The predictive value of such extensive testing required of targeted serum screening has not been validated, as recognized in current Codex guidelines [Codex Alimentarius, 2003]. Exceptions are the highly sequence conserved (> 50% identity) homologs of the pathogenesis-related birch pollen allergens Bet v 1; Bet v 2, a profilin; and maize lipid transfer protein, which act as cross-reactive allergens for a few individuals [Goodman and Hefle, 2005].

In general, even positive IgE-binding in serum screening is not necessarily indicative of clinical allergy. *In vitro* cross-reactivity does not necessarily correlate with clinical cross-reactivity. Many individuals without noticeable symptoms of specific allergy may have detectable levels of specific IgE. Conversely, some individuals with challenge-proven allergies do not have detectable IgE. There are also very few food allergen-specific quantitative IgE assays that have been validated for predicting positive clinical responses [Goodman and Hefle, 2005]. A negative or equivocal *in vitro* IgE assay may be followed by clinical testing (skin prick test and food challenge) to confirm a lack of allergenic effects with consenting adults who have been shown to be allergic to the source [Metcalfe et al., 1996]. This recommendation assumes a sufficient population of proven allergic volunteers and permission by an institutional ethics review board. Functional assays, such as *ex vivo* basophils or mast cells activation test that measures biological activity [Dibbern et

al., 2003; Abassi et al., 2004] may question or confirm the clinical relevance of a positive IgE-binding test. Functional tests show promise for refinement and validation as a useful complement to serum screening, especially where skin prick test is not practical, although its inclusion as part of standard testing at present has not yet been supported [Dearman et al., 2005].

In the near term, paying attention to the quality of IgE binding tests, such as ensuring specificity of binding and establishing clear selection criteria for serum donors, will be important in reducing uncertainty related to the clinical relevance of the test. IgE binding to pepsin-digested food extracts may better characterize or differentiate the risk of a positive *in vitro* IgE test [Akkerdaas et al., 2005]. In the longer term, development of assays that measure IgE affinity and the number and spacing of IgE epitopes on allergens could enhance our ability to assess allergenic risk [Goodman and Leach, 2004; Goodman and Hefle, 2005].

To address the current gap in methods for testing the allergenicity (sensitizing potential) of new proteins without a history of food use, the development of *in vitro* tests and animal models is encouraged. Prediction of food allergy from animal or *in vitro* tests requires a better understanding of the underlying mechanisms, including gastrointestinal immunology and how it is influenced by dietary components.

Unvalidated *in vitro* tests are available for predicting contact allergy caused by LMWCs. No tests for oral allergens, particularly those of proteinaceous nature, are yet at a sufficiently mature stage for validation. Methods to identify T-cell epitope are at an early stage of development [Stickler et al., 2000, 2003]. Their potential to be applied to the assessment of novel proteins in GM foods requires evaluation. *Ex vivo* restimulation of (memory) T-cell appears a feasible approach that could possibly exclude the need to conduct a challenge test in the case of a positive response [Eisenbrand et al., 2002]. Considerable resources have been spent on the development of mixed lymphocyte primary human cell culture assays with the goal of predicting sensitizing potential. However, given the complexity in the immunostimulatory process, it remains doubtful whether an *in vitro* cell-based assay could mimic systemic processes underlying tolerance or sensitization to external antigens [Goodman and Hefle, 2005].

In developing an animal model, it is important that the model identifies the same allergenic structures or epitopes as those relevant in humans when the allergen is eaten. Because digestibility is a factor in food allergy, a reliable animal model should include the oral route of sensitization. Models should be able to distinguish between immunogenicity (involving non-IgE and cellular immune responses) and allergenicity (IgE-mediated responses) [Barlow et al., 2002; Germolec et al., 2003]. No such model has been developed to date. Animal models with clinical manifestations similar to those seen in humans will be useful for studying mechanisms of food allergy. In the absence of generally accepted methods for testing the sensitizing potential of new proteins introduced into GM foods, some authors argue for the need to include animal testing on the basis of the correlation between immunogenicity and allergenicity, despite the absence of validated animal models for assessing allergenicity [Spök et al., 2005].

In summary, methodological improvement and validation of the predictive value of currently available methods would enhance confidence in their validity in preventing the introduction of allergenic proteins into GM foods. There is a critical need for the development and validation of new functional tests that overcome current constraints regarding research ethics and the availability of sera from qualified donors. Challenges remain in predicting the allergenicity of novel proteins that may lack similarity in structural or physico-chemical properties to known allergens. Further, there are also challenges in assessing membrane-bound enzymes that cannot be isolated or purified in its native form for conducting tests of pepsin stability and IgE-binding.

Despite possible improvements in testing methods, allergenic risks cannot be expected to be entirely predictable. Fundamentally, assessment of the allergenicity of novel proteins is complicated in two aspects. First, the causation of atopy occurs at a variety of levels: generalized hyper-IgE responsiveness, IgE response to specific allergens or epitopes, and clinical disease expression [Hopkins, 1997, cited in IPCS, 1999, p. 125]. Second, the intrinsic allergenicity of a protein can only be predicted to a very limited degree, as IgE immunogenicity depends to a large extent on factors other than the protein itself [Aalberse, 2005]. Any protein can sensitize some individuals under the right circumstances [Goodman and Hefle, 2005]. Therefore, in spite of possible improvements in assays in the future that may better predict the allergenic potential of novel substances in foods, the need for postmarket surveillance of GM foods will remain (see section 6.3.8).

6.3.2. Reproduction and development

The general purpose of reproductive and developmental toxicity assays is to evaluate the competence of breeding pairs to produce phenotypically normal offspring and includes a broader category of endpoints than any other kinds of toxicity testing because of the multiple stages of exposure and the variability of possible effects [NRC, 2006]. Four types of reproductive and developmental studies may be distinguished by testing objectives, duration, and stage of development. They include: screening level reproductive toxicity, prenatal developmental toxicity and teratology, generational tests, and reproductive assessment with continuous breeding. The last test protocol is conducted almost exclusively by the National Toxicology Program in the U.S. [NRC, 2006].

While a tiered approach for the testing of chemicals has been implemented in Europe based on production volume (including screening and both one- and two-generation tests), an approach based on progressively more extensive testing has not been adopted for food-related substances. According to FDA guidelines, the two-generation test including a teratology phase is a required test for direct food additives in concern levels II and III. Expanded tests may be indicated based on results from the reproduction study with a teratology phase. An *in utero* phase is also required for one of two carcinogenicity studies with rodents for food additives in concern level III.

Chemicals with endocrine disrupting activity may also have reproductive and developmental implications, which current animal protocols are not designed to address. Therefore, short term tests for endocrine disrupting activity have the potential to serve as screening tests for adverse reproductive and development effects [Barlow et al., 2002]. A tiered approach to the screening and testing of chemicals for endocrine-disrupting activity is being pursued as part of the Endocrine Disruptor Screening Program of the EPA [EPA, 2005a]. A parallel conceptual framework is also being developed by the OECD [OECD, 2004].

Reduced animal testing for human reproductive and developmental risks may be considered for chemicals present in the diet at very low levels. Examples include veterinary pharmaceutical products used to treat food-producing animals. Very low residue levels are generally present in the tissues of slaughtered animals, as required by protocols for drug administration in food producing animals. A two-tier approach has been proposed for this category of food-related chemicals. The tiered approach begins with developmental toxicity testing in a rodent species,

preferably the rat. If teratogenicity is observed, then no testing in a second species is required, except where an acceptable daily intake (ADI) would be set from the rat developmental toxicity study. If a negative or an equivocal result for teratogenicity is observed, or if there are other signs of developmental toxicity in the rodent, then a developmental test in a second species, preferably the rabbit, is conducted [Hurtt et al., 2003]. This proposal was based on an analysis of relative sensitivity of laboratory animals to reproductive and developmental toxicity of 105 compounds representing a variety of chemicals and pharmacological classes with data on more than one species. A tiered approach to life-stages testing for agricultural chemical safety assessment has also been published [Cooper et al., 2006]. It calls for enhanced endpoints in a rat reproduction and developmental study and a rabbit development study.

Currently available animal toxicity tests for reproductive and developmental endpoints may be limited in their ability to predict human risk due to species differences in the underlying biology, timing of developmental events, and critical period of susceptibility [Barlow et al., 2002; NRC, 2006]. Looking to the potential for new technical approaches, the Committee on Developmental Toxicity convened by the U.S. National Research Council proposed a multidisciplinary, multilevel, interactive approach to developmental toxicity testing [NRC 2000b]. The proposal was presented at a conceptual level without providing a detailed plan for change, or specifying how the current testing paradigms could be modified and expanded to incorporate the new science [NRC, 2006].

6.3.3. Genotoxicity and carcinogenicity

Current genotoxicity tests aim to assess the potential of a chemical or its metabolite to alter or cause mutation at one of three levels of the genetic makeup of an organism: DNA, genes, and chromosomes [NRC, 2006]. Mutations can also occur at the genome level (numerical chromosome changes leading to aneuploidy). Each of these levels of mutation may play a role in inherited disorders and cancer.

Despite wide acceptance of a recommended battery of *in vitro* and *in vivo* short-term tests for genotoxicity screening, the value of mutagenicity tests in assessing food ingredients remains debatable [IPCS, 1987; Poulsen, 1990; Eisenbrand et al., 2002]. The test battery generally consists of (1) a test for induction of gene mutations in bacteria, (2) a test for induction of gene mutations in mammalian cells, and (3) a test for induction of chromosomal aberrations in

mammalian cells. Except in the case where a chemical is bioactivated *in vivo* through pathways not reproduced by standard activating systems used in *in vitro* assays, the requirement for an *in vivo* assay as part of standard test battery is not supported by current experience [Eisenbrand et al., 2002]. A reduced battery, or even no testing, could be acceptable in cases where consideration of physico-chemical, structural, and pharmacokinetic or metabolic properties indicates a lack of genotoxic activity. On the other hand, supplementary tests may be indicated when these considerations lead to additional concerns or where an aneugen is suspected [Eisenbrand et al., 2002].

Following a positive result *in vitro*, further testing is normally required to ascertain whether the compound is also active in the whole animal, unless it can be adequately demonstrated that the positive *in vitro* findings are not relevant for the *in vivo* situation [Barlow et al., 2002]. The *in vivo* tests usually undertaken are the rodent cytogenetic test and/or the rat liver unscheduled DNA synthesis tests, depending on the type of assay that was positive *in vitro*. Alternatively, if the chemical has little or no systemic bioavailability and is not a directly acting electrophilic molecule, then studies at the main site of contact, such as gut tissue, may be more appropriate. In most cases, a chemical that is positive in an *in vivo* somatic cell mutagenicity test will be assumed to be both a potential genotoxic carcinogen and potential germ cell mutagen, without further testing.

In marked contrast to assessing genotoxicity, there are no generally accepted methods for identifying non-genotoxic carcinogens. Given the plethora of mechanisms involved, it would seem impossible that a single *in vitro* test could ever serve this function [Eisenbrand et al., 2002]. Several mechanisms are associated with carcinogenic potential, including: persistent cytotoxicity accompanied by proliferative regeneration, chronic inflammation, hormones, ligands for xenobiotc induction receptors, and DNA methylation [Eisenbrand et al., 2002]. Among the likely non-genotoxic carcinogens, most act either as hormonal agonists (proestrogens) or provoke a persistent inflammatory reaction. Other carcinogens induce regenerative proliferation as a consequence of chronic cytotoxicity or irritation. The development of *in vitro* systems to detect compounds acting by these major mechanisms could greatly improve our ability to assess the risks associated with exposure to non-genotoxic carcinogens. Avenues for future efforts include the integration of *in vitro* systems with quantitative structure-activity relationships and

other computational techniques, along with the application of gene arrays and other approaches that include transcription profiling and monitoring downstream events.

In order to achieve more efficient use of animals in toxicity testing, non-conventional assays for both genotoxicity and carcinogenicity are being developed. Examples include the Comet assay for genotoxicity and a mouse transgenic carcinogenicity model with high sensitivity to non-genotoxic chemicals. The relevance of these approaches to human cancer risks remains to be established. There is increasing recognition of the need to understand the mode of action of suspect carcinogens and its relevance to human health risk assessment [Meek et al., 2003; Bolt and Degen, 2004]. The influences of other diet components in the response to carcinogens in animal studies also require attention [Barlow et al., 2002].

6.3.4. Metabolism and biokinetic studies

Metabolism studies are used to identify potential biotransformation products and the potential for accumulation of compound or metabolites in tissues with repeated dosing of test chemicals in experimental animals. Metabolism studies describe the absorption, distribution, metabolism and elimination (ADME) of xenobiotics. Biokinetic studies can evaluate the time course of changes in concentrations in the body. Both types of study are useful for comparing species differences and for understanding mechanisms of toxicity. In addition, toxicodynamic analysis examines the interactions between molecules of the chemical in question (or its metabolites) and biologically important molecules such as DNA and cellular receptors. Generally speaking, less is known about the toxicodynamic than about biokinetic aspects [Health Council of the Netherlands, 2001].

A tiered approach to the conduct of ADME studies has been applied in the testing of chemicals such as drugs and pesticides. For the review of pesticides, EPA [1998] has established guidelines for a 2-tier approach to metabolism and toxicokinetic studies. Tier 1 consists of a single dose of a radiolabelled test chemical to evaluate material balance, identify metabolism, and assess distribution and persistence of the radiolabel in various tissues. Tier 2 studies are required on the basis of other toxicity test results or tier 1 results in order to answer questions about chemical disposition.

Other approaches to tiered metabolism and pharmacokinetic studies have been proposed, including a recent proposal from ILSI, as noted previously [Wilson et al., 1994; Barton et al., 2006]. The objectives of tier 1 testing in different proposals are similar, consisting of a minimum data set that can be conducted in an economic manner at an early stage of toxicity testing. The minimum data set aims to address three fundamental questions: Is the chemical absorbed? Is the chemical metabolized? Does the chemical persist? Factors influencing the need to obtain more than minimum data include: significant findings in tier 1, the need for data in the design and/or interpretation of toxicity studies with respect to understanding mode of action and dose-response relationships. The advantage of conducting ADME studies early in the process is that it can help define a systemic or internal dose (as distinct from the external or administered dose) and allows follow-up studies to be tailored to the particular toxicity endpoint of interest. Data from ADME studies have the potential to contribute to the replacement, reduction, and refinement of animal studies [Eisenbrand et al., 2002].

In recent years, there has been growing support for pharmacokinetic modelling approaches in risk assessment. In particular, physiologically based pharmacokinetic (PBPK) models (a series of equations to describe each of the physiological processes involved in ADME) permit route-to-route, high-to-low-dose, and interspecies extrapolation, beyond the conditions of the experiment, with significant utility in improving human health risk assessment [Wilson et al., 1994; Eisenbrand et al., 2002].

As noted at the end of section 6.2.3, complex mixtures such as whole foods are not suited to biokinetic studies. However, a thorough understanding of the metabolism, physiology, and nutrition of major components of concern is indispensable. The parameters to be evaluated in single and repeated dose studies have been outlined [LSRO, 1999].

6.3.5. Non-animal models and emerging technologies

The demand for alternative methods to classical *in vivo* animal testing has grown in parallel with concerns for animal welfare, chemical safety, and resource constraints [Meyer, 2003]. Concurrent new scientific and technological developments in the area of genomics also provide opportunities for advances in testing methods, with potential application to the testing of GM foods [Cellini et al., 2004; König et al., 2004; NRC, 2004a].

a) Non-animal models

Non-animal models include the use of *in vitro* studies as well as computational and bioinformatic approaches. Structure-activity analysis and biokinetic modeling are examples of computational approaches. The application of these approaches in the assessment of specific endpoints is discussed in preceding sections. In this section, the discussion will focus on the integration of animal and non-animal approaches to health risk assessment.

Toxicity testing using laboratory animals traditionally requires large doses of the test material at levels much higher than those experienced by humans. While this approach facilitates the identification of adverse effects and reduces the need to use very large numbers of animals to detect small effects, extrapolation of the observed dose-response relationship to lower doses relevant to human exposure creates a source of uncertainty. An integration of *in vitro*, toxicokinetic and toxicodynamic studies, through a parallelogram approach, coupled with the use of predictive markers for toxicity in subchronic *in vivo* studies, has been proposed to improve current approaches to toxicological testing [Huggett et al., 1996].

An integrated testing strategy has been defined as "any approach to the evaluation of toxicity which is based on the use of two or more of the following: physicochemical, *in vitro*, human (epidemiological, clinical case reports), animal data (as available or if unavoidable), computational methods, such as (quantitative) structure-activity relationships ([Q]SAR), structural alerts and toxicokinetic models" [taken from Blaauboer et al., 1999]. While the ideal of complete replacement or minimal acquisition of animal data may not be feasible or optimal for human risk assessment in many cases in the near future [Greim et al., 2006], the underlying "fit for purpose" principle in the conduct of studies or data acquisition is desirable and widely applicable regardless of the nature of the test substance. "Fit for purpose" principle centres on asking relevant questions, such as the following.

- What do we need to know?
- When do we need to know this?
- What is the most appropriate method to obtain the knowledge?
- What will we do with the knowledge obtained?

This principle, among others, provides a basis for a generic four-stage scheme for evaluating a compound's local and/or systemic toxicity. The four stages are as follows:

- assessment based on physicochemical properties and chemical functionality;
- biokinetic/biodynamic modelling and basal cytotoxicity testing;
- conduct of specific cytotoxicity and cell-specific functional toxicity tests in the case of high tissue concentrations; and
- expanded tissue-specific *in vitro* tests and limited well-targeted *in vivo* testing as indicated by other considerations (such as intended use, prior knowledge, regulatory considerations, and results of previous stages).

A useful feature of the generic strategy is the presence of multiple decision points for further testing at each branch point of the scheme [Blaauboer, 2002, 2003]. The scheme allows for expert judgement to be made at different stages. The development of transparent criteria for decisions will be necessary.

b) Emerging technologies

It is expected that when supported by emerging approaches such as genomics, transcriptomics, and proteomics, the sensitivity and relevance of hazard characterization and the predictability of current methods will be further enhanced [Eisenbrand et al., 2002]. The limitations of using separate "omics" technologies, particularly gene expression alone, must be recognized. In the near term, gene expression analysis can be useful for screening and priority setting [EPA Science Policy Council, 2005; Boverhof and Zacharewski, 2006]. Patterns of DNA expression that are associated with specific types of toxicity may provide early markers of toxicity [Pennie, 2002]. As a predictive tool, toxicogenomics would be valuable in providing early biomarkers of effect, classifying chemicals based on their gene and metabolite profiles using pattern recognition techniques, and in determining the relevance of certain animal models based on mechanistic information. Genes whose profiles show correlation can be further examined for coordinated regulation and for mapping toxicant-affected pathways and biological networks [Hayes and Bradfield, 2005]. There are expectations that toxicogenomics could enhance human health risk assessment by providing insights into a chemical's mode of action of toxicity, inter-species differences and individual sensitivity [NRC, 2005, 2006]. Significant impediments also exist. They include technical, interpretation and implementation issues [Adelman, 2005; Boverhof and

Zacharewski, 2006]. In particular, the full potential of the "omics" technologies may be constrained by our inability to tackle complex physiological processes [Adelman, 2005]. Despite reservations about the extent to which genomic and computational approaches might be able to replace traditional toxicology in the foreseeable future, there is considerable optimism that new technologies will revolutionize toxicological evaluation and the protection of human health [Bhopal et al., 2005; NRC, 2005].

An integrated approach to the toxicity testing of GM foods with a focus on assessing the introduced protein has been explored [personal communication cited in König et al., 2004]. The study investigates GM rice containing lectins using 90-day rat study that included multiple control groups. Toxicity endpoints from the *in vivo* study were examined in parallel with *in vitro* experiments on the digestibility and cytotoxicity of the recombinant proteins. Using epithelial cell lines derived from humans and rats, changes in gene expression profiles in rat and human cell lines exposed to subcytotoxic levels of the protein and its peptic-tryptic digests were also determined and compared to expression profiles in intestinal samples from *in vivo* feeding studies. Despite the testing of the whole food, the study design is tailored to assessing the introduced protein and not unintended effects.

6.3.6. Human studies

Three major categories of human data may be distinguished in the context of product safety assessment: epidemiologic studies, case reports, and clinical or controlled-exposure studies [NRC, 2006]. While epidemiologic studies generally are not useful in the premarket assessment of novel products, they can provide valuable safety-in-use information, particularly in population groups experiencing higher exposure levels (e.g., occupational groups). The use of epidemiologic studies is further discussed in section 6.3.8 (postmarket monitoring).

Case reports are relevant in investigating exposures on which there is little or no prior human toxicity information [NRC, 2006]. In the area of food safety, case reports have been a major source of information on acute adverse health effects of natural toxicants in foods. Although not human data, case reports of poisoning in domestic animals have also provided an extensive database on plants containing harmful substances. Case reports can also provide a source of data in postmarket surveillance programs.

In contrast to pharmaceutical products, clinical or controlled-exposure studies are not commonly conducted as part of premarket assessment of other chemical entities. With the development of novel complex food substances (e.g., macronutrient substitutes), there is a general recognition of the need for human studies in the testing of these substances that are consumed at high levels (sections 6.2.2 and 6.2.3).

The purpose of clinical trials in humans is to confirm the absence of adverse metabolic and physiological effects, particularly those that would be difficult to detect in animals, and to assess the relevance of experimental findings in animals to conditions of use in humans [Meulenbelt et al., 1998; LSRO, 1999; Wilks, 2001; Food Standards Agency, 2002; FDA, 2003]. For example, endpoints such as allergenicity and gastrointestinal function and tolerance cannot be adequately assessed using common animal models. For complex substances that can alter gastrointestinal function, there may be the need to examine their effects on gut microflora and the absorption of other nutrients; such effects should be examined in susceptible population groups with gastrointestinal disorders or other metabolic disorders related to the complex substance being assessed. Novel food ingredients used in food products that are major sources of nutrition in the diets of susceptible or vulnerable population groups (e.g., infant formulas or products for individuals with a metabolic disorder) would also require testing in humans to ensure safety [IOM, 2004]. For simple chemically defined substances, improvement in the accuracy of science in regulatory decisions related to the size of the interspecies uncertainty factor used to establish a reference dose is another motivation for the use of human studies in risk assessment, provided that stringent conditions for ethical conduct are met [NRC, 2004c; EPA, 2005b]. (A reference dose is an estimate of the daily exposure dose that is likely to be without deleterious effect even if continued exposure occurs over a lifetime.)

Human studies are conducted only after extensive preclinical testing with no identifiable risk or with reasonable certainty of no harm to participants. The types of human studies that are particularly relevant include metabolism and biokinetic studies which do not require test substances to be administered at high doses. Depending on the substance and its expected conditions of use, testing may also involve incorporating the substance in a food matrix in which the substance is typically consumed. Endpoints to be examined should be early or acute biomarkers that reflect inherently reversible effects. Consideration should also be given whether or not the compound under investigation and its metabolites are also produced by the human

body itself [Meulenbelt et al., 1998]. The following two sections discuss additional aspects of characterization of food use in humans: dietary exposure assessment and postmarket monitoring.

6.3.7. Dietary exposure assessment

Exposure assessment is a key component of the risk assessment process. Only intake of toxicologically significant amounts can lead to adverse health effects, even with a relatively toxic substance [Kroes et al., 2002]. As illustrated by the concept of a threshold of toxicological concern, the level of exposure can influence the type and extent of testing of a chemical with limited toxicity data. As with other types of testing, dietary risk assessment is also amenable to a tiered approach [WHO, 1997; Kroes et al., 2002]. A three-tier process is outlined.

Tier 1 – Screening

Several tools are available for screening purposes, including the threshold of toxicological concern (section 6.2.1c), the budget method, and the per capita approach. The *budget method* is based on the concept of a "maximum physiologically possible intake of foods", effectively a food group consumption model based on assumptions about the concentration of food chemicals contained in individual food groups with high dietary intake. In the *per capita approach*, the total amount of the chemical of interest available on the market (production plus imports minus exports) is divided equally across the entire population (or a subpopulation, if it can be clearly and meaningfully identified). Additional assumptions can be made about the percentage of the population consuming the foods containing the substance. This approach is suitable for chemicals added to foods. More refined data based on household purchases of the foods in question can also be used. Depending on the quality and amount of data and the precision of results needed, approaches in tier 2 may be pursued.

Tier 2 – Exposure assessments based on specific data

This tier involves the analysis of actual data on food consumption and on the levels of the chemical present in food, either based on analytical results, on known amounts added, or on levels legally permitted. Estimates of either or both of the consumption of foods containing the chemical of interest and the levels of chemical in the foods can be obtained. Estimates can be presented as "point estimates", reflecting the most likely value of the parameter of interest, or "simple distributions" taking into consideration variability in intakes among individuals in the

population. Further refinement can be achieved by conducting probabilistic analyses to characterize inter-individual variability in exposure and uncertainties in exposure estimates. This distributional approach accounts for all the possible values that each variable could take on and weights each possible outcome by the probability of its occurrence. A distinct advantage of this approach is its capacity to provide sensitivity analysis with respect to uncertainties in parameters. Where appropriate, the relative merits of different interventions that can alter exposure levels can be compared.

Tier 3 – Confirmatory methods

Confirmation refers to the acquisition of data that reflect actual consumption. Confirmation can be achieved using validated biomarkers of exposure or by conducting duplicate dietary intake studies to estimate mean exposure [Kroes et al., 2002]. These approaches are considered "confirmatory" methods because they provide more direct measurements of intakes of food constituents than the theoretical estimates developed with any of the indirect methods in tier 1 and tier 2. However, both the analyses of biomarkers and dietary intakes using laboratory methods are subject to sampling and measurement errors.

The pragmatic tiered approach to exposure assessment outlined above could be replaced by a more sophisticated method if greater accuracy or precision is needed. However, in the proposed RBCS, dietary exposure using theoretical estimates is consistent with the screening step in the tiered approach, as proposed by Kroes et al. [2002]. More refined methods in tier 2 can be applied to substances classified beyond level I in Part A of the proposed scheme.

6.3.8. Postmarket monitoring

Postmarket surveillance or monitoring can complement a thorough premarket assessment of exposure and risk. It is considered to be important for new foods and food substances to be consumed at high concentrations in the diet [LSRO, 1999]. Surveillance can be described as "the ongoing systematic collection, analysis, and interpretation of selected health-related data closely integrated with the timely dissemination of these data to those who need to know" [Cheney, 1996]. In this discussion, the terms postmarket surveillance (PMS) and postmarket monitoring (PMM) are used interchangeably as the distinction is not generally made in the literature.

The need for PMM, as with the need for human studies discussed previously, is due in part to difficulties and limitations of testing complex materials at concentrations with safety factor traditionally deemed to be large enough for animal to human extrapolation. Historically, PMM has served two primary functions: to collect and investigate reports of adverse health outcomes, and to confirm premarket estimates of exposure. PMM has been widely used to examine adverse reactions or unexpected side effects of pharmaceutical products, but is less commonly used to assess the potential risks of dietary alterations. PMM can also be used to assess premarket predictions of adverse effects. The nutritional impact of macronutrient substitutes that have the potential to be consumed as a large part of daily dietary intake and that may affect the intake of other nutrients may also be assessed. Such impact can only be assessed after the substance has been approved for use in foods, and there is widespread use by the population over time [Cheney, 1996].

Proactive monitoring or surveillance requires the implementation of well-designed epidemiological studies with clearly defined objectives to enhance the likelihood of demonstrating causality [Hlywka et al., 2003]. Accurate assessment of exposure is a key element of such an approach. With respect to GM foods and foods containing GM ingredients, additional challenges in ascertaining exposure include the ability to trace the origins of the GM food, and all products derived from the modified food, along the entire production chain [Ritter, 2002]. Effective monitoring would also require the development of a central coding system, with linked barcode and ingredient information that is updated regularly [Robertson et al., 2003]. Integrated systems to enable the flow of validated information among different stakeholders are also important [Wal et al., 2003].

Two types of health effects have attracted attention as they relate to PMM of GM foods: the potential for allergenicity and chronic health effects. Passive monitoring of case reports of allergenic reactions that are acute in nature may be considered, given our limited ability to assess the sensitizing potential of a novel substance via the oral route (as discussed in section 6.3.1). Our current ability to adequately assess the allergenic activity or the likelihood of cross-reactivity of a new substance is also limited by the prevalence of reactions to the allergen in question and the ability to recruit sufficiently large samples with clinically validated allergenic response. Targeted monitoring of highly exposed groups may also be useful (e.g., farm workers exposed to anemophilous GM crops, and workers in facilities processing the GM products). This type of

targeted monitoring may be considered for potential aeroallergens or contact allergens that could sensitize susceptible individuals or promote allergic reactions to an ingested form after repeated exposure [Khalili et al., 2003; Strid and Strobel, 2005; Holsapple et al., 2006].

The methodological tools available to monitor and detect long-term adverse health effects are limited, and likely to be of only marginal value in establishing a meaningful relationship between a dietary component and a specific health endpoint [Ritter, 2002; Hlywka et al., 2003]. Where the concern is related to long-term unintended adverse health effects, resources would perhaps be better devoted to the development of methods that can reliably detect unintended effects with the potential for adverse health effects before the product is approved for general release [Ritter, 2002]. For a GM food with intentionally altered composition or other characteristics to provide health benefits, their perceived added value would facilitate the implementation of practices that permit product tracing, such as identity preservation and labelling. The need for PMM will be triggered by considerations similar to those discussed for complex food ingredients and novel foods in general.

6.3.9. Biochemical and molecular characterization

This section focuses on the role of biochemical and molecular characterization in the assessment of unintended effects in GM crops and its application to predicting potential adverse health effects. Molecular characterization has received considerable attention in the assessment of foods derived from GM crops modified by rDNA technologies. Biochemical characterization is also important in capturing other unintended effects resulting from epigenetic and gene interactions (discussed in section 3.3.2) not readily assessed directly. Biochemical characterization is applicable to assessing all types of GM crops regardless of the method of modification.

As noted in section 3.1, unintended effects can be predictable or unpredictable. Effects are deemed unpredictable when they fall outside our present knowledge of how an organism responds to genetic, metabolic, and environmental perturbations and how these disruptions interact at the system level [Fiehn and Weckwerth, 2003; Nielsen and Oliver, 2005]. Concerns about current methods for assessing the safety of GM foods are primarily related to their perceived inadequacy in detecting unpredictable effects. Advances in genomic technologies have played a key role in promoting the need for untargeted approaches to identifying unpredictable

unintended changes in GM crops. Untargeted approaches refer to the use of transcriptomics, proteomics, and metabolomics as analytical tools. However, advances in our ability to detect increasingly larger pools of analytes with greater sensitivity and speed cannot be translated to increased level of health protection at present because of our inability to interpret the biological significance of data derived from these types of analysis [NRC, 2004a].

a) Biochemical characterization

Current limitations in the routine application of "omics" technologies in the safety assessment of GM crops are recognized [König et al., 2004; NRC, 2004a]. Major limitations relate to our inability in interpreting the health significance of unintended compositional changes in foods, given that a large proportion (70-90%) of secondary metabolites in plants that are present as minor substances in foods are unknown or cannot be identified using available standards [Bino et al., 2004; NRC, 2004a, p. 90]. Their homoeostatic ranges are largely undetermined. It is very difficult to interpret or predict the effects on human health of changes in the composition of a single food in the context of the total diet. This difficulty in interpretation is amplified by the growing body of data suggesting that the variation between non-GM cultivars/genotypes is much greater than the differences between the GM lines [Noteborn et al., 2000; Roessner et al., 2001; Chen et al., 2002]. As well, other sources of variability also exist in food production chains beyond agricultural production, including changes arising from food storage, processing and preparation [Dekker and Verkerk, 2003].

Challenges of a technical nature in using these "omics" techniques outside of a research setting have been reviewed. While the use of these methods may be encouraged during the development phase of a product, their immediate applicability as a required component of safety assessment of GM crops has been questioned [Cellini et al., 2004; ILSI, 2004, p. 41; NRC, 2004a, pp. 84-85]. Among the challenges are:

- the lack of standardized methods for extraction, separation, and quantification of metabolites;
- a lack of spectral libraries that would allow the unambiguous identification of a peak from a given analysis;
- a lack of commercial availability of authenticated standards for many known natural products; and

- the need for improved data management and data-mining systems in order to derive useful information.

To address these challenges, collaborative research has been initiated and progress is being made [Bino et al., 2004; Weckwerth et al., 2004; Kopka et al., 2005]. In the future, it is likely that advances in functional genomics and systems biology can be translated into predictive or interpretive tools that will promote best practices in the genetic modification of crops, particularly in the design of plant metabolic engineering in reducing or minimizing unintended effects. These tools should also be useful in bridging the gaps that currently exist in assessing unintended effects in modified crops.

b) Molecular characterization

Current guidelines on molecular characterization aim to establish that the integrity of the transferred DNA has not been modified as a result of the transformation process. Requirements may differ among jurisdictions, but essentially consist of identifying whether there are significant alterations in DNA sequence to the native gene in the expression cassette used in transformation and in the DNA sequence inserted in the modified plant [ILSI, 2004; König et al., 2004]. Data on the size and copy number of all detectable inserts are required, typically determined by Southern blot analysis. Sequence data extending into the host plant genome are required in some jurisdictions to identify insertion into, and interruptions of, known or suspected open reading frames (ORFs or coding regions) [EFSA, 2004]. However, it is also recognized that comparative sequence analysis may not always be possible due to limited genomic databases for the crop species in question; furthermore, not all the functions and/or sequence patterns of plant genes and non-coding sequences are known [EFSA, 2004]. Sequencing of flanking regions serves an additional purpose as a marker for tracing a transgenic event. If potential chimaeric ORFs are identified, bioinformatics and transcript analyses will be required to investigate the possibility of any expression of fusion proteins and the extent of sequence similarity with known toxins or allergens. Stability of the introduced DNA must also be demonstrated.

Southern blot analysis is a commonly used molecular method in the identification of the copy number and loci of gene insertion, as part of the risk assessment of transgenic plants. This method has been criticized as being insensitive in identifying all insertion-site mutations and the extent of genomic disruption [Wilson A. et al., 2004]. Increasingly other complementary

methods, such as polymerase chain reaction (PCR) or DNA sequencing, are used in addition to Southern blot hybridization. Other cytogenetic techniques such as genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH) have been used to complement Southern blot analysis in research studies. Results from FISH analysis identified significantly larger and more complex transgene loci in greater numbers than previously determined by Southern blot analysis [Svitashev et al., 2000; Makarevitch et al., 2003].

The molecular characterization methods typically used in evaluating bioengineered crops are generally not amenable to assessing changes at the DNA level in plants modified by non-rDNA techniques when the target gene may not be known. Other molecular methods used in conventional plant breeding may be useful for risk assessment. For example, cytogenetic techniques such as GISH and FISH provide a direct visual method for distinguishing parental genomes and analyzing genome organization in interspecific and intergeneric hybrids. Sequential GISH or FISH approach coupled with chromosome-specific cytogenetic DNA markers can reveal the number and identity of alien chromosomes and their homologues in the breeding lines of crops modified by wide hybridization [Kato et al., 2005]. For plants that lack extensive genomic data, techniques such as cDNA-amplified fragment length polymorphism (AFLP) can be used in transcript expression analysis as this method does not require prior sequence information [Goossens et al., 2003]. At present however, there are few regulatory requirements for safety assessment of conventionally bred food crops and there are no incentives to develop new methods or adapt existing methods for risk assessment of this category of GM crops.

6.4. Summary and conclusions

Several observations from the extensive review above on major testing strategies are summarized below. Underlying principles that can be applied to the development of a graded approach to the testing of GM foods follow.

Testing strategies

To meet a growing need for more efficient testing to achieve health risk assessment objectives, there has been a trend towards the adoption of tiered approaches in hazard identification and characterization. Tiered testing may be seen as a pragmatic approach where more extensive (and expensive) methods are applied in higher levels only after simpler methods in lower tiers identify

and prioritize areas of concern. Testing in lower tiers can include a range of methods. They serve a screening function by covering a spectrum of indicators or endpoints relevant to the product being assessed. Subsequent data needs may be guided by the totality of information rather than by the results of any single test.

A tiered approach can be used in combination with other approaches to achieve a balance of structure and flexibility. Testing based on a standardized test battery may offer some advantages in terms of comparability of study results and transparency through uniformity. Alternatively, standardization may be achieved through meeting a core or minimum set of requirements, while specific testing methods may be tailored to the product being assessed. This provides flexibility as new methods evolve. While core or minimum testing or requirements are useful as a starting point, successive levels of testing will necessarily require tailoring to address specific questions identified in the preceding tests. One question that may be addressed in testing in higher tiers is the relevance of toxicity findings to human health risks. Data on the mode of action can help data interpretation in this regard.

Tailored testing that is guided by predictive and confirmatory tools and that is concern-based (fit-for-purpose) should provide a more efficient approach than a standard "blackbox" approach. However, in the context of testing for regulatory purposes, the selection of a testing approach is also constrained by considerations other than testing efficiency. Other considerations include: legislative or regulatory mandate, availability of scientific tools and resources, and risk management objectives [Yokota et al., 2004; NRC, 2006]. Regardless of the testing strategy, reliable characterization of the product prior to toxicological testing is fundamental.

Factors influencing the extent and nature of testing of food-related product categories

For food-related substances, multiple factors influence the extent and nature of testing. They include: exposure levels, history of food use, chemical structural similarity with dietary substances or similar substances with known toxicity, and whether innocuous metabolites can be expected. For complex substances, additional factors such as digestibility and the similarity of digested profile to dietary constituents are also considered. Routine comprehensive toxicity testing is not justified. However, except for the general criteria of dietary levels and chemical structure (such as those developed for chemically defined food additives), few clearly defined

criteria or decision rules are available for the further testing of complex substances within a regimented testing scheme. Explicit decision criteria would increase transparency and decrease the subjective nature of the decision process without sacrificing flexibility.

Testing models

Traditionally, animal models have been the cornerstone of toxicological evaluation. There appears to be a higher standard for the validation of non-animal models, even though the relevance of animal models to health risk assessment in humans is not assured. Few data exist for comparative analysis, except for pharmaceutical products and chemicals with high exposures in humans. The relevance of animal models is dependent on their judicious use, with consideration given to similarities and differences in physiology and metabolism between test animals and humans.

Non-animal models hold promise for reducing our reliance on animal testing in keeping with growing concerns about animal welfare. Emerging "omics" technologies will likely make predictive markers for toxicity more accessible. Progress is continuously being made in these areas. Their general acceptance for regulatory purposes depends on method validation. In particular, the use of a parallelogram approach that integrates *in vitro*, toxicokinetic, toxicodynamic studies, and "omics" technologies, should improve efficiency in animal toxicity testing to better reflect relevance to human toxicity.

Application of testing principles to GM food assessment

In developing a graded approach to testing GM foods, one needs to consider the types of compounds that are likely to be synthesized by crops for food use through genetic modification. It is expected that many substances intentionally introduced or modified in GM foods will be similar to those already present in the food supply, or have a naturally occurring counterpart in living organisms. This is generally the case for crops modified for food use to enhance nutritional or other health benefits. In comparison, gene products from organisms without a history of food use may be introduced for pesticidal purposes, typically at relatively low expression levels. However, biologically derived substances are generally more specific in their activity than novel compounds that are chemically synthesized without a biological analogue. Therefore, the most likely health impact of genetic modification of food crops is the alteration or broadening of the scope of food sources of inherent food constituents, thus increasing

human exposure. Principles of assessing enrichment or fortification of foods using approaches other than genetic modification are in place and are equally applicable to assessing GM foods.

Further, the principles of toxicology used in assessing defined simple chemical substances and those established for complex food substances are also generally applicable to the assessment of intended effects of gene products introduced or modified in GM foods. The assessment of allergenic potential of new substances is an area of development for toxicology in general. The methodological concerns and limitations are not unique to the assessment of GM foods.

The assessment of unintended effects of GM foods would benefit from a rational approach based on probability or risk (not just the possibility of the existence of a hazard). Complementary predictive and confirmatory tools made available through advances in plant functional genomics and systems biology should guide the assessment of unintended changes with a greater focus on biological rather than statistical significance in comparison with an unmodified counterpart.

Box 6-1. Technical information related to human health risks required in premarket assessment of GM foods [Source: Adapted from EFSA, 2004, Annex III].

INFORMATION RELATING TO THE GENETIC MODIFICATION

1. Description of the methods used for the genetic modification

2. Nature and source of vector used

3. Source of donor DNA, size and intended function of each constituent fragment of the region intended for insertion

INFORMATION RELATING TO THE GM PLANT

1. Description of the trait(s) and characteristics which have been introduced or modified

2. Information on the sequences actually inserted or deleted

    (a) The copy number of all detectable inserts, both complete and partial

    (b) In the case of deletion(s), size and function of the deleted region(s)

    (c) Chromosomal location(s) of insert(s) (nucleus, chloroplasts, mitochondria or maintained in a non integrated form) and methods for its determination.
    (d) The organisation of the inserted genetic material at the insertion site including sequence data of the inserted material and of the flanking 5' and 3' regions.
    (e) All sequence information (in electronic format) including the location of primers used for detection.

3. Information on the expression of the insert

    (a) Information on developmental expression of the insert during the life cycle of the plant

    (b) Parts of the plant where the insert is expressed

    (c) Expression of potential fusion proteins

    (d) Methods used for expression analysis

4. Information on how the GM plant differs from the recipient plant in: reproduction, dissemination, survivability

5. Genetic stability of the insert and phenotypic stability of the GM plant

6. Any change to the ability of the GM plant to transfer genetic material to other organisms

    (a) Plant to bacteria gene transfer

    (b) Plant to plant gene transfer

7. Information on any toxic, allergenic or other harmful effects on human or animal health arising from the GM food/feed

    7.1 Comparative assessment

    7.2 Production of material for comparative assessment

        (a) Number of locations, growing seasons, geographical spread and replicates

        (b) Statistical models for analysis, confidence intervals

        (c) The baseline used for consideration of natural variations

    7.3 Selection of material and compounds for analysis

    7.4 Agronomic traits

    7.5 Product Specification

    7.6 Effect of processing

    7.7 Anticipated intake/extent of use

    7.8 Toxicology

        7.8.1 Safety assessment of newly expressed proteins

        7.8.2 Testing of new constituents other than proteins

        7.8.3 Information on natural food and feed constituents

        7.8.4 Testing of the whole GM food/feed

    7.9 Allergenicity

        7.9.1 Assessment of allergenicity of the newly expressed protein

        7.9.2 Assessment of allergenicity of the whole GM plant or crop

    7.10 Nutritional assessment of GM food/feed

        7.10.1 Nutritional assessment of GM food

        7.10.2 Nutritional assessment of GM feed

    7.11 Post-market monitoring of GM food/feed

8. Mechanism of interaction between the GM plant and target organisms (if applicable)

9. Potential changes in the interactions of the GM plant with the biotic environment resulting from the genetic modification

Table 6-1. Concern levels for direct food additives.

Degree of concern High ←----------→ Lower		
Concern level III	**Concern level II**	**Concern level I**
Structure C and > 0.25 ppm[a]	Structure C and 0.0125-0.25 ppm	Structure C and < 0.0125 ppm
Structure B and > 0.5 ppm	Structure B and 0.025-0.5 ppm	Structure B and < 0.025 ppm
Structure A and > 1.0 ppm	Structure A 0.05-1.0 ppm	Structure A and < 0.05 ppm

[a] All concentrations listed in the table are estimated concentrations in the total diet.

Source: Adapted from FDA, 2003 and NRC, 2006.

Table 6-2. Summary of the toxicity tests recommended for food additives.

Toxicity tests[a]	Direct food additives Concern levels			Indirect food additives Dietary levels (ppm)		
	I	II	III	< 0.05	> 0.05	> ~1
A. Acute oral study with rodents		x[e]	x[e]	x	x[e]	x[e]
B. Short-term toxicity tests with rodents (min. 28 days)	x[b]					
C. Subchronic toxicity tests with rodents (90-day) with *in utero* exposure					x	
D. Subchronic toxicity tests with rodents (90-day)		x[b]	x[b,c]			x[e]
E. Subchronic toxicity tests with non-rodents		x[b]			x	
F. Lifetime (2-year) chronic toxicity/carcinogenicity study with rodents with *in utero* exposure			x[c]			x
G. Lifetime (2-year) chronic toxicity/carcinogenicity study with rodents			x[c,d]			x
H. One-year toxicity tests with non-rodents			x			x
I. Reproduction study with teratology phase (min. 2 generations)		x[b]	x[b]		x[f]	x
J. Teratology study		x[f]	x[f]		x[f]	x[f]
K. Short-term tests for genetic toxicity	x	x	x		x[g]	x[g]
L. Metabolism and pharmacokinetic studies			x[g]			x[g]

[a] Not including dose range-finding studies, if appropriate.
[b] Including neurotoxicity and immunotoxicity screens.
[c] An *in utero* phase is recommended for one of the two recommended carcinogenicity studies with rodents, preferably the study with rats.
[d] Combined study may be performed as separate studies.
[e] If needed as preliminary to further study.
[f] If indicated by available data or information.
[g] Suggested.

If carcinogenicity is suspected as a problem, carcinogenicity studies will be done no matter what the circumstances.

Source: Adapted from FDA, 2003 and http://www.cfsan.fda.gov/~dms/opa-tg1.html (accessed on April 3, 2006).

CHAPTER 7 - GRADED TESTING OF GM FOODS

In this chapter, a graded approach to the testing of GM foods is presented. The term "testing" is used broadly to include a range of methods and approaches to the risk assessment of GM foods. The graded approach corresponds to the concern levels in the RBCS described in Chapters 4 and 5. While the approach is primarily based on currently accepted testing methods, it also includes a proposal for a tiered approach to the use of emerging technologies in the assessment of unintended effects in GM foods.

The term "graded" is used here to describe the proposed testing approach in general that corresponds to the concern levels in the proposed RBCS, whereas the term "tiered" retains the meaning that has recognized usage in toxicity testing and other assessments relevant to health risk appraisal.

7.1. Rationale for a graded approach to testing GM foods

Current testing requirements described in guidelines on the premarket assessment of GM foods focus on foods derived from crops that are genetically engineered using rDNA techniques. Major elements of assessment include: molecular characterization of the modified crop, structural-functional properties and allergenic potential of the introduced protein gene product, as well as chemical composition of the derived food. The agronomic-morphological characteristics of the plant are also considered. Nutritional and toxicological assessment using animal studies has also been conducted to provide "an additional measure of safety assurance" [ILSI, 2004]. Justification for the routine inclusion of animal studies in the assessment of bioengineered foods remains equivocal [Domingo, 2000; Hodgson, 2001; Pryme and Lembcke, 2003; Freese and Schubert, 2004].

Questions have also been raised whether current testing methods would be adequate for assessing genetically engineered crops and derived foods that are modified by altering the plant's metabolism and composition [GAO, 2002; SOT, 2003]. Particular concern relates to plants with engineered regulatory, metabolic, and signalling pathways [Wolfenbarger, 2002]. This category of bioengineered crops contrasts with the majority of crops modified by rDNA techniques that have been evaluated and commercialized to date. Most of the initial wave of genetically

engineered crops involve the introduction or modification of a limited number of pesticidal or enzymatic proteins that are expressed at very low levels and are believed to have minimal interactions with the plant's metabolism. Several classes of crystal proteins from *Bacillus thuringiensis* dominate pesticidal proteins in genetically engineered crops introduced to date. The majority of enzymatic proteins introduced to confer herbicide tolerance involve well-defined and limited direct reactions in primary metabolism. Metabolites of the reactions catalyzed by these enzymatic proteins are also not expected to have major metabolic effects in the modified crop. A task force of the International Life Sciences Institute reviewed the applicability of current testing methods for nutritionally modified crops, and concluded that existing assessment processes are suitable for this category of modified foods. However, there may be the need for additional studies, such as nutrient bioavailability studies [ILSI, 2004].

As discussed in Chapters 2 and 3, the modification of the genetic makeup of food crops through genetic engineering using rDNA techniques is among a wide range of methods of genetic modification used in agricultural biotechnology. However, crops modified by conventional genetic modification methods have received little attention with respect to health risk assessment. While there is recognition that the scientific basis of requiring premarket assessment of crops and derived foods based exclusively on a particular method of genetic modification is questionable [Stirn and Beusmann, 2003; Cellini et al., 2004; NRC, 2004a; Wilson A. et al., 2004], no systematic approach has been developed to review or revise this policy.

The above observations support the need for a more rational, graded approach to the testing of GM foods based on transparent criteria regardless of the method of genetic modification. The following sections describe a proposal to meet this need.

7.2. Development process

As part of the process of developing a graded approach to testing GM foods, an extensive literature review was conducted (Chapter 6). It compares testing approaches for several food-related product categories, as well as tiered approaches to assessing different toxicological endpoints. The use of tiered approaches to dietary assessment and biochemical characterization was also reviewed. Alternative testing models and emerging technologies were discussed. The relevance of the review to assessing intended and unintended changes in GM foods was

summarized. Based on the principles and objectives of testing analyzed in this review, drafts of a proposed graded approach to testing GM foods were developed.

Early drafts of the proposal were reviewed by a multidisciplinary group of experts and two groups of toxicologists, totalling 28 experts from eight countries in two consultations (3 and 4) in Phase 2 of the project. Detailed reports of consultations 3 and 4 are included in Appendices C and D in Volume II of the thesis. Consultation 3 included responses to a structured questionnaire from experts with different expertise relevant to GM food risk assessment, as well as specialists in toxicology with or without prior direct involvement in GM foods. Based on comments received from Consultation 3, a modified proposal was prepared, and reviewed by another group of toxicological experts in Consultation 4. Participants' comments were discussed in one group interview and one individual interview. Written comments were also provided by four other participants.

Areas for improvement of the draft proposals were suggested by the expert participants in the two consultations. It was suggested that the proposal should keep the use of currently accepted methods separate from emerging or unvalidated methods. Respondents also preferred a testing approach that is more flexible and tailored to the gaps in information to be addressed. The integration of the testing of intended and unintended toxic and antinutritional effects should also be considered.

7.3. Proposed graded approach to testing GM foods

7.3.1. Principles

Ideally, a testing framework should be structured with clear criteria for the need of specific types of testing. At the same time, the structure should also be flexible and amenable to answering specific questions regarding the product being assessed. As explained below, structure and flexibility are not mutually exclusive [Health Council of the Netherlands, 2001].

The concern levels of the RBCS provide a structure for the extent of testing. A basic premise underlying concern levels in the RBCS is that the level of concern about the health risk of a GM food is based on the type and extent of prior information one has about the GM food in question. This basic information then determines the type and extent of further testing that will

be required to fill critical information gaps. Compared to a product or a substance in the same chemical group in a lower concern level, testing in a higher concern level can be expected to be broader in scope, or to include testing in a higher tier according to a *tiered* approach for a specific endpoint. For reasons explained in section 6.2.2.b, protein substances have low potential for chronic, mutagenic, or carcinogenic toxicity. Differences in the range of testing in levels II and III for this chemical group are small.

Flexibility in testing is also possible by taking into consideration the factors and criteria that determine the concern level to which a product is classified. This means that the nature or the type of specific testing can be *tailored* to fill the information gaps identified. Where a specific potential health effect is identified in the classification process, specific testing in a higher tier may proceed without going through tier 1 testing. Tier 1 testing is most useful where limited prior information is available. Tier 1 testing is typically designed to screen for the most sensitive adverse health effect on which further testing will be focused. Readers are referred to section 6.2 in which the concepts of tiered and tailored approach to testing are described.

It should be emphasized that the tests included in the **Tables 7-1, Parts A, B, and C** (Tables 7-1A, 7-1B and 7-1C) serve primarily to illustrate that a gradation of testing can be applied in parallel with the proposed RBCS. The list of tests is not meant to be exhaustive. The proposed RBCS and associated graded testing approach are intended primarily as an analytical framework for identifying and organizing critical factors to be addressed in minimizing or identifying uncertainty and enhancing transparency in GM food safety assessment. This is *not* intended to be a practical guide in meeting regulatory requirements that may vary among jurisdictions. There is also no attempt to specify test protocols, which are outside the intent and scope of the proposed framework for graded testing.

Testing suggested in the proposed framework in Table 7-1 generally reflects minimum testing for the specific issues identified in the respective concern levels. The rationale and objectives for specific testing are included as part of "considerations". This provides flexibility in modifying the objectives or types of testing where warranted in dealing with products with exceptional characteristics.

Suggested testing focuses on currently accepted methods. Where current methods may be limited in their ability to address uncertainty, new methods are suggested. At present, the use of new methods has not generally been accepted as part of premarket assessment of GM foods. Their inclusion does not necessarily reflect their status or potential as an accepted method. Other methods in early stages of development are also not included (e.g., toxicogenomics and cell-based assays for allergenicity testing).

7.3.2. Overview

In this proposal, testing methods typically used in the safety assessment of GM foods at present serve as a baseline. Current requirements provide a reasonable starting point for data gathering and product-specific testing. Products that meet baseline requirements are classified as Level I and generally require very limited or no further testing. Limited further testing for products in Level I of Part A of the RBCS may entail confirmatory compositional analysis using samples representative of post-harvest handling and processing. Confirmatory compositional analysis applies to gene products expected to have negligible dietary exposure as defined by criteria in the RBCS.

In addition to a standard list of factors that are the core elements of the proposed RBCS, "other considerations" are added to ensure that factors specific to individual GM products are not overlooked. For example, further assessment of products classified as Level I in Parts A and C of the scheme may be triggered by the intended use of the product as it relates to special population groups. *In vitro* digestion assays have become one main pillar of the evaluation process for recombinant proteins in GM foods. However, current *in vitro* methods for assessing protein digestibility may not be relevant in assessing potential adverse health effects of certain types of novel protein substances in individuals with impaired gastric function [Jensen-Jarolim and Untersmayr, 2006]. In addition, the potential for new sources of pollen-borne allergens mediated by GM crops in sensitizing previously unexposed individuals is addressed in few current guidelines [Scientific Institute of Public Health, 2003; EFSA, 2004; Moneret-Vautrin, 2006].

Health concerns about transgenes are most relevant for those of microbial origin. No health concerns were identified in a review of microbially derived transgenes that have been introduced into GM crops and have received regulatory approvals for food use [Kleter et al., 2005]. While

health concerns of transgenes introduced into GM crops do not require routine comprehensive assessment for every GM food, a case-by-case assessment through "other considerations" would be appropriate for GM foods classified as Level I for intended effects (Part A). This proposal is supported by the detailed review of health considerations regarding horizontal transfer of transgenes conducted by Kleter et al. [2005].

An outline of the graded approach for each part of the RBCS is given below with details provided in **Tables 7-1A, 7-1B, and 7-1C**.

Part A – Testing for potential toxic and antinutritional effects from intended changes

A diversity of primary and secondary gene products are considered when testing for potential toxic or antinutritional effects from intended changes. Their testing requirements are differentiated on the basis of several critical factors: dietary history of food use, level of expression and expected change in dietary exposure, prior knowledge of toxicity profile, and structural-functional comparability to known dietary constituents. Other factors that determine testing requirements include the potential for synergistic and interactive effects affecting toxicity of stacked gene products and the potential to affect bioavailability of essential nutrients. A gene product that meets the criteria for both critical and other factors is classified as Level I with the need for limited further testing. However, the intended use of the GM food for vulnerable population groups may trigger additional need for assessment, as explained above.

Level II includes gene products that have a dietary history, but fail to meet the criteria for one of the core factors. A limited group of gene products without a history of food use may also be classified as Level II. They are either pesticidal or enzymatic proteins at low expression level, or other substances below a specified dietary exposure level and without identifiable toxicological concerns with respect to mutagenicity or carcinogenicity. Testing will focus on obtaining information that is missing or limited.

Level III includes gene products that fail to meet criteria for more than one critical factor, or one of the other factors, or any combination of critical and other factors. As expected, testing will be proportionally more extensive for gene products in Level III than those in Level II.

However, for both levels II and III, testing can be tailored to the nature of the gene product, namely, its structural complexity (low or high molecular weight), expected exposure level, and structural comparability to compounds with known toxicity profiles, as detailed in **Table 7-1A**.

Part B - Testing for potential toxic and antinutritional effects from unintended changes

The GM food itself (as opposed to the gene products intentionally introduced or modified) is the focus of Part B of the proposed scheme. GM foods are differentiated into two concern levels on the basis of whether they meet the criteria for critical factors that include: absence of transfer of undesirable characteristics from the donor organism, adequacy of the scope of biochemical characterization, and health significance of changes in key nutrients and known toxicants/antinutrients. For a modified trait that is intended to provide nutrition or health benefits, adequacy of the quality control of the consistency in the expression level of the targeted gene product is also critical. This is of particular importance when the health consequence of instability of the modified health trait is potentially severe. Examples include the reduction of an allergenic protein or toxic substance. Required information and testing for Level I classification are detailed in **Table 7-1B**. GM foods that fail to meet the criteria for any one of the critical factors will be classified as Level II.

Testing of GM foods in Level II can range from expanding the scope of biochemical data, to nutritional or toxicological testing using animal studies (**Table 7-1B**). At present, a targeted approach to conducting metabolite analysis has been advocated, for the reasons discussed in Chapter 6. When coupled with backcrossing and selection based on phenotypic traits and agronomic performance, targeted metabolite analysis can provide a reasonable screen for unintended changes associated with known plant toxicants, nutritive substances, and metabolites of pathways expected to be affected by the genetic modification. An untargeted approach to metabolite analysis has not generally been accepted because of our limited ability in interpreting the health significance of unintended compositional changes in a large proportion of secondary metabolites in food crops.

At present, there are no criteria for determining the adequacy of the scope of analytes with respect to assessing unintended effects for health risk assessment. There are, however, general

guidelines for a targeted approach to the analysis of metabolites in GM foods (e.g., OECD consensus document series on compositional considerations for new plant varieties). The current targeted approach focuses on analyses of single constituent compounds, including macro-and micro-nutrients (particularly those of which the modified crop is a significant dietary source), key toxicants and anti-nutrients. These substances have the highest relevance to assessing potential adverse health effects. Compositional analytes are part of a highly integrated and interconnected set of regulated pathways [FIFRA SAP, 2004]. When compared to unmodified comparator plants grown under the same conditions, metabolic disruptions would be indicated if the levels of selected metabolites are outside of normal ranges.

The current targeted approach generally analyzes over a hundred metabolites in different primary and secondary metabolic pathways. In addition to key nutrients and known toxicants and antinutrients specific to the crop in question, if an enzymatic protein is introduced, a review of all related metabolic pathways that could be directly affected by the enzyme's presence, altered levels, or substrate specificity would also identify other analytes to be included [ILSI, 2004]. For example, if an enzyme is introduced to alter the biosynthesis of β-carotene, the levels of other carotenoids should be measured. Additional assessment may also be undertaken of other key intermediate metabolites at the branch points of the carotenoid and closely related pathways. This current approach provides part of the basis for determining the adequacy of the scope of metabolites analyzed, which is critical in meeting a criterion for Level I for Part B of the proposed RBCS. Further, if there is evidence that genetic modification can affect the plant's susceptibility to endophytic infections or the uptake of heavy metals, additional analyses would be required.

This proposal extends the current concept of targeted analysis to include a tiered approach to an expanded biochemical characterization for GM foods that fail to meet the criteria for Level I. The proposed tiered approach consists of the use of rapid "fingerprinting" of metabolites as an initial step to guide more detailed profiling of metabolites where significant differences are suspected [Catchpole et al., 2005].

The tiered approach to biochemical characterization suggested here should be judiciously applied where such an approach can add value to enhancing our ability in assessing the health risk potential of a GM food with respect to unintended effects. Factors to consider include: the

dietary role of the GM food being assessed, and uncertainty in the breeding history of the modified crop. In the case of poorly characterized genetic modification (e.g., wide-cross hybridization or mutagenesis induced by a chemical agent or by irradiation), a tiered approach to biochemical characterization would also be valuable if the safety of the donor plant is uncertain.

The remainder of this section will examine the rationale for a tiered approach to biochemical characterization of GM foods in Level II, along with practical considerations for and implications of its adoption. Metabolite analysis is discussed in detail because of the diversity of metabolites in crop plants and limited information on their natural variation and their biological significance. Five themes will be addressed:

a) the need for a tiered approach;

b) the scope of analytes in a tiered approach;

c) the predictability of the extent of unintended effects;

d) designing metabolically engineered crops with reduced metabolic disruptions; and

e) integrating biochemical characterization with toxicity testing.

a) Need for a tiered approach

It has been estimated that a typical plant cell and tissue might contain between 5,000 to 10,000 metabolites, while up to 200,000 different metabolites are estimated to occur in the plant kingdom [Fiehn, 2001; NRC, 2004a]. Different analytical platforms are available for metabolomics, including: GC-MS (gas chromatography-mass spectrometry), LC-MS (liquid chromatography-MS), FT-ICR-MS (Fourier transform-ion cyclotron-MS), and NMR (nuclear magnetic resonance) spectroscopy. Detailed reviews on the advantages and disadvantages of these analytical platforms have been published [Fiehn, 2001; Sumner et al., 2003; Cellini et al., 2004; Goodacre et al., 2004; ILSI, 2004; NRC, 2004a; Dunn et al., 2005; Krishnan et al., 2005; Ratcliffe and Shachar-Hill, 2005]. Several studies have shown that 100 to 500 individual compounds can be reproducibly resolved in a single GC-MS analysis of plant tissue extracts [NRC, 2004a, p. 85]. Even though some analyses may yield up to 1,000 different chemical species [Weckwerth et al., 2004], this is still a fraction of the total number of compounds present in a plant extract [Stitt and Fernie, 2003; Sweetlove et al., 2003; NRC, 2004a, p. 91; Weckwerth et al., 2004].

The choice of analytical method is a trade-off between metabolite coverage and quantification as a function of compound resolution, identity, sensitivity, and accuracy [Sweetlove et al., 2003; Dunn et al., 2005; Rischer and Oksman-Caldentey, 2006]. Analytical platforms also have intrinsic biases against certain classes of compounds [Weckwerth and Fiehn, 2002]. Unique compounds in complex mixtures that coelute in the analysis will not be identified [NRC, 2004a, p. 91]. Global analytical techniques can improve comprehensiveness of coverage, although the coverage is not exhaustive. Therefore, setting priorities for analysis by using a tiered approach is needed to improve testing efficiency.

Further, a truly global or exhaustive comparative analysis is an impractical goal for hazard identification. The scope of analytes is by necessity selective as one moves through a tiered approach. A selective scope of analytes may be described as "targeted". Contrary to the views of some critics [e.g., Millstone et al, 1999], a targeted approach to compositional analysis is not necessarily "anti-scientific". A targeted approach, when supported by prior knowledge, a testable hypothesis, and predictive or confirmatory tools, would be more consistent with scientific principles than an untargeted "blackbox" approach.

b) Scope of analytes for a tiered approach

Strategies are needed to more reliably determine the adequacy of the scope of analytes for GM foods classified as Level II, beyond known nutrients, toxicants, and antinutrients in the plant species being modified. Two strategies may be considered. The first strategy emphasizes a total diet perspective in setting priorities for identifying and ranking the relative health significance of food constituents at an aggregate level. Focusing on the modified crop, the second strategy identifies pathways and metabolites that are broadly related to the pathway being intentionally modified, based on knowledge of metabolic networks. In other words, the first strategy aims to establish a priority list of naturally occurring food constituents for which changes in a modified crop would trigger further assessment. This addresses concerns about our limited ability in interpreting the health significance of unintended compositional changes in a large proportion of secondary metabolites in food crops. The second strategy complements the first strategy in determining the need for analysis of priority substances in the modified plant being assessed. The rationale for these two strategies is elaborated below as a starting point for further expert discussion. The development of these testing strategies and associated underlying research

requires contributions from an interdisciplinary group of experts in plant sciences, toxicology, molecular biology, analytical chemistry, and health risk assessment.

(i) Identification of health-relevant metabolites from a dietary perspective

There is a need to set priorities for analyzing secondary metabolites in plants beyond key nutrients and known toxicants and antinutrients that are specific to the crop in question. This need is based on a balance between two considerations: (1) the type of unintended changes in modified foods with a high probability of health risks, and (2) our limited knowledge about chronic or delayed adverse health effects of naturally occurring food constituents [NRC, 1996, p. 36; Speijers and van Egmond, 1997; Essers et al., 1998; Gold et al., 2001].

Concerns about the disruption of normally unexpressed genes in GM crops have prompted critique of focusing analysis on only changes in known nutrients, toxicants, and antinutrients as an indication of unintended effects in GM crops. At issue are two questions: (1) how likely would the disruption of unexpressed genes affect those encoding proteins or enzymes that catalyze reactions that would lead to an increased synthesis or decreased catabolism of toxic metabolites in food crops?, and (2) how likely would changes in "unknown" metabolites lead to adverse health effects? While these questions cannot be answered with quantitative estimates of probability, knowledge about the biosynthesis of secondary metabolites in plants and plant-related toxicants in foods can provide a rational basis for addressing these questions, as discussed in section 4.3.3, Part B.

Briefly, natural products in plants belong to several major chemical classes with pivotal backbone structures. It is the subsequent decoration of the backbone structures that generates the huge diversity of plant secondary products [Jørgensen et al., 2005]. This knowledge can provide a structure-guided approach to priority setting for assessment. Further, the production of secondary metabolites with potent biological activity is likely a rare property of any one molecule [Jones and Firn, 1991; Firn and Jones, 2003]. In addition, many known toxic metabolites are species- and tissue-specific [Cheeke, 1998; Osbourn et al., 2003]. Their biosynthesis requires highly coordinated mechanisms of channelling and compartmentalization [Croteau et al., 2000; Jørgensen et al., 2005]. Unlike prokaryotes in which genes are regulated as clusters (operons), genes encoding enzymes in specific pathways can be located on different loci or frequently even on different chromosomes in eukaryotes [Hadacek, 2002]. This property of

eukaryotes suggests that multiple linked metabolic steps would have to occur concurrently or sequentially to modify specific groups of plant metabolites to effect health-relevant changes. From a dietary perspective, despite the vast diversity of metabolites in the microbial, animal and plant kingdoms, about 200 natural toxicants are known, with less than nine out of the 21 food-borne toxic substances being of plant origin [IFBC, 1990]. These observations support a rational approach with a focus on the probability, rather than the mere possibility, of the potential for adverse health effects of unknown metabolites. This means that priority in assessing unintended effects at the metabolic level should be given to existing plant metabolites, as opposed to completely novel compounds.

Other factors that most likely affect altered expression of endogenous substances should also be considered. As discussed in section 4.3.3, Part B, enzyme substrate specificity can help to predict, in part, the formation of new "hybrid" compounds between the introduced or modified enzyme and endogenous compounds in the host plant [Laurila et al., 1996]. A variety of methods that currently exist can provide information on substrate specificity of gene products and detect changes before developing the transgenic plants. These methods include supplementation experiments, inhibitor application, and enzymological assays using extracts of plants expressing heterologous genes [Martens et al., 2003]. These biochemical methods are important tools in developing metabolically engineered crops and would have been used in screening modified lines for further development.

This proposal contrasts with the current targeted analytical approach that focuses only on changes in metabolite levels in the modified plant relative to respective ranges of natural variation. Statistical significance has been a key criterion in this comparative approach in determining whether the difference is within the range of natural variation. Different approaches to determining statistical equivalence have been used [e.g., Catchpole et al., 2005; Hothorn and Oberdoerfer, 2006]. Determining an acceptable range of natural variation for an expanded scope of plant metabolites will be increasingly challenging. Whether the range of natural variation is a meaningful reference point or indicator for unintended changes may be questionable, particularly if natural variations among unmodified crops are larger than variations within GM crops of the same species [e.g., Catchpole et al., 2005; Lehesranta et al., 2005]. Further, adaptive responses may not be easily distinguished from metabolic disruptions of relevance to human health risks. Limited information is available on the duration of exposure to and health risk implications of

plant metabolites that are induced only in response to biotic or abiotic stresses as part of normal adaptive plant physiology. An alternate dietary focus on metabolites that have the potential for adverse health effects as a starting point for assessment seems justified.

From an implementation point of view, the concept and methodology for priority setting and ranking potential toxic health hazards among naturally occurring food chemicals for toxicity testing is not new [NRC, 1996; Gold et al., 2001]. Databases on food plant toxicants are being developed [Knudsen et al., 2005]. Toxicity databases also exist for other plant-derived substances, such as natural flavouring compounds, that have been evaluated for food use. Screening methods for synthetic chemicals may also be applied to increase the efficiency of testing. However, caution is needed in interpreting results of toxicity testing of isolated food constituents, as their health effects should also be viewed from a dietary context.

(ii) Identification of related pathways and metabolites

Current targeted approach to compositional analysis already focuses on "predictable" changes in metabolites in the modified crop based on existing knowledge of metabolic pathways expected to be directly affected by the enzymatic protein(s) introduced or modified. Expanding the current targeted approach to include changes in metabolites that may not be predicted based on prior knowledge would require the use of emerging techniques. With advances in functional genomics and systems biology, predictive tools are evolving based on information gained from the development of protein interactions and metabolic correlations [McDermott and Samudrala, 2004; Morgenthal et al., 2006]. These predictive tools have the potential to inform the extent of cross-talk among metabolic pathways and the types of metabolites that should be included in an expanded biochemical characterization. To a limited extent, predictive ability in this regard may be enhanced with the identification and use of "marker metabolites" of pathways of interest without the extensive use of functional genomics data [Foyer et al., 2003; Tarpley et al., 2005]. Using predictive tools to identify pathways that could be indirectly affected by the genetic modification and the chemical classes of metabolites implicated is also necessary because analytical platforms have intrinsic biases against certain classes of compounds.

A variety of analytical platforms are already being used as part of screening in GM crop development [GAO, 2002; ILSI, 2004]. The data generated can provide further support as part of premarket safety assessment strategy. Examples of techniques suitable for rapid fingerprinting

of crude extracts with minimal treatment of samples include: FT-ICR-MS, NMR, FIE-MS (flow injection electrospray ionization-MS), and FTIR (Fourier transform infrared spectroscopy) [Rischer and Oksman-Caldentey, 2006]. A range of profiling platforms may be distinguished based on coverage and quantification by class of compounds, association with a specific pathway, or comprehensive inclusion of diverse classes of compounds [Fiehn, 2001; Dunn et al., 2005]. Gas chromatography and liquid chromatography, coupled to MS detectors, are among the major techniques used in the identification and quantification of as many compounds as possible [Rischer and Oksman-Caldentey, 2006].

c) Predictability of the extent of unintended effects

The magnitude of unintended effects is in part a function of the gene product being introduced or modified and the extent of connectivity of the pathway being modified with other pathways in the organism. On theoretical grounds, certain types of proteins introduced into food crops to date are expected to have minimal interactions with the plant's metabolic pathways [Ellis, 2001; ILSI, 2004, p.51], while other proteins that mediate global responses would have a higher likelihood of inducing pleiotropic effects. Examples of proteins in the former category include marker genes and pesticidal proteins that do not act by inducing systemic responses. Proteins in the latter category include those with metabolic, regulatory, or signalling functions, such as transcription factors, regulatory proteins, and enzymes that cause molecular modification of key proteins (e.g., kinases), or that catalyze the synthesis of signalling molecules (e.g., hormones, peptides, small carbohydrates).

Available data appear to support theoretical tenets that the magnitude of unintended effects may be predicted qualitatively, even though the underlying factors may not be known precisely [Le Gall et al., 2003; Defernez et al., 2004; El Ouakfaoui and Miki, 2005]. If the observations in these studies could be replicated and generalized to a broader range of crop types and gene functions, the strategy for analyzing plant metabolites for unintended effects can be further refined. Current targeted approaches may be used judiciously in the absence of extensive metabolite fingerprinting and profiling without necessarily increasing uncertainty, as explained below. Predictive tools that inform metabolic pathway interconnectivity could also help to confirm or refute theoretical propositions.

d) Designing metabolically engineered crops with reduced metabolic disruptions

While heterologous proteins with minimal or global interactions with the metabolism of the host plant can be reasonably predicted, the magnitude of interactions among genes and enzymatic proteins that participate in biosynthetic or catabolic pathways will be more difficult to predict based on current knowledge. However, recent developments suggest that it may be possible to design metabolically engineered plants with reduced unintended effects. Enhancing design capability can improve efficiency in safety assessment. Two examples of such developments are outlined below.

It has been suggested that the most successful metabolic engineering approaches are those that have introduced new pathways into plants [Trethewey, 2004]. A notable example is the introduction of the entire biosynthetic pathway from *Sorghum bicolor* for the tyrosine-derived cyanogenic glucoside (dhurrin) into a model plant [Tattersall et al., 2001; Kristensen et al., 2005]. Unintended effects were assessed by morphological changes and targeted and non-targeted analysis of the transcriptome and metabolome. It was concluded that significant changes could be reduced by inserting the three enzymes required in dhurrin synthesis, when compared to insertion of parts of the pathway by introducing less than the full complement of the required enzymes. There was also a correlation in the extent of changes between observable morphology and biochemically characterized metabolites.

The metabolic activities of a plant are highly coordinated through mechanisms such as the channelling of substrates to their target enzymes through compartmentation of cells into different organelles and sub-structure. There is growing evidence in support of the formation of metabolons as a means by which plants can redirect the synthesis of secondary metabolites with flexibility and precision. Metabolon formation refers to the anchoring to a membrane of multiple enzymes by either membrane-bound structural proteins that serve as "nucleation" sites, or by other membrane-bound proteins such as cytochrome P450s that directly catalyze one or more of the sequential channelled reactions carried out by the metabolon [Jørgensen et al., 2005]. It is expected that a better understanding of metabolic channelling and protein trafficking processes in plant metabolism can enable more effective designs of metabolically engineered plants that reduce, minimize, or avoid unintended metabolic perturbations.

In summary, a range of information acquired or generated as part of designing the modified crop described above may strengthen the current approach to targeted analysis of unintended changes induced by the introduction of non-enzymatic proteins in modified crops. For crops modified using enzymatic proteins or metabolic engineering approaches that are predicted to have low level of interactions with other pathways, predictive tools, such as the ones described here, may be used to justify the adequacy of the scope of metabolite fingerprinting or profiling analysis proposed for GM foods in Level II.

e) Integrating biochemical characterization with toxicity testing

According to current guidelines, for GM foods with complex changes in metabolites or changes with biological significance, toxicity or nutritional testing would likely be indicated [c.f. EFSA, 2004]. As guidelines for animal nutritional testing have been developed [ILSI, 2003; OECD, 2003], this discussion will explore the rationale for an expanded biochemical characterization from the perspective of integration with strategies for assessing the toxicity of mixtures. This is an aspect of the testing of unintended effects of GM foods that has not received much attention in the scientific literature to date.

As noted in section 6.2.3, toxicity testing of whole food presents special challenges for some types of GM foods (e.g. vegetables and fruits), as a much lower margin of safety may be unavoidable when compared to that traditionally accepted for single chemically defined entities. Therefore, the issue of sensitivity of animal bioassays must be addressed. Testing of a food extract may also be considered if the food fraction containing the substance(s) of interest can be identified. In some cases, animal bioassays may have value only as an additional measure of safety assurance when considered in concert with other risk assessment elements [ILSI, 2004, p. 41]. This contrasts with the use of animal toxicity testing as a pivotal component of hazard identification for chemically defined substances. Whether animal bioassays are used as a primary tool for hazard identification or as a secondary confirmatory tool in assessing unintended adverse health effects of GM foods, reliable biochemical characterization of the test food is needed to ensure meaningful interpretation of assay results.

Reliable biochemical characterization is important in animal bioassays for several reasons. First, proper study design requires knowledge of whether any substance of concern is present in the food in sufficient amounts to induce toxicity; i.e., testing sensitivity must be considered [ILSI,

2004]. Without ensuring sufficient sensitivity, no-effect observations in toxicity studies would provide only limited assurances of safety. Second, study design for testing a complex mixture based on a component-based strategy would require knowledge about the identity of either representative chemicals of each class or a proxy representation of a certain class (section 6.2.3). Third, information on a properly characterized test mixture would be valuable information even when whole-mixture testing is the preferred option. Such information can be used in determining whether another similarly produced mixture would require toxicity testing and to what extent. As well, the efficient use of animal models is consistent with the principles of animal welfare in regulatory toxicology. Further, knowledge of the identities of the components in a test mixture would facilitate the use of toxicogenomics as a predictive tool in identifying biomarkers of effect through pattern recognition.

At a practical level, as both intentionally and unintentionally altered substances are part of the composite food to be consumed, it may not always be possible to completely separate the design and the outcome of testing for the two categories of substances. A testing regimen that can assess both categories of changes is especially relevant where interactions among intentional gene products or with food components are of concern. Integrating the assessment of both categories of changes is also appropriate when sufficient quantities of a gene product cannot be purified from the modified crop, or reproduced in an alternate host organism without causing structural or functional changes to the gene product. Membrane-bound proteins are one category of gene products that would require testing in its native form in the modified crop.

Integrating the assessment of both intentionally and unintentionally altered substances in GM foods by way of testing the whole food has been suggested. As noted in section 6.3.9.a, this may be the preferred option if the discontinuation of product development were to follow positive findings in toxicity testing. Conversely, negative findings may not provide assurance of no-effect in the absence of other supporting data, particularly with respect to unintended changes.

Part C - Testing for potential allergenic effects from intended and unintended changes

As with Parts A and B, baseline criteria in determining relative allergenic potential of introduced protein and GM foods are based on generally accepted methods [FAO/WHO, 2001; Codex

Alimentarius, 2003]. Proteins and GM foods that meet the criteria for all critical factors will be classified as Level I. Critical factors include: allergenicity of the donor organism and of the host plant, comparison of amino acid sequence identity with known allergens, and protein digestibility. Proteins and GM foods that fail to meet the criteria for all critical factors are classified as Level II. According to current guidelines, confirmation of negative results from accepted methods may be required of proteins or GM foods classified as Level I.

One generally accepted method for assessing the allergenic potential of proteins or GM food is an *in vitro* test for IgE binding using sera from individuals with clinically diagnosed reactions to the suspect allergenic protein or its food source (specific serum screening). In the proposed testing scheme, this method is recommended for proteins or GM foods that are classified as Level II. A negative outcome from specific serum screening generally requires confirmation with a skin-prick test (SPT) or histamine release assays [FAO/WHO, 2001; Goodman et al., 2005]. Despite the fact that the false positive rate for specific serum IgE binding test can be high and can be affected greatly by test conditions and quality [Goodman and Leach, 2004], a positive outcome from this test would likely lead to termination of product development. Another type of *in vitro* IgE binding test, targeted serum screening, has been recommended in the guidelines of the EFSA [2004] and FAO/WHO [2001] as an additional test or as follow-up to a negative result from specific serum screening. Targeted serum screening of IgE binding is a broadly based test using sera from individuals who are allergic to species distantly related to the source (e.g., if the gene was derived from a dicotyledon, testing using sera from latex-, birch-pollen-, and peach-allergic individuals is required). The usefulness of targeted serum screening has been questioned, as no study has confirmed the predictive value of such a test [Goodman et al., 2005]. In the Codex guidelines [2003], targeted screening is not recommended as a routine practice for assessing GM products. Targeted serum screening is also not included in this proposed testing scheme. Methods under development such as cell-based assays are also not included for reasons discussed in section 6.3.1.b.

Few generally accepted methods are available for assessing the allergenic potential of proteins that are from a source that is not known to be allergenic, or that are deemed to be resistant to pepsin digestion. In addition, no distinctions are presently made about the applicability of the standard pepsin digestibility test with respect to the level of the protein in the GM food, the food matrix, or individual physiological conditions (discussed in Part A). Other types of

immunogenicity of proteins from non-food sources are not considered. There is no standard requirement for assessing new sources of protein for GM crops that are pollen-borne for their potential to sensitize previously unexposed individuals through inhalation. It is also not clear what level of change in endogenous allergenic protein in a known allergenic food should be considered clinically significant.

Given the complex interactions between the host and the allergenic agent, as well as limitations in testing methods for premarket assessment, the monitoring of identifiable high risk groups (e.g., occupationally exposed individuals) may be justified. It may also be necessary to develop risk management strategies (other than food labelling) to address issues that arise in the event of perceived or confirmed cases of allergic reactions associated with a marketed GM food. Such occurrence may not be entirely preventable despite conservative decision-making in the approval process [Goodman and Hefle, 2005].

7.4. Discussion

7.4.1. Key aspects of proposed graded approach to testing GM foods

The graded testing of GM foods presented here provides a structured approach to the assessment of this category of products. The suggested graded testing parallels the respective concern levels in the proposed RBCS. The graded approach can be broadly divided into two main parts: baseline requirements for Level I classification, and further data acquisition and testing for products in Level II or higher. Further testing is tailored to address specific questions that result in the classification of the GM food into the particular concern level for each part of the RBCS. A structured approach based on explicit criteria that allow tailored testing provides flexibility without compromising transparency. This contrasts with published guidelines that are typically descriptive in nature and may lack explicit criteria for additional testing on a case-by-case basis.

Baseline requirements for Level I classification are largely based on testing generally accepted for premarket GM food safety assessment. Testing for potential toxic and antinutritional effects of primary and secondary gene products from intended changes in Levels II and III is generally consistent with that for the applicable chemical class. Choices about the extent and type of testing should also take into account dietary history, level and expected change in dietary

exposure. Further data acquisition and testing beyond Level I suggested in **Tables 7-1 A-C** are partial in nature, and are presented for illustrative purposes only. New methods in toxicity and allergenicity testing are continuing to evolve and have not been included as part of the approaches suggested for further testing.

In contrast, there is less general agreement on the testing of potential toxic and antinutritional effects from unintended changes. A broad-based targeted approach to compositional analysis is proposed as baseline requirements for Level I. An additional requirement to justify the scope of analytes is triggered for products not meeting criteria for secondary factors. The testing approach presented here considers the likelihood of unintended changes with adverse health effects in GM crops with a history of food use. For GM foods in Level II, a tiered approach to an expanded biochemical characterization is presented, taking into consideration developments in emerging methods in genomics and metabolite fingerprinting and profiling. It is expected that these new techniques will assume growing importance in the design and development of GM crops, particularly those involving extensive metabolic engineering. Therefore, a testing approach that keeps pace with technical developments should not present unacceptable barriers to adoption of new test procedures when the methodological aspects are validated for hazard identification purposes. The rationality of the routine use of animal studies in assessing unintended changes is questioned. Whole food toxicity testing that takes into account principles of testing mixtures is suggested.

The assessment of the allergenic potential of introduced proteins and GM foods presents a different type of challenge. The predictive value of the basic tests for assessing allergenicity has not been rigorously evaluated. Bioinformatics techniques are evolving. Few studies have attempted to define statistically significant predictive values for allergen-specific *in vitro* IgE assays or skin-prick test that would circumvent the need for *in vivo* challenge tests [Goodman et al., 2005]. Positive predictive values ranged from 25 to 75%, depending on the food in question and the disease prevalence [Germolec et al., 2003]. Predictive values for the pepsin digestion assay reported in one study [Bannon et al., 2002] did not explain the basis for the cut-off level defining pepsin stability, nor did it include considerations of test accuracy, sensitivity, specificity, and how the reported predictive values may be influenced by prevalence of allergenic reactions to the proteins tested [Hoffmann and Hartung, 2005]. The clinical significance of changes in the level or potency of endogenous allergens in GM foods is also unclear. Given that allergic

reactions are in large part a function of individual susceptibility, ethical concerns about testing in humans further limit the acquisition of relevant data.

7.4.2. Comparison with current approaches to testing GM foods

As the majority of GM foods that have been marketed to date would be classified as Level I using the proposed scheme, no comparison can be made regarding the relative efficiency of the proposed vs. published approach. However, several qualitative differences between the proposed approach and published guidelines exist, principally in the application of targeted biochemical characterization and repeated dose animal toxicity testing in the assessment of unintended effects. An analysis of these two aspects of the proposal is presented in detail in this chapter. As noted previously in Chapter 6, there is a reliance on animal testing in the assessment of unintended effects by authors who are critical of current approach that does not mandate animal testing. As well, there have been critiques of the unscientific nature of a targeted approach to compositional analysis perceived based on a poorly understood or articulated concept of substantial equivalence.

It is expected that the majority of GM foods submitted for premarket safety assessment would be classified as Level I. However, a scheme such as the proposed RBCS should be anticipatory in nature, and should be capable of identifying products requiring further testing, despite the low probability of this occurring at the premarket stage. The proposed RBCS and graded testing approach should have value in guiding structured assessment and testing at an early stage of product development. As product safety is not merely an activity or outcome at the end of product development, product safety should be a design criterion from the outset. A structured approach to evaluating all critical factors included in the proposed RBCS should contribute to safe product design.

7.4.3. Potential limitations

A limitation of the proposed testing approach is that it is primarily based on available and generally accepted methods. The suggested approach does not integrate the testing of intended and unintended toxic/antinutritional effects. This would likely require consideration of test designs, which is beyond the scope and objective of this phase of the research. The suggested approach also did not elaborate on the integration of *in vitro* and *in vivo* animal and human testing. Future directions in toxicological evaluation will be the subject of a NRC report due for

publication next year [NRC, 2006]. It would be worthwhile to revisit the graded approach to testing GM foods presented here in the light of emerging testing approaches in the new NRC framework.

Questions may be raised regarding the "correctness" of the proposed graded approach to testing. Limitations of current testing methods are identified in the literature review. However, there is no gold standard against which the adequacy of a testing approach or strategy can be compared. While complementary tests in a test battery may be evaluated for their collective predictive value, none of the published tiered testing strategies presented in the literature review has been subjected to validation for "correctness".

The proposed approach may also be criticized for not including decision points with clear criteria for determining whether or not to proceed with testing. In the context of testing as part of premarket safety assessment of GM foods, uncertainty in meeting criteria for Level I classification in any one part of the proposed RBCS would likely lead to one of the following scenarios:

- discontinue further testing and product development;
- continue testing to clarify or refute a potential "false positive" finding from a previous test; or
- continue testing to better define an adverse health endpoint in terms of setting acceptable exposure limit.

To a large extent, the nature and extent of testing depends on the type of action that can be taken following testing. This consideration is especially relevant in testing for potential adverse health effects of unintended changes. Practically, assessing the toxicological significance of unintentional novel substances or substances at unexpected altered levels with a view to continue product development is meaningful only if the substance(s) of concern can be removed or reduced in level in the modified food. Similarly, withdrawal from further product development is probably the most likely outcome for a GM food that contains an uncertain introduced protein with respect to allergenic potential, as already noted. The potential for severe health consequences of allergenic reactions in sensitive individuals promotes a conservative

approach to decision making. From the product developer's perspective, the decision to discontinue product development would preferably be taken early using more efficient and effective screening methods.

In conclusion, testing approaches should remain flexible in light of advances in technologies and should tailor to the substance or the food to be assessed. In this context, the graded testing approach presented in this chapter is offered as a starting point to stimulate discussion of the strengths and limitations of current approaches and how they can be enhanced for purposes of population health protection.

Table 7-1. Data and testing requirements by concern levels.

PART A - Potential for toxic and antinutritional effects from intended changes in gene products introduced or modified in GM foods

Types of data/testing	Baseline requirements
Ia - Gene products with a prior dietary history	
Prior knowledge Demonstration of prior dietary history.	Naturally occurring constituent in foods, or other substance with a history of consumption from foods without reported adverse health effects.
Structure-function ***Protein substances*** Bioinformatic comparison of amino acid sequence identity with known mammalian toxins. Pepsin digestibility under standard conditions. Physical-chemical properties – electrophoretic mobility and/or molecular size Functional tests – immunoreactivity, bioactivity (target specificity or enzyme substrate specificity). ***Non-protein substances*** Comparison of chemical structure with authenticated standard.	Structurally similar and functionally equivalent to the native form of dietary substance. Structurally identical to standard.
Exposure estimates Tier 1 dietary assessment (theoretical) based on levels of proteins/non-proteins from biochemical characterization.	No expected change in total dietary exposure (consumption ratio > 1) with wide margin of exposure.
Prior knowledge Information to support the following properties of the gene product: a/ Specificity of mode of action not relevant to mammalian toxicity, or b/ Simple chemical structure suggestive of low order of oral toxicity. AND c/ No unusual digestion and degradation products in modified crop and in mammalian metabolism from consumption of the gene product. d/ No synergistic toxic effects expected between gene products introduced or modified and no interactions between gene products and other food.	*Primary gene products (protein substances) and secondary gene products (metabolites of enzymatic protein introduced or modified)* - low safety concern based on known biological activity of the gene product of interest or other substances with similar chemical structure. - comparable processing conditions between food derived from the modified crop and from the gene source. *Additional requirements for secondary gene products (metabolites of enzymatic protein introduced or modified)* - known metabolism (including catabolism) in the same or genetically related plant species and in mammalian organisms - not known to interact with other compounds in the modified food in comparable dietary context.

Types of data/testing	Baseline requirements
Ib - Gene products with negligible dietary exposure	
Biochemical characterization Transcript and protein analysis of the edible parts supporting the absence of expression of target gene product(s).	Gene product not expressed, detected, or changed in the edible parts of the modified plant through gene silencing or tissue targeted expression, and a/ Gene product has no nutritional role, or b/ Edible part is used as a food ingredient in limited form as a highly refined and well-defined chemical entity.
Exposure estimates Tier 1 dietary assessment (theoretical).	Total dietary intake of gene product(s) below the threshold of toxicological concern for the chemical class where applicable. *Not applicable to protein substances and several categories of chemicals and endpoints for which threshold levels have not been determined at present* [Barlow, 2005].
Ic - Gene products produced by well-characterized GM process based on prior experience	
Biochemical characterization Transcript and protein analysis.	Gene products expressed at expected levels based on prior experience.
Exposure estimates Tier 1 dietary assessment (theoretical).	No expected increase in total dietary exposure.

Part A - Gene products classified as Level I (meeting baseline requirements) - suggested further data acquisition/testing*

Types of data/testing	Considerations
Ia - Gene products with a prior dietary history	
Dietary assessment (tier 2) Probable level of substitution in the diet. Changes in intakes of all or selected nutrients. Population groups who are most likely to be significantly affected.	*The gene product is a complex (high molecular weight) substance consumed at appreciable level in the diet in a modified food that is intended or likely to* - replace specific foods in the diet, or - lead to unintended changes in nutrient intakes and/or changes in existing dietary habits.
Metabolism and Toxicology Acute toxicity test	*Pesticidal proteins* Acute toxicity in rodents is normally required for confirmatory purposes in some jurisdictions.
Dietary assessment (tier 2) Anticipated intakes of the gene product(s) and related substances in the modified food for susceptible groups.	*Other considerations* - e.g., Are there concerns with special population groups? Special population groups may include: - individuals who have intakes higher than the "average consumer" not covered by theoretical estimates - individuals who have special physiological or

Types of data/testing	Considerations
	metabolic conditions, - the modified food being used as a major ingredient in infant foods.
Applicable data/tests e.g. Alternate digestibility test under conditions that simulate gastric and intestinal conditions of target user group. e.g. Information on natural prevalence/distribution, extent of similarity to or divergence from other naturally occurring microbial genes.	Are there concerns with the relevance of the protein digestibility data for or other groups with impaired gastric function? If the transgene is from a microbial source, are there characteristics of the transgene and its introduction into the modified plant that would trigger the need for special assessment relative to human health risks?
Ib - Gene products with negligible dietary exposure	
Chemistry Analysis of target gene product(s) in - representative post-harvest samples - processed ingredient: conformance with established ingredient specifications.	Analytical confirmation of the absence of target gene product(s) is required to ensure that no safety concerns would arise from commingling with non-edible parts of the modified crop during harvesting, or through concentration during processing if the modified food is used as a source of food ingredient.

Part A - Gene products classified as Level II - suggested further data acquisition/testing*

Types of data/testing	Considerations
Gene products in Level II have limited or equivocal information on one of three critical factors: expected change in dietary exposure (A.3), toxicological profile (A.4), or structure-activity characterization related to pepsin digestion only (A.5). Further assessment of one of these applicable concerns is indicated.	
IIa - Substances with a prior dietary history i) For gene products with dietary history in Level II that have limited data on expected changes in dietary exposure, further assessment of this group of substances will focus on dietary assessment. Different extent of dietary assessment is suggested, depending on the nature of the substance.	
Dietary assessment (tier 2) ***Substances with known toxicity*** Detailed dietary exposure assessment using a probabilistic approach.	A probabilistic approach to dietary assessment (with variability and sensitivity analysis) is recommended to increase confidence that estimated intakes are below established upper limit.
Substances with limited empirical toxicity data Assessment of probable expected intakes. Assessment of the extent of normal variation in dietary intakes of the substance of interest.	Data are needed to verify that no appreciable increase in intake of the substance is expected in the context of normal variations in intake in the diet.

Types of data/testing	Considerations
ii) For gene products with dietary history in Level II that have limited or questionable data regarding toxic or antinutritional properties, information on their potential for mammalian toxicity or antinutritional effects under normal conditions of consumption is limited, but their chemical structures and predicted metabolism suggest low order of oral toxicity. Information on the expected change in dietary exposure of this group of substances is considered sufficient. Further assessment of this group of substances will focus on metabolism and toxicology. The extent and type of toxicity-related testing depends on the nature of the gene product and prior knowledge.	
Physicochemical characterization	Prior to conducting metabolism and toxicity testing, detailed characterization of the physical and chemical properties of the test substance is necessary. This is particularly relevant for non-protein substances for which detailed characterization is not part of the baseline requirements.
Metabolism and Toxicology ***Non-protein (low molecular weight) substances*** Absorption/distribution/metabolism and excretion (ADME) study.	ADME study facilitates the design and interpretation of toxicity studies, but is not suitable for high molecular weight substances that are readily digested.
Repeated dose toxicity study in mammalian species (90 day minimum).	Repeated dose toxicity study is indicated where ADME study suggests that metabolites of the substance are not innocuous, or the substance can accumulate in body tissues.
Short-term tests for genotoxicity.	*In vivo* genotoxicity tests are not indicated unless a substance is bioactivated *in vivo* through pathways not reproduced by standard activating systems used in *in vitro* assays. A reduced battery, or even no testing, could be acceptable in cases where considerations based on physico-chemical, structural and pharmacokinetic or metabolic properties would indicate lack of genotoxic activity. On the other hand, supplementary tests may be indicated where these considerations lead to additional concerns or where an aneugen is suspected [Eisenbrand et al., 2002].
Protein substances expressed at high levels Comparison of proteolytic profiles between recombinant and native proteins.	*Dietary protein that is resistant to pepsin digestion in a standard test* (without other safety concerns) - Comparison of stability characteristics between the recombinant and native protein is suggested. If the proteolysis profiles between the recombinant protein and the source protein in its native form are similar, no further study would be required. *If there are concerns about digestibility of the recombinant protein relative to the source protein, repeated dose toxicity study may be indicated.*
Protein substances at low expression level Repeated dose toxicity study in mammalian species (14-28 day).	*Pesticidal or enzymatic protein at low expression level* - Toxicity testing may not be required if the protein is denatured under conditions of processing or

Types of data/testing	Considerations
	preparation of the modified food. Reliable stability data may be sufficient without further testing for such a protein substance.
IIb - Substances with a questionable dietary history (food constituents substantially modified) ***IIc - Substances without a dietary history (novel substances with limited/defined structural concerns)*** Substances in these 2 subcategories in Level II are either i) pesticidal and enzymatic proteins at low expression level and have *no* structural, functional, or other characteristics suggestive of toxic or antinutritional properties, or ii) non-proteins without identifiable toxicological concerns with respect to mutagenicity or carcinogenicity and estimated level of exposure in the diet (< 0.5 ppm for low molecular weight chemicals, and < 0.1% for high molecular weight chemicals).	
Dietary assessment (tier 2) ***Pesticidal and enzymatic proteins and non-protein substances*** Assessment of the extent of exposure to the new protein/substance.	To assess likely exposure to a new protein/substance in the diet, data are needed with respect to: - variability in the levels of protein expression in the edible part(s) of the modified crop - aggregate exposure in all food crops potentially containing the new protein/substance.
Metabolism and Toxicology ***Pesticidal and enzymatic proteins*** Prediction and identification of peptide fragments of concern using bioinformatic and analytical techniques.	*Novel pesticidal or enzymatic protein that is resistant to pepsin digestion in a standard test* - It may be necessary to predict and identify the peptide fragments using bioinformatics prior to conducting an acute toxicity test.
Acute toxicity.	A novel protein in Level II does not have structural-functional similarity to proteins that have known toxic or antinutritional properties. Since the majority of known toxic proteins act through acute mechanisms, high dose, acute testing would generally be sufficient as an initial toxicity test.
Non-protein substances ADME Repeated dose toxicity (90-day). Short-term tests for genotoxicity.	Suggested testing is similar to that for non-protein substances with dietary history (IIa).
Reproduction and developmental toxicity.	Additional studies (e.g., reproduction and developmental toxicity) may be required if preceding tests (physicochemical characterization, ADME, or repeated dose study) indicate other concerns (e.g., the substance is likely to cross placental barrier, or is structurally similar to known reproductive/ developmental toxicants).

Part A - Gene products classified as Level III - suggested further data acquisition/testing*

Types of data/testing	Considerations
Gene products in Level III have limited or equivocal information on more than one characteristic related to dietary exposure, toxicological profile, and structure-activity (factors A.3, A.4.1/4.2, A.5 in the proposed RBCS). Further assessment in more than one of these areas would be triggered.	
Data and testing listed for Level II More than one type of data or testing suggested for Level II.	Applicable to gene products that do not meet the criteria for more than one factor (A.3, A.4.1/4.2, A.5).
The following illustrates other types of data or testing that may be required if the criteria for one or more of factors A.6 and A.7 in the RBCS are not met.	
Plant metabolism ***Non-protein low molecular weight substances*** Investigation of the catabolic routes of the substance of interest.	Information on the catabolism of the secondary gene product (metabolic product of the introduced enzyme) may be indicated when - the introduced enzyme and/or its metabolic product is expressed at high levels in the modified crop and the metabolic product(s) of the enzyme have structural characteristics suggestive of toxicity - the enzyme is introduced to metabolize a known toxic substance (e.g., herbicide, mycotoxin)
Mammalian metabolism and Toxicology ***Non-protein substances*** Absorption/distribution/metabolism and excretion (ADME) study of the purified form of the substance, if not already indicated for testing under Level II.	Information on the digestion or degradation products of the secondary gene product is indicated when there are concerns that they are not innocuous substances. *Complex (high molecular weight) non-protein substance consumed at high levels* - Additional testing may include, e.g., effects on gut microflora, if the substance is not readily digestible. - Complex substances that are digested similarly to well known sequences of amino acids, mono- and di-saccharides, and fatty acids may not require chronic toxicity test unless they are expected to be consumed in large amounts or have widespread use.
Effect of gene product on absorption/ bioavailability of key nutrients.	If interactions between gene products and other food constituents are of concern, comparison of absorption/ bioavailability of key nutrients of concern in the food matrix with and without the gene product is recommended. If the stability of the gene product, which is a known inhibitor of nutrient absorption, is affected by heat, issue of bioavailability or antinutritional effects should be considered. - Where the food derived from the GM crop is processed or prepared dissimilarly from the food

Types of data/testing	Considerations
	derived from the gene source (e.g., food derived from the gene source is eaten in a cooked or processed form, while the modified food is primarily eaten raw), concern for bioavailability would require further assessment.
Pesticidal and enzymatic proteins ***Proteins expressed at higher levels*** Repeated dose toxicity (28-day).	Proteins in Level III include those that are structurally or functionally similar to proteins with known toxic or antinutritional properties (e.g., protease inhibitors and lectins). Even for these proteins, toxicity is generally manifest after short-term dosing for 1-2 weeks. A 28-day study (including immunotoxicity and neurotoxicity screening) is generally considered sufficient. *Proteins expressed at levels higher than those typical for pesticidal and enzymatic proteins* - Further assessment of cell-mediated immune response and functional immunogenicity may be indicated based on results from the 28-day test, or concerns regarding stability to digestion or processing.
Protein and non-protein substances Repeated dose toxicity (90-day) study of synergistic toxic effects of more than one gene products introduced or modified.	Toxicity study of synergistic toxic effects between gene products introduced or modified is indicated when prior knowledge suggests that the gene products might interact in amplifying their toxic effects.
Specific toxicity tests (e.g., reproductive and developmental toxicity, immunotoxicity).	Specific toxicity tests may be indicated by findings in 28- or 90-day toxicity test, or triggered by a property or structure of the substance that is similar to those in chemical classes that are toxicants of known endpoints.

* Suggested further data acquisition/testing is provided only to illustrate that the range of testing can vary with concern levels classified using the proposed RBCS and with the nature of the substance being assessed. Appropriate testing should be tailored to all prior information available on the substance. See Chapters 4 and 5 for detailed descriptions of Levels I, II, and III.

PART B - Potential for toxic and antinutritional effects from unintended changes in GM foods

Types of data/testing	Baseline requirements
Ia and Ib - GM foods derived from crops transformed by well characterized or poorly characterized gene modification	
Secondary data/testing is marketed with * (for all GM crops) or § (for poorly characterized genetic modification processes only). Where secondary data/testing is judged to be limited or equivocal, the adequacy of the scope of biochemical characterization should be justified.	
Prior knowledge Safety of donor organism, or genetically related organisms in the same genus (poorly characterized gene modification). *Safety of host plant.	Donor organism is not a source of known toxicant, antinutrient or pathogenicity/infectivity. *If the safety of the donor organism is uncertain, further assessment would be required. This may include the need for experimental evidence, as suggested below for characterized or poorly characterized gene modification.* *If the safety of the host plant is uncertain, adequacy of the scope of analytes in biochemical characterization should be justified.*
Characterization of genetic material being transferred Characterization of DNA insert and absence of expression of known toxins associated with the donor organism, or Characterization of removal of genetic material from donor organism.	*Characterized gene modification* Absence of transfer of undesirable characteristics to the host plant is demonstrated. *Poorly characterized modification* Sufficient backcrossing in removing large percentage of genetic material from the donor organism is demonstrated.
Prior knowledge and experimental evidence *Function of the expression product of the introduced/modified gene. *Extent of effect of the gene product in the modified plant. *Substrate specificity of enzymatic gene product.	The following parameters are used as indicators of low potential for novel/altered patterns of metabolites. Protein gene product has well-characterized function. Gene product does not have a systemic effect on the metabolism of the modified plant. Gene product has narrow substrate specificity and is unlikely to interact with endogenous substrates to form novel compounds.
Characterization of DNA sequences inserted §In the introduced/modified insert. *Bordering site of insertion/modification.	The absence of open reading frames (ORFs or unknown DNA sequences) is used as an indicator that the interruption of coding or regulatory DNA sequences in the modified plant is unlikely. *If ORFs are identified, further data should be provided to demonstrate that they are unlikely to be transcribed or expressed.*

Types of data/testing	Baseline requirements
Characterization of biochemical and other relevant parameters Core targeted analysis - key nutrients - known toxicants/antinutrients - where applicable, other metabolites directly related to the modified metabolic pathway and known characteristics of the modified crop relevant to the safety of the GM food	Composition of the food derived from the modified crop and physical chemical properties and biological activity of relevant parameters (e.g., heat stability of protease inhibitors) should demonstrate that the analytes are either - within acceptable ranges of natural variation, or - not expected to have adverse nutritional, metabolic, or toxicological effects.
Other product-specific data Consistency in lot-to-lot expression of the targeted gene product.	For a modified trait that is intended to provide nutrition or health benefits, evidence should be provided to demonstrate adequate quality control. Priority consideration may be given to those health-related traits that are intended to reduce toxic or allergenic substances in crops for human consumption.
Ic - GM foods derived from crops modified by well-characterized modification process based on prior experience.	
Molecular characterization Characterization of DNA sequences inserted. **Biochemical characterization** Targeted analysis - key nutrients - known toxicants/antinutrients - where applicable, other metabolites/parameters considered critical based on prior experience	No unusual patterns are observed based on prior experience. Targeted analysis is needed to verify that relevant metabolites of concern are present at expected levels in the modified food based on prior experience.

Part B - GM foods classified as Level I (meeting baseline requirements) - suggested further data acquisition/testing*

Types of data/testing	Considerations
In general, no further data and testing would be required for GM foods classified as Level I for unintended effects. However, if the gene modification involves targeted gene integration, excision, or expression, additional types of data/testing may be required, depending on concerns related to: stability of insert, binding-site specificity if a recombinase is used in marker gene removal, or specificity and mode of action if a nuclease or DNA repair protein is used (discussed in section 2.4.2, 2.4.3, and 3.4.4). GM foods classified at Level I for unintended changes meet baseline requirements for critical factors related to the safety of donor organism and of host plant, absence of unknown DNA sequences or their expression in the introduced/modified insert. Where uncertainty exists with respect to secondary data/testing, adequacy of the scope for biochemical characterization can be reasonably justified. When compared to the unmodified counterpart, biochemical characterization reveals no changes of health significance. Where the modified trait is intended to provide nutrition or health benefits, data on quality control is sufficient to ensure stability of the modified trait. Where applicable, data are sufficient to address issues related to targeted gene integration, excision, or expression.	

Part B - GM foods classified as Level II - suggested further data acquisition/testing*

Types of data/testing	Considerations
GM foods in Level II have limited or equivocal information on one or more of the following: - absence of transfer of undesirable characteristic from the donor organism - adequacy of the scope of biochemical characterization - health significance of changes in key nutrients, known toxicants/antinutrients - for a modified trait that is intended to provide nutrition or health benefits: adequacy of quality control of lot-to-lot consistency in the expression level of the targeted gene product. Further data and testing requirement depends on the type of uncertainty associated with the GM food, as outlined under "considerations".	
Applicable toxicity testing Testing of unintentionally expressed toxin.	Where a suspected toxin from the donor organism is expressed in the modified plant, toxicity testing of the toxin will be triggered. However, a likely outcome would be the discontinuation of product development and testing.
Animal nutritional study	Animal nutritional study (e.g. using rapidly growing broiler chickens) may be indicated if there is limited or questionable information on the health significance of changes in key nutrients or antinutrients.
Animal toxicity study Testing of whole food or food extract.	Toxicity testing is indicated if there is limited or questionable information on the health significance of increase in known toxicants/ antinutrients, or alterations in the levels of compounds with limited information on toxicity or antinutritional effects.
Other product-specific data Quality control plan and its implementation. Consistency in expression level of target gene products.	Limited or questionable information on the adequacy of control of seed quality.
The following types of analysis have not generally been required or accepted as part of pre-market safety assessment of GM foods at present.	
Tiered biochemical characterization Metabolite fingerprinting/profiling	Tiered biochemical characterization (see section 7.2.3, Part B) is suggested for GM foods in Level II for which criteria for factor B.5.3 are not met. This factor requires a reasonable justification of the adequacy of the scope of metabolites analyzed.
Animal toxicity study Component-based testing	Component-based toxicity testing can be applied to a mixture for which the number of components of concern is limited, or for which a proxy representation of chemicals in the mixture can be selected (see section 6.2.3).

PART C - Potential for allergenic effects from intended changes in gene products and unintended changes in GM foods

Types of data/testing	Baseline requirements
Gene sources **Prior knowledge** Allergenicity of donor organism. Allergenicity of host plant.	Donor organism is not a known source of allergenicity (including common and less common sources) and not a known source of gluten-sensitive enteropathy. Host plant is not a common source of allergenicity or gluten-sensitive enteropathy.
Protein gene product **Bioinformatic analysis** Comparison of amino acid sequence identity with known allergens.	The protein is unlikely to cross-react with known allergens. - No sequence identity of ≥ 35% over 80 or more amino acids with known allergens based on a sequential search. Requirement for no identical matches in amino acids in a short sequence (e.g., 6-8 contiguous amino acids) may be considered questionable as recent evidence does not support its use [Goodman and Hefle, 2005].
Data specific to the protein being assessed *In vitro* pepsin digestibility test.	No stable peptide fragments within accepted time limits and under standard testing conditions, depending on the prior knowledge of the protein. *If a protein has only questionable digestibility characteristic under standard test conditions, the following secondary factors would also be considered.*
Glycoprotein structural analysis.	The protein does not have structures most associated with cross-reactivity (e.g., α(1,3)-fucosyl and β(1,2)-xylose residue).
Prior knowledge Functional relationship to specific protein families or functional categories commonly associated with allergenicity.	The protein is not related to protein families or functional categories of prolamin, Bet v 1, cupin, profilin, lipocalins, parvalbumins, seed storage proteins of the 2S albumin type, pathogenesis-related protein families (2, 3, 4, 5, 8, 10, and 14 comprising β-1,3 glucanases, chitinases of other classes I and III, and lipid transfer proteins).
Heat stability.	This is *not applicable* to lipid transfer proteins which are generally heat labile and are allergenic.
Abundance (level of protein).	The role of level of protein in the modified food in contributing to allergenic potential is recognized. However, no upper level can be defined across types of food and protein. For illustrative purposes, the following provisional cut-off levels are suggested. Pesticidal and enzymatic proteins: < 0.1% protein.

Types of data/testing	Baseline requirements
	Other proteins: < 1% total protein.
Biochemical characterization Protein profiles (including known allergenic proteins) in the modified plant.	*All* No change in protein profiles. *If the host plant is a common source of allergenicity or gluten-sensitive enteropathy.* - The GM food should also be classified as having low potential for unintended effects (Level I in Part B).

Part C - Protein gene products classified as Level I (meeting baseline requirements) - suggested further data acquisition/testing*

Types of data/testing	Considerations
Protein gene products in Level I meet the criteria for all critical factors related to the allergenicity of the gene source, amino acid sequence identity with known allergens, and resistance to pepsin digestion. Where the host plant is a common source of food allergen or gluten-sensitive enteropathy, no unintended changes in known endogenous allergenic proteins are identified. In addition, the potential for unintended changes in other metabolites is also judged to be low based on biochemical characterization. Therefore, no further testing is recommended for recombinant proteins that are expressed in the edible parts of the modified crop, unless the recombinant proteins are pollen-borne. However, baseline requirements may not be sufficient to evaluate allergenicity if the protein gene product is a novel protein or a protein from a gene source without prior dietary history to sensitize exposed individuals through an oral route. If a protein is expressed in the pollen, the following questions may be asked. - Is the modified plant considered anemophilous? - Are population groups, other than occupational groups, likely affected? Other considerations regarding protein gene products may include - Are the protein digestibility data relevant to individuals with impaired gastric function?	

Part C - Protein gene products classified as Level II- suggested further data acquisition/testing*

Types of data/testing	Considerations
Protein gene products in Level II include proteins derived from known allergenic sources, judged to have the potential to cross-react with known allergens, or being resistant to pepsin digestion and having other structural-functional characteristics associated with known allergens. Endogenous proteins in a modified crop that is a common allergenic source and is classified as Level II for unintended changes are also classified as Level II with respect to allergenic potential. Further testing is outlined below.	
Specific serum screening for IgE binding, using - food extract	Specific serum screening test is applicable to a protein that is

Types of data/testing	Considerations
- pepsin-digested food extract, and/or - purified protein	- from a known allergenic source or has amino acid sequence identity with a known allergen. - resistant to pepsin digestion and has at least one other characteristic associated with known allergens. - modified food derived from a common allergenic source and classified as Level II for unintended changes. The test is conducted using serum from individuals with a clinical diagnosis for the known allergen. Testing of food extracts can evaluate matrix effects, potential differences in folding, or processing between pure and native proteins. Pepsin-digested extracts may be used to identify IgE antibodies against potentially dangerous stable allergens, which have not been identified [Akkerdaas et al., 2005]. Purified protein is to be used if it is at low level in the food to avoid false negative results. However, for many foods, purified allergens are not yet available.
Functional assays - skin-prick test, or - *ex vivo* basophil or mast cells activation test	A positive result from serum screening will generally lead to discontinuation of product development or testing. A negative finding in serum screening test may require confirmation, as IgE-binding test lacks clinical relevance. A negative skin-prick test or other functional assays may be used for confirmatory purposes. While skin-prick test has clinical validity, its use may be limited by the availability of ethics approval, or of qualified test subjects in the case of an uncommon allergen.
Immunochemical assays Comparative quantification of endogenous allergenic proteins between food derived from modified vs. unmodified crops	Quantification of endogenous allergenic proteins is indicated for the modified crop that is a common source of food allergens for which the allergenic proteins are known and protein-specific assays and test reagents are available. This test aims to determine whether there are unintended changes in the levels of endogenous allergenic proteins.
The following testing has not generally been required or accepted as part of pre-market assessment of GM foods at present.	
Digestibility test Comparability of digested protein fragments between native and recombinant proteins. Alternate digestibility under conditions that	*Dietary proteins not known to be allergenic* Where both the recombinant protein and the protein in its native form are resistant to pepsin digestion, additional tests may be indicated.

Types of data/testing	Considerations
simulate gastric and intestinal conditions of target user group. Comparison of IgE binding using pepsin digested food extracts.	Additional test may compare the profiles of the digested fragments, or an alternate digestibility test may be used. *Dietary proteins from common allergenic sources* Comparison of IgE binding using pepsin digested food extracts may also be indicated if the protein is derived from a common allergenic source where both the native and recombinant proteins are resistant to pepsin digestion.
Animal model for assessing other immunogenic responses	*Novel proteins (without dietary history) from sources of known immunogenic proteins (e.g., certain types of microorganisms), present at appreciable level in the GM food (e.g., ≥ 0.1% protein for pesticidal and enzymatic proteins, or ≥ 1% protein for other types of protein), and resistant to pepsin digestion.* Other aspects of immunogenicity may need to be assessed (see Part A, Level III).
Post-market monitoring Serum screening of high risk occupational groups.	Occupational group with high inhalation exposure to pollen-borne novel recombinant proteins may be considered to have higher risk than the general population for developing allergic reactions to a novel protein. Therefore, monitoring high risk occupational group would be more efficient than monitoring the general population. However, positive IgE binding in serum screening may indicate the need for a more clinically relevant test (e.g., skin prick test) before an assessment about allergenic risks can be made.

* Suggested further data acquisition/testing is provided only to illustrate that the range of testing can vary with concern levels classified using the proposed RBCS and with the nature of the substance being assessed. Appropriate testing should be tailored to all prior information available on the substance.
See Chapters 4 and 5 for detailed descriptions of Levels I and II.

CHAPTER 8 - APPLICATION

In this chapter, the application of the proposed RBCS is illustrated by means of a specific product example. The product is a food derived a GM crop modified for nutritional enhancement that involves extensively engineered gene products. This is one of the reference products for which agreement in the classification of concern levels in all three parts of the RBCS was obtained by two independent raters in the concordance study (see Chapter 9). **Table 8-1** and the accompanying text provide a profile of the product, which is an abridged version of the product profile used in the concordance study. The following text explains the rationale for the concern levels assigned and proposed testing based on concern levels and those factors for which criteria are not met. It should be emphasized that classification of concern level is based solely on the information provided in the product profile, which may be incomplete in terms of describing the intrinsic characteristics of the product.

8.1. Overview of product profile

The product being assessed is a field corn to which a mutant gene from barley encoding chymotrypsin inhibitor-2 (CI-2) was introduced. The DNA sequence transferred was engineered to confer multiple lysine substitutions as a means to generate a lysine-enriched protein for the purpose of enhancing the nutritional quality of field corn for feed use. Field corn is also consumed by humans, mainly in the form of food ingredients.

Information for assessing potential toxic and antinutritional effects from intended changes (Part A)

Limited data are available on the level of CI-2 type protein from grains and on current intakes. Dietary exposure to the recombinant CI-2 protein is expected to vary widely, depending on the role of maize in the human diet. For the majority of the Canadian population, corn is consumed primarily through refined ingredients (such as starch and sweeteners). A nutritionally significant increase in the intake of lysine from bioengineered CI-2 protein in modified maize is unlikely. However, maize consumption can be high in some population groups for whom maize is a staple food. Assessment of the introduced protein will focus on the safety implications of its altered structure.

Information for assessing potential toxic and antinutritional effects from unintended changes (Part B)

Both the gene source (barley) and the modified plant (maize) are cereal grains with a long history of breeding and safe food use. Both crops are consumed by humans only in processed form. Their inherent constituents are not known to be toxic or antinutritional under common conditions of consumption. The introduction of the modified CI-2 protein will affect amino acid metabolism in the modified crop. However, the protein is not expected to have other direct interactions with the metabolic pathways in the modified crop. Targeted biochemical characterization focusing on key nutrients (including amino acids), known toxicants, and antinutrients, as suggested in the OECD consensus document for the food use of maize would be considered sufficient in assigning a concern level.

Information for assessing potential allergenic effects from intended and unintended changes (Part C)

Both the gene source and the modified crop are sources of known allergens. Barley is also a source of proteins associated with gluten-sensitive enteropathy. The engineered CI-2 protein also demonstrated resistance to pepsin digestion assay.

8.2. Application of RBCS

Part A

Assignment of concern level III is determined by insufficient or equivocal information provided for more than one critical factor (A.4, A.5). In addition, criteria for other factors (A.6 and A.7.2) are also not met.

Factor A.4 - Prior knowledge of safety/mode of action

The protein (barley-derived CI-2 protein) was extensively engineered with multiple lysine substitutions prior to transfer to the modified maize. While the safety of the native protein is not in question, the same conclusion cannot be made for the engineered protein based on the information provided.

Factor A.5 - Structure-activity characterization

The modified CI-2 protein was resistant to pepsin digestion. When compared to the native protein, differences in several aspects of structure-activity characterization (thermodynamic stability and inhibitory activity against proteases) were observed.

Factors A.6 and A.7.2

The protein's resistance to digestion also raises questions about its metabolic fate in mammalian metabolism, its bioavailability and potential effect on the bioavailability of other food constituents.

Part B

Concern Level I is assigned because the criteria for a combination of critical factors are met: a history of safe food use of the gene source (B.1); absence of unknown DNA sequences in the post-transformation insert (B.4.1); and absence of biologically significant differences in the levels of key nutrients and known toxicants when compared with unmodified maize (B.5.1 and B.5.2). There is no concern with human health consequences of instability of the modified trait (B.6) as the nutritional modification is intended primarily for animal feed. Meeting the criterion for factor B.5.3 is not considered essential because criteria are met for all secondary factors (B.2, B.3, B.4.2) related to the safety of the host plant, potential for novel/altered patterns of metabolites (as indicated by the function of the gene product) and the absence of unknown DNA sequences in the regions flanking the transgene insertion site.

Part C

Failure to meet two critical factors (C.1 and C.3) contributes to the assignment of concern Level II for the CI-2 protein. In addition, the protein is expressed at a high level, equivalent to 10% of total seed protein (C.4.3). However, the potential for alterations in the allergenicity of the modified maize would be considered to be low based on assessment of Part B.

C.1 - Allergenicity of donor organism

Barley, the gene source of CI-2 protein, is a known and common source of hordeins and hordenins responsible for inducing gluten-sensitive enteropathy (C.1). It is also a source of other allergenic proteins.

C.3 - Pepsin digestion and C.4.3 - Abundance

The recombinant protein is resistant to pepsin digestion and expressed at a high level (10% of total seed protein).

8.3. Suggested further data acquisition/testing

Naturally occurring variations exist in the structure and function among proteins with considerable tolerance to amino acid substitutions without deleteriously affecting protein function [Bowie et al., 1990]. Data are lacking that changes in characteristics such as stability and substrate specificity without alteration in conformation will convert a non-toxic protein into a toxic protein [Pariza and Johnson, 2001]. However, given that the CI-2 protein is resistant to pepsin digestion, a comparison of its proteolytic profile with the native protein and an animal nutritional study should be conducted in order to set priorities for further assessment of its potential for toxicity and antinutritional effects.

In addition, since the CI-2 protein is derived from an allergenic source and the protein is extensively modified and introduced at a high level, the potential for allergenicity of the recombinant protein requires careful scrutiny. A negative result in a commonly used test (i.e., specific serum screening for IgE binding) may not be sufficient in this case, particularly if differences are observed in the proteolytic profiles between recombinant and native proteins.

Suggested testing is outlined below.

- Comparison of proteolytic profiles between recombinant and native proteins.
- Animal nutritional study to assess the bioavailability of the protein and nutritional quality of the modified food.
- If there are *no differences* in the proteolytic profiles between the two proteins, further testing will focus on assessing the allergenic activity of the recombinant protein.
    - Specific serum screening for IgE binding to known allergenic proteins in barley.
    - Confirmatory tests following negative result in specific serum screening (skin prick test, or other functional assays).

- If *there are differences* in the proteolytic profiles between the two proteins, short-term toxicity test would be recommended before continuing product development.
    - Repeated dose toxicity (14-28 day) including immunogenicity screening (tier 1).

8.4. Discussion

One strategy of genetically modifying crops for nutritional enhancement is to alter the structure and/or functionality of the expression product of the introduced gene. This may involve extensive engineering of a dietary protein, as illustrated in the above example. Opinions may differ as to whether a modified gene product with an intended change in its amino acid sequence without changes in conformational structure or detrimental changes in functionality should be considered as a substance with prior dietary history. Pepsin digestibility is a key element in the safety assessment of GM food. However, few guidelines provide interpretive guidance on the results of such an assay, particularly in the case where both the native and recombinant proteins are resistant to pepsin digestion and the native protein is a dietary protein that is not a known toxin, antinutrient, or allergen. Current guidelines also do not consider to what extent the level of the recombinant protein in the modified food should guide further testing or assessment, presumably because the threshold for sensitization is not known. A threshold for toxicological concern for proteins has not been determined. Past experience would suggest that a GM food containing a stable protein would more likely be refused market release, despite many unanswered questions about the relationship between protein stability in gastric and intestinal digestion and allergenicity and immunogenicity. This example highlights some of these unanswered questions.

Table 8-1. Product profile: maize modified with CI-1 protein.

Modified trait: Lysine enrichment for nutritional enhancement of feed by the introduction of a gene that encodes for a lysine-enhanced protein.

Intended food use of modified crop (host plant): Little whole kernel or processed maize or field corn (*Zea mays*) is consumed by humans worldwide when compared to maize-based food ingredients. Maize-based ingredients have wide applications in food and include starch, sweeteners, flaking grits, flour, and oils from wet or dry milling processing.

Gene (source) / Promoter (source): A mutant gene from barley (*Hordeum vulgare L.*) encoding chymotrypsin inhibitor-2 (CI-2) was engineered to incorporate multiple lysine substitutions. The engineered gene was introduced under the control of the endosperm-specific storage protein promoter derived from barley. Lysine substitutions were engineered at residue positions where they would be more likely to tolerate substitutions.

Transformation method: Microprojectile bombardment.

Metabolic reactions / pathways: Not applicable.

INTENDED GENE PRODUCTS TO BE ASSESSED
Primary gene products: CI-2 protein.
Secondary gene products: The intended change in lysine content in the modified crop will be considered, although it is not an expression product of the mutant gene.

* M = Criteria met N = Criteria not met NA = Not applicable ? = Product information insufficient or equivocal

Factors	Rating*	Summary of product profile
PART A		
A.1. Expression of gene product(s) in edible part(s) of the modified crop	N	**CI-2 protein**: 10% of total seed protein with an increase in total protein in the maize grain to 14% from 9%. **Lysine**: Doubled to 0.50% from 0.25% of the grain. As % total protein, lysine increased only slightly to about 3%.
A.2. Prior history of consumption from foods	M	**CI-2 protein**: Appeared to be structurally comparable to the wild-type protein derived from barley based on the parameters analyzed although functional differences are noted (see A.5). **Lysine**: A common constituent in protein-containing foods, ranging from 0.3-0.5% of the grain (2-4% of total protein) in major cereals.
A.3. Expected change in total dietary exposure (theoretical estimates)	M	**Food derived from the modified crop**: No changes in the consumption of the modified maize are expected. It will replace unmodified maize in the same uses. **CI-2 protein**: Limited data on the level of the native CI-2 protein in barley and the levels of similar proteins in other cereal grains, but expected to be lower than in modified maize. Depending on the level of maize consumption, CI protein can contribute between 0.5 - 5 g protein to the diet (0.02-0.2% of diet). This represents

Factors	Rating*	Summary of product profile
		1 - 10% of typical protein intake for adults, assuming 100% lysine-enhanced maize, which is a gross over-estimate. **Lysine**: No physiologically significant change in lysine intake is expected in a varied diet where maize is not a staple food. Given that the lysine and protein content of the modified grain is comparable to that in other cereal grains, no further assessment of lysine will be made.
A.4. Prior knowledge of safety/mode of action	?	The native protein is believed to play a significant role in plant defense against insects and other pests by inhibiting the activity of digestive enzymes of insect pests. Despite its protease inhibitory activity, the protein is not known to have serious antinutritional activity in humans, probably because it is heat labile. The safety of the extensively modified CI-2 protein has not been studied in detail. Preliminary data are summarized below.
A.5. Structure-activity characterization	?	No amino acid identity with known mammalian toxins was identified. In standard pepsin digestibility test, the protein was partially digested at the end of 30 min, and fragments were visible up to 60 min. Sequences and molecular mass were as predicted (based on N-terminal sequencing, MALDI mass spectrometry, and SDS-PAGE). Comparable conformation to native protein. Lower thermodynamic stability than native CI-2, but comparable to many naturally occurring globular proteins. Considerably reduced inhibitory activity against chymotrypsin and subtilisin and lower resistance to trypsin digestion compared to native CI-2.
A.6. Digestion and degradation products	?	No unusual protein fragments in the modified crop identified by Western blot analysis. No unusual protein breakdown products expected in mammalian metabolism, but profile of proteolytic fragments from pepsin digestibility not analyzed.
A.7. Synergism and interactions		
A.7.1. Synergism between gene products	NA	Not applicable.
A.7.2. Interactions between gene products introduced/modified and food constituents	?	Bioavailability of the protein and potential effect on the absorption of other food constituents not known.
A.8. Prior experience with gene products introduced/modified	NA	Not applicable.
Concern level	**III**	
PART B		
B.1. Safety of donor organism *Applicable if donor organism and host plant are different.*	M	**Barley**: Long history as a food source. Physiological significance of several proteases, amylase inhibitors and lections in barley is unknown. In processed form

Factors	Rating*	Summary of product profile
		normally consumed, barley has not been associated with human toxicity.
B.1.1. Characterized gene modification	NA	Not applicable - safety of donor organism not a concern.
B.1.2. Poorly characterized gene modification	NA	Not applicable - gene modification of the crop was well-characterized.
B.2. Safety of host plant	M	**Maize**: Long history of consumption as food. Does not contain significant toxicants or antinutritional factors (see also B.5.2).
B.3. Potential for novel/altered patterns of metabolites		
B.3.1. Knowledge of gene product function	M	Native CI-2 protein has defense function in plants.
B.3.2. Impact of gene product function in the modified plant	M	Not expected to affect regulatory, metabolic, or signalling pathways in the modified maize.
B.3.3. Substrate specificity of gene product	NA	Not applicable. Does not function as an enzymatic protein in plant metabolism, despite enzymatic activity in target pests.
B.4. Potential for disrupting coding or regulatory DNA sequences in modified plant		
B.4.1. In the inserted/modified DNA	M	Integration of one transgene copy at a single genetic locus identified by Southern hybridization and RT-PCR. No unknown DNA sequences in the insert identified using bioinformatics analysis.
B.4.2. In genomic sequences bordering site of insertion/modification	M	The absence of unknown DNA sequences in the regions flanking the insertion site (about 500 bp) was supported by sequence and bioinformatics analyses.
B.5. Biochemical analysis of edible part of modified crop		
B.5.1. Key nutrients	M	Analysis included proximates, amino acid, and fatty acid profiles, as well as several minerals and vitamins, as suggested in the OECD consensus document for food use of maize. Levels of most nutrients analyzed in the modified and unmodified control are comparable, except for amino acids. Changes in amino acid content are expected, given the nature of the modification. These changes are not considered to be of nutritional significance, given the natural variation in amino acid content in cereal grains and other protein-containing foods.
B.5.2. Known toxicants and antinutrients	M	Trypsin and chymotrypsin inhibitors, as well as phytic acid and raffinose were analyzed, as suggested in the OECD consensus document. No differences were noted in their levels in the modified and unmodified control. Analysis of DIMBOA (2,4-Dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one) was not recommended because of the high variability of its levels among maize varieties and the fragmentary knowledge of its toxicology.
B.5.3. Other metabolites/parameters *Applicable if criteria for factors B.2, B.3,*	NA	Analytes included furfural, ferulic acid, and p-coumaric acid, which are notable secondary metabolites in maize,

Factors	Rating*	Summary of product profile
and B.4.2 are not met.		as described in the OECD consensus document. The protein is not expected to function in metabolic pathways in the modified plant. In concert with observable phenotype and function, analysis of key nutrients synthesized in primary metabolic pathways (B.5.1), known toxicants/antinutrients (B.5.2), and notable metabolites for the crop (B.5.3) arising from secondary metabolism, would be considered to be sufficient in scope to provide a reasonably sensitive indication of unintended effects of direct heath relevance.
B.6. Health consequences of instability of the modified trait *Applicable if the modified trait is intended to provide nutrition or health benefits.*	NA	The modified trait is primarily for feed use - not intended to affect human health.
Criteria 1.3 of Part A	NA	Not applicable.
Concern level	**I**	
PART C		
C.1. Allergenicity of donor organism *Applicable where donor organism and host plant are different.*	N	**Barley**: Source of hordeins that are associated with gluten-sensitive enteropathy. It is also a source of a minimum of 4 allergenic proteins.
C.2. Potential for protein gene product to cross-react with known allergens	M	No overall sequence identities of > 35% were found, nor were matches of greater than eight consecutive amino acids identified.
C.3. Pepsin digestion	N	As noted in A.5, the protein was partially digested at the end of 30 min, and fragments were visible up to 60 min.
C.4. Secondary factors *Applicable if criteria for factor C.3 are not met.*		
C.4.1. Structural/functional characterization	M	Native CI-2 protein not classified as part of the protein families associated with allergenicity. No glycans detected in glycoprotein assay.
C.4.2. Heat stability	M	Comparable to native protein, which is heat labile. Heat stability (at 90°C for 30 min) was indicated by inactivation of inhibitory activity on proteases.
C.4.3. Abundance	N	CI-2 protein expressed at 10% of total seed protein.
C.5. Allergenicity of host plant	M	Not a common source of food allergen, but allergenic proteins in maize have been identified. Cases of skin, gastrointestinal and respiratory symptoms have been reported.
Criteria 1.3 of Part A	NA	Not applicable.
Concern level	**II**	

References.

1. OECD (2004) Consensus document on compositional considerations for new varieties of barley (*Hordeum vulgare L.*): key food and feed nutrients and antinutrients. Series on the Safety of Novel Foods and Feeds. No. 12. ENV/JM/MONO(2004)20.
2. Roesler KR and Rao AG (1999) Conformation and stability of barley chymotrypsin inhibitor-2 (CI-2) mutants containing multiple lysine substitutions. Protein Engineering 12(11), 967-973.
3. Roesler KR and Rao AG (2001) Rapid gastric fluid digestion and biochemical characterization of engineered proteins enriched in essential amino acids. Journal of Agricultural and Food Chemistry 49, 3443-3451.

CHAPTER 9 - EVALUATION OF PROPOSED RBCS

This chapter covers five aspects of evaluation of the proposed RBCS: objectives and plan for the evaluation, development of a panel of reference GM food products, development of the evaluation tools, and data analysis. A discussion of the utility of the proposed scheme with respect to the characteristics evaluated concludes the chapter.

9.1. Evaluation objectives

The evaluation of the proposed risk-based classification scheme (RBCS) aims to assess the following components:

a) degree of concordance in concern levels assigned by two independent raters,

b) applicability of the proposed scheme to a range of GM foods with different characteristics,

c) ability of the scheme to differentiate GM foods into concern levels as defined in the scheme, and

d) ease of use of the scheme

9.2. Evaluation plan

Evaluation of the proposed RBCS consisted of the following steps:

a) development of a panel of reference GM food products;

b) development of evaluation tools;

c) pilot testing of the clarity of descriptions of reference products and of the evaluation tools; and

d) evaluation by two independent raters of the RBCS using reference products (concordance study).

9.2.1. Measurements

Measurements of the utility of the RBCS included the following indicators:

a) Degree of concordance - Inter-rater agreement was assessed by descriptive statistics reporting observed proportion of agreement in concern levels for the three parts of the RBCS separately and for the three parts combined. Kappa statistics were also calculated using SAS

version 9.1 (SAS Institute Inc., Cary, N.C., U.S.A.). The kappa coefficient is a commonly used statistic in assessing inter-rater agreement or consistency. It is a summary statistic that verifies agreement beyond chance level between two judges rating N objects [Cohen, 1968; Soeken and Prescott, 1986]. Kappa values can range between positive one to negative one, with positive value of one indicative of perfect agreement, which is rare.

b) Applicability - The applicability of the proposed scheme was judged by the range of characteristics represented by products in the reference panel, as indicated by: the types of target traits modified, mode of action of the target gene product in conferring the desired trait, and distribution across concern levels.

c) Discriminating ability - The ability of the scheme to differentiate a GM food into a concern level was assessed independently by two different qualified raters in the concordance study. The raters were also asked to identify the reasons if they were unable to provide a rating for a factor in the scheme, as well as any assumptions or default decision rules used in applying the scheme. The raters also provided comments to the question "In your opinion, does the scheme clearly differentiate a GM food into a concern level in each of the 3 categories of health effects?". Raters' responses to these questions were used as indicators of the ability of the scheme to differentiate among GM foods.

d) Ease of use - Ease of use of the scheme was indicated by the length of time required to complete concern levels for the reference products by the two raters. Quantitative rating of ease of use was also provided by the raters (on a numeric scale from 1 to 7, very easy to very difficult).

9.2.2. Recruitment and selection of raters

Separate recruitments of raters were conducted for the pilot test and the concordance study. A brief description of each recruitment process follows.

a) Pilot test

Eighteen participants and contacts from previous phases of the research project who indicated an interest in evaluating the proposed RBCS were contacted for their availability to participate. Participants from previous phases were purposively selected based on expertise relevant to the

objectives of the project. Potential participants received an information letter sent electronically that described the scope of the evaluation and estimated length of time required. This was followed-up with a phone call. Nine participants agreed to participate. Each individual was assigned two products. Seven individuals returned completed evaluation of 13 products. The recruitment protocol was reviewed by the University of Ottawa Health Sciences and Science Research Ethics Board and approved as part of Phase 2 of the project (certificate of approval included in Appendix C to the thesis).

b) Concordance study

A list of qualified consultants considered having general expertise in toxicology and health risk assessment was prepared. The first two individuals contacted who indicated an interest and availability for the work participated in the evaluation. Each individual was compensated with an honorarium of $3000 CDN for completing an evaluation of the proposed RBCS. The consultants were provided with the following information for the evaluation.

I. Instructions for completing product rating.
II. One sample rated product and corresponding product profile.
III. Supplementary information for applying the risk-based classification scheme.
IV. Form for overall assessment.
V. Product profiles for 20 GM food products.
VI. Combined risk-based classification scheme-product rating form for assigning product concern levels (1 copy for each product).

9.3. Development of a panel of reference products

9.3.1. Selection of reference products

The GM food products in the reference panel were selected to reflect a range of characteristics that are relevant to their classification into concern levels using the proposed risk-based classification scheme. They include existing products and future GM crops at different stages of research and development and for which information at varying levels of detail may be available. Efficacy of the modified trait for the intended purpose and the potential for commercialization were *not* considered in product selection for the reference panel.

Included in the final reference panel were four "current" products and 16 "new" products with sufficient information for constructing meaningful product profiles. Seven categories of traits were included. A focus was placed on traits related to pathogen resistance and nutrition/food

quality to illustrate different modes of action for the same trait. One factor (prior experience with gene products introduced/modified) in the RBCS was not evaluated. Raters were asked to treat all products that meet the criterion of prior experience as new products. New products were emphasized in order to assess the potential of the proposed scheme to be applied to emerging categories of GM foods. Crops modified by conventional methods were underrepresented in the reference panel. Information obtained from the public literature was limited on key characteristics of these crops for the development of product profiles for classification purposes. **Table 9-1** summarizes the major characteristics of the products in the reference panel.

9.3.2. Development of product profiles

Product profiles were descriptions of reference products that provided information on the factors in the RBCS so that raters could judge whether the criteria for applicable factors are met. Based on the ratings for individual factors (yes, no, or uncertain), the rater assigned a concern level for each part of the RBCS using aggregation rules provided to the rater. Complete profiles of the 20 reference GM foods are included in Appendix F to the thesis.

Product profiles varied in length from five to eight pages. The information provided in product profiles was not intended to be exhaustive, but was considered sufficient for classification purposes. Only essential "core" information relevant to product classification using the proposed scheme was included in the product profiles in order to keep respondent burden reasonable. It is a subset of the information typically submitted or required in premarket assessment. For example, the use of reasonably well-established marker genes was not included in the product profiles as part of the evaluation. Excluded from the core information was "prerequisite" information that must be met by all GM crops. As noted previously (section 4.3.2), such information does not provide a basis for differentiating products into concern levels for testing. In particular, issues of the quality of molecular characterization data have been addressed in other guidance documents [e.g., ACRE, 2001]. In applying the RBCS, one assumes that these prerequisite criteria have been evaluated and are met. For some products, only limited information was provided for specific factors, either intentionally to vary the completeness of information available, or out of necessity because the information was unavailable or inaccessible. Unavailable or inaccessible information includes information that is specific to proprietary products and requires product-specific testing. Examples of these types of

information include: molecular and compositional data on the modified crop, pepsin digestibility, and heat stability of protein gene products. For new products that are in the early stages of research and development, these types of information are generally not reported in the public literature, or are of insufficient scope or specificity for applying the RBCS.

All product information was obtained from public sources, including peer-reviewed scientific literature and product assessment reports on websites of regulatory agencies. Different processes were used in developing profiles for current vs. new products.

a) Current products

For current products already approved or reviewed by at least one country, information was drawn from the Agbios database available at http://www.agbios.com and supplemented by the scientific literature. The Agbios database provides links to decision documents (in English) of national authorities. Out of over 90 products in the database that were screened, four products were included in the reference panel. The four selected products represent the spectrum of typical modified traits and crop types in the so-called first generation of GM crops. The information for these products provided in the database was considered sufficient for classification using the RBCS. In some cases, composite information was pooled from similar traits and crop types in developing the product profiles. Current products provide a baseline for assessing the utility of the proposed scheme.

b) New products

For new or future products, information on trends in GM crop research and development was reviewed, using special reports [e.g., Pew Initiative on Food and Biotechnology, 2001; AEBC, 2002; Lheureux et al., 2003; ILSI, 2004], field trial database websites[5] [e.g., European Commission Biotechnology and GMOs Information; Information Systems for Biotechnology; United States Regulatory Agencies Unified Biotechnology Website], and scientific papers on future trends [e.g., Cockburn, 2004; Dunwell, 2005]. Based on this information, additional literature searches were conducted in five categories of modified traits: pathogen resistance, nutritional/food quality, abiotic stress tolerance and others (including reduction of undesirable

[5] European Commission Biotechnology and GMOs Information [http://gmoinfo.jrc.it]; Information Systems for Biotechnology [http://www.isb.vt.edu]; United States Regulatory Agencies Unified Biotechnology Website [http://usbiotechreg.nbii.gov].

substances, modification of crops for the extraction of food or functional ingredients, herbicide tolerance, and non-rDNA modifications). Within each category of modified trait, major strategies for achieving target traits were identified. Where applicable, a minimum of two scientific papers describing the same or similar traits and modification strategies were identified. A "simulated" product profile was developed based on this trait- and modification-specific information. Other relevant supplementary information from the scientific literature was added as necessary. The final reference panel consisted of 16 new or future products, selected from drafts of profiles on over 30 new or future products.

9.3.3. Assumptions used in developing product profiles

Where information on the required parameters was not available in the public literature, assumptions and default information were used to fill in the information gaps. This was done to enable product classification using as much information as possible. For example, protein substances from food crops or sources not known to be toxic would not be considered to have amino acid sequence identity similar to that of known mammalian toxins. Unless the structure of a protein has been determined to be similar to one that is known to be heat stable or resistant to digestion, it would be assumed not to possess these characteristics. In other cases (e.g., molecular, compositional, and protein digestibility data), variations of "model statements" were developed that represent different levels of adequacy of information relative to meeting the criteria in the RBCS. For less critical factors (e.g., heat stability), conclusions may be presented without data-derived supporting evidence.

9.3.4. Additional sources of information

Several types of information are required in applying the RBCS. They include dietary exposure, safety, and allergenicity of gene products and of their source organisms, as well as that of the host plants. The basic reference sources used in developing these types of information as part of product profiles are listed in **Box 9-1**. Additional references used and obtained from the general scientific literature are included in the individual product profiles.

9.4. Development of evaluation tools

9.4.1. Rating forms

In order to provide a structured approach to applying the proposed RBCS in assigning concern levels for the reference GM foods, a combined RBCS-product rating form was developed (Appendix F). The version included in the Appendix was the one used in the concordance study. It provided a detailed stepwise guidance for product rating in an effort to reduce ambiguities identified in a simpler version used in the pilot test.

A form for overall assessment was also provided to raters in the concordance study (Appendix F). There were six questions on this form related to: ease of applying the proposed scheme, clarity of differentiation by concern levels, usefulness of supplementary information provided to raters, use of assumptions or default decision rules in applying the scheme, general comments, background information on rater's expertise, and preference for acknowledgement of contribution to the project.

9.4.2. Resources for raters

Three types of resources were provided to raters in the concordance study: (1) instructions for completing product rating, (2) explanatory and interpretive notes on the combined RBCS-product rating form, and (3) supplementary information as a separate document (Appendix F). Instructions provided general information on the meaning of the different ratings possible for a given factor in the proposed scheme (i.e., yes, no, equivocal, and not applicable). A sample of a rated product and corresponding product profile complemented the instructions. Explanatory and interpretive notes elaborate on the factors and criteria in the RBCS. They were included in the rating form for ease of reference. A separate document on "Supplementary Information" included background information on the rationale, the context, and the development process of the scheme. An overview of the conceptual framework was presented, as well as the factors and criteria underlying the scheme and the principles of aggregation of the factors. The process used and assumptions made in developing product profiles were also described. "Supplementary information" was provided to facilitate a common understanding for the raters in applying the proposed classification scheme.

9.4.3. Pilot testing

Participants in the pilot test evaluated 13 products in the reference panel of products using a draft version of the combined RBCS-product rating form. Results of the pilot test guided the revision of rating form with respect to formatting and suggested the need to include explanatory

notes on the form for easy reference, and the need to clarify several criteria. Participants' responses also prompted the editing of product profiles to improve clarity. Results of the pilot test are detailed in Appendix E.

9.5. Data analysis

An analysis of the results on the four evaluation components obtained from the concordance study is presented below. Also included is an overall assessment of the proposed RBCS provided by the two raters in the study. A general discussion of the results follows.

9.5.1. Concordance between two raters

a) Data verification

Before descriptive and kappa statistics were calculated, product ratings provided by the two raters were checked for internal consistency among products for which the ratings for the same factors should be identical. For example, the ratings for "safety of the host plant" should be identical where the host plant is the same for several products. The extent to which variations of model statements affected ratings for specific factors was also evaluated. Minor internal inconsistencies were noted, but they had no influence on the assignment of concern levels of the products affected. Both raters were contacted for clarification regarding apparent errors and absence of ratings for applicable factors. An example of apparent error would be a rating of "yes" for a factor for which no information was provided in the product profile. A response from one rater indicated that the individual's expert knowledge was used, since the information was not provided in the product profile. None of the responses from both raters to the request for clarification resulted in changes to their assignment of concern levels.

The raters were asked to provide the reasons for any factors given a "no" rating or a rating of "product information insufficient or equivocal". This information was used to determine raters' judgement and interpretation of product profiles in the application of criteria in the RBCS. Only one rater provided the reasons as requested on the rating form. There was no other attempt to seek clarification on ratings that may be related to expert judgement or interpretation.

Corrections were made by this researcher to concern levels that could be attributed to errors in applying the aggregation rules. This correction is justified because this type of error could be

avoided if the aggregation process had been automated and the raters were not required to apply the aggregation rules manually. The number of products requiring correction in concern levels was small. The correction added one product in Part A and removed one product in Part B from the number of products for which agreement in concern levels was reached by both raters.

b) Inter-rater agreement

The proportion of agreement in concern levels and simple kappa coefficients were calculated for Parts A, B, and C separately and for the three parts combined. Results are presented for concern levels as assigned by the raters and for concern levels after correction (**Table 9-2**). Tests of marginal homogeneity (McNemar's and Bowker's tests for 2 and 3 categories, respectively) did not detect significant differences in the propensity with which the two raters classified the products (data not shown). As the differences in proportion of agreement between concern levels as assigned and after correction were minor (a change in one product each for Parts A and B), the following discussion and tables will focus on concordance for corrected concern levels only.

Based on descriptive statistics, good concordance (80% and higher) was observed for Parts A and C. For Part A, concern level differed by more than one level for only one product. Proportion of agreement was slightly lower for Part B (75%). However, the proportion of products that received identical concern levels for all three parts combined was lower still (40%). Out of the 12 products with discordant concern levels, only one product received discordant levels in more than one part of the RBCS. Kappa coefficients for Parts A, B, and C, and for all three parts combined were 0.69, 0.29, 0.60, and 0.29, respectively. Confidence limits were wide for all three parts, in part due to the small number of products being evaluated.

The interpretation of kappa values is subject to some debate, as it can be affected by factors such as balance across categories in the sample and assumptions about the decision-making of raters [Uebersax, 1987; Byrt et al., 1993; Brenner and Kliebsch, 1996; Hoehler, 2000; Hripcsak and Heitjan, 2002]. Kappa values reflecting good to strong agreement above chance suggested by some authors range from 0.6 to 0.75. Kappa values indicative of fair to moderate agreement above chance range between of 0.2 to 0.4 [Landis and Koch, 1977; Fleiss, 1981, p. 218; Altman, 1991, p. 404]. Using these suggested values for interpretation, reasonable agreement was reached

between the two raters for Parts A and C, and agreement was at best fair for Part B and the three parts combined.

Factors contributing to discordance in concern levels were examined (**Table 9-4**).The factors in Part A that were rated differently between the two raters were: expected change in total dietary intake (A.3) and structure-activity characterization (A.5). The latter contributed singly to two out of the three products with discordant concern levels for Part A and was also one of the contributing factors for the third product. Out of the six products with discordant concern levels for Part B, factors that were rated differently were: health consequences of instability of the modified trait (B.6), characterization of the inserted/modified DNA (B.4.1), and characterization of other metabolites/parameters (B.5.3), in descending order of frequency. For Part C, different ratings for allergenicity of the host plant (C.5) contributed to discordant concern levels for two of the four products. Factors contributing to discordant concern levels in the other two products included allergenicity of the donor organism (C.1) and structural/functional characteristics (C.4.1).

9.5.2. Applicability

The distribution of concern levels across the 20 reference products by concordance and discordance is shown in **Tables 9-3 and 9-4**, respectively. A summary is provided in **Table 9-5**. The majority of the products were classified as Level I for Parts A and B, whereas a more even distribution between Levels I and II was obtained for Part C. This trend also holds when the distribution of concern levels was compared between products on the concordant and discordant lists. It should be noted that concern levels can vary among the three parts of the RBCS for the same product. This highlights the importance of differentiating the different aspects of GM foods with respect to their potential for health risks.

When concern levels were examined with respect to the major product characteristics identified in **Table 9-1**, no generalizations can be made. This suggests that the determination of concern levels may require more detailed product information than what was presented in the product profiles for the concordance study. One characteristic appears to promote concordance between the two raters in assigning concern levels for all three parts in the study. Four of the five products with complete concordance in concern levels (**Table 9-3**) are derived from crops modified for insect resistance with insecticidal proteins expressed at low levels and not expected to interact with metabolism of the host plant.

9.5.3. Discriminating ability

In response to the question "does the scheme clearly differentiate a GM food into a concern level in each of the three categories of health effects", one rater answered "yes" while the other rater provided a "no" answer.

The reason given for a "yes" answer by rater 1 was that the product examples showed a range of differences within each category of health effects. The rating to be given to a factor was evident when the information provided for a product moved the rating beyond a comfortable range from "yes" (criteria met) to "no" (criteria not met) or "?" (product information insufficient or equivocal).

The reasons provided by rater 2 for a "no" answer to the question fall into two categories.

1) Disagreement with the criteria provided in the RBCS - Rater 2 described three factors for which the criteria were considered questionable. The rater questioned why different criteria were used for determining allergenicity of the donor organism (source of known allergen) vs. the host plant (source of common allergen) in Part C (factors C.1 and C.5). The second questionable criterion is the requirement for adequate quality control in ensuring the stability of a modified trait that is intended to provide nutrition or health benefits (factor B.6). The rater considered that failure to meet this criterion alone should not be a sufficient condition for classifying a product as Level II in Part B. Also suggested in the response was that the loss of a beneficial health-related trait should not be considered a health risk. The third criterion questioned by the rater was that a dietary protein, which is resistant to pepsin digestion (factor A.5 and C.3), should not be considered to have the same level of concern as other characteristics of a novel protein.

2) Ambiguity in the principles underlying the aggregation of factors into concern levels - Rater 2 thought that the aggregation patterns provided on the combined RBCS-product rating form did not include a number of logical combinations, and that some products did not fall obviously into the aggregation patterns provided. This suggests that the basis of the aggregation rules was not clear to this rater and would require elaboration in future revision of the rating form and supplementary information.

9.5.4. Ease of use

On a scale of 1 to 7 (1 being "very easy" and 7 being "very difficult"), both raters provided similar ratings (4.7±0.4, mean±s.d.) and required similar length of time to complete product assessments (31±11, 34±10 minutes, respectively, for rater 1 and rater 2). Both raters found the scheme easier to use with practice.

Specific areas of difficulty in using the scheme can be summarized as follows.

1) Counter-intuitive wording of some criteria (raters 1 and 2) - A rater was asked to give a "yes" rating for a factor with a criterion worded in the negative (e.g., "*not* a source of allergenicity").

2) Confusing aggregation rules (rater 2) - The table format used in the combined RBCS-product rating form was considered confusing. The table format displayed specified combinations of ratings for the different factors for each concern level in each part of the scheme. A descriptive explanation of the aggregation rules was considered to be simpler to understand than the format used. Automation of the aggregation algorithm (e.g., using an EXCEL spreadsheet) would also simplify the task of aggregation.

3) Lengthy combined RBCS-product rating form (rater 1).

4) Some criteria not mutually exclusive (not specified, rater 1).

In general, both raters considered the overview of background materials provided in the resource "supplementary information" useful. This document explained the rationale for the RBCS and the factors to be considered in decision-making regarding the safety of GM foods.

9.5.5. Overall assessment by two raters

The two raters were asked to provide general comments and suggestions for improvement of the RBCS, product profiles and/or the supplementary information. Their comments are summarized below.

1) The way exposure is included in determining the three concern levels in Part A can be confusing. The consideration of exposure should perhaps be done separately.

2) The criteria for expected change in total dietary exposure (factor A.4) are inappropriate for a generally benign product that is intended to be consumed in large amounts for the purposes of enhancing nutrition (e.g., rice in some Asian countries). If a high consumption product would be

considered "moderate-high" risk based on consumption alone, that rationale should be further explained. Conversely, a criterion of "within natural variation" may not be adequate in cases where natural variation includes varieties that can be toxic or otherwise problematic.

3) It is not clear why different criteria are used for the allergenicity of donor organism vs. host organism (factors C.1 and C.5). This comment was also included as part of the rater's response to the question related to the discriminating ability of the RBCS (section 9.5.3). The rater suggested that an explanation of this difference would be useful.

4) It is possible that GM crops that are modified by nucleic acid techniques may not be fully characterized. The rater suggested that the criteria for poorly characterized gene modification as currently defined to include only non-nucleic acid techniques (factor B.4.1) should be expanded to include poorly characterized genetic modification based on techniques such as rDNA.

5) Repeating a comment made in response to the question of the discriminating ability of the proposed RBCS, the rater noted that the absence of quality assurance data (factor B.6) should not be the sole trigger for classifying a product into a higher level in Part B.

6) The implications of the different levels for testing are unclear. The rater agreed that the products classified into higher concern levels generally require more in-depth evaluation than products classified as level I. However, more assessment does not necessarily mean more testing.

9.6. Discussion

Based on the parameters evaluated using a reference panel of 20 GM foods, the proposed RBCS shows considerable promise for continued refinement for broader application. Specific issues and suggestions for improvements identified in the concordance study are discussed below.

9.6.1. Sources of disagreement in concern levels between two raters

A number of reasons could contribute to discordant concern levels between the two raters. The reasons can be grouped into three categories: rater's expertise and use of judgement, completeness and clarity of product information, and attributes of the proposed RBCS.

Rater's expertise and use of judgement. Both raters who participated in the concordance study were toxicologists with experience in health risk assessment of food-related product categories. However, one rater may be considered to have less direct or less recent experience in GM food safety assessment than the other. This may have influenced the stringency with which they followed the criteria as stated in the RBCS, as explained below. They also had different specialized training (genetics vs. molecular biology). As already discussed (section 9.5.1.a), in seeking clarification on the initial product ratings and comments received from the raters, it was noted that one rater did not rely entirely on the information provided in product profiles.

Notably, in response to a specific question as part of overall assessment, the raters indicated the use of different assumptions or default decision rules in applying the scheme. Rater 1 indicated an emphasis on the need for clear or convincing evidence with respect to genetic characterization and allergenicity (Parts B and C). However, this level of demand for certainty was not specified for assessing the factors in Part A by this rater. The default decision rule regarding genetic characterization and allergenicity used by rater 1 was one of "guilty until proven innocent" and could probably account for a more conservative rating of factor C.5 (allergenicity of host plant) by rater 1 compared to rater 2 for three products (A, G, and O). Differences in the ratings of these factors resulted in different concern levels for Part C. A difference in the application of default decision rules between the two raters could be one factor contributing to discordant concern levels for Part C for these products because strict adherence to the criteria as stated in the RBCS would have resulted in ratings for the factors that were different from those provided by rater 1. In addition, a more conservative approach with respect to allergenic risk adopted by rater 1 was also evident in how the rater used expert judgement in interpreting the criteria for the factor related to structural-functional characteristics in Parts A and C (factors A.5 and C.4.1) in one product (D). For rater 2, an assumption was made that adequate gene characterization meant that the presence of open reading frames was not evident, even though this was not explicitly stated as a criterion in the RBCS. Using this assumption, rater 2 was more consistent than rater 1 in interpreting product information related to molecular characterization of inserted sequences for two products with discordant concern levels for Part B (products F and K).

Given that the more contentious aspects of GM food safety assessment are related to unintended changes, it is not surprising to have lower concordance in the classification of the

reference products with respect to unintended toxic and antinutritional effects (Part B) compared to intended effects (Part A) and allergenic effects (Part C). There were also a higher number (7) of factors in Part B for which there was disagreement in rating compared to the other two parts (3 factors in each part). This may be a reflection of the multi-dimensional nature of the factors that need to be considered in determining the potential for unintended changes in GM foods most relevant to health risk assessment. Two individual raters with expertise in primarily one area relevant to GM food safety assessment (toxicology) could differ in their application of the proposed RBCS to the interpretation of criteria that require knowledge in plant sciences. As previously noted, rater 1 adopted a conservative default decision rule regarding the molecular characterization of inserted sequences. Two of the six products with discordant concern levels for Part B are related to this factor (B.4.1). Another factor (B.6) regarding health consequences of instability of the modified trait contributed to three of the remaining four discordant concern levels in Part B. In all three cases, rater 1 provided a "yes" rating while rater 2 provided a rating based on the stated criteria and product information provided.

Completeness and clarity of product information. The raters were presented only with product summaries without "raw" data. While the information may be sufficient for product classification purposes, it is not complete and may be considered limited by other standards. Reliance on limited data requires considerable use of expert judgement [FAO/WHO, 2006]. Raters were asked to provide the reasons for giving a rating of "product information insufficient or equivocal" to a factor. It is not obvious from the reasons provided that the clarity or completeness of product information can be entirely dissociated from the clarity of the criteria in the RBCS as understood by the rater.

Attributes of proposed RBCS. Both raters noted the counter-intuitiveness of the wording of some criteria. This may account for an apparent incorrect rating of one factor related to the allergenicity of donor organism and discordant concern level for Part C for one product (product A). This was the first product assessed by one rater. Judging from the comments of the two raters provided to the question on the discriminability of the RBCS (discussed in section 9.5.3), it is not clear if this aspect of the RBCS had a major effect on the level of concordance and discordance observed in the study.

9.6.2. Potential limitations

Potential limitations of the concordance study related to the product profiles and the selection of raters can be identified. In the preparation of the product profiles, only publicly available information was used and no content input from experts was solicited. Data were presented in summary format that did not permit raters to draw conclusions from raw data. The product information provided, while sufficient for the purpose of classification using the RBCS, may be incomplete in describing the product. It was necessity to keep the product profiles to a manageable length and respondent burden reasonable. Products modified by conventional genetic modification methods were underrepresented in the reference panel. As already noted, the RBCS was evaluated by experts representing only one scientific discipline. In summary, the study did not fully reflect the typical situation that would occur in assessing the risks of GM foods in regulatory practice. However, the study provided useful information for an exploratory evaluation of several functional aspects of the proposed RBCS, which was its primary objective.

9.6.3. Addressing the issues identified

Several limitations of the concordance study are recognized. Due to time and resource constraints, the study was conducted under less than optimal conditions. Product profiles were prepared without expert input and review. For pragmatic reasons, profiles lacked raw data and were limited in detail. In addition, it was necessary to recruit consultants who assessed the products as individuals without the benefits of a team approach. A multidisciplinary perspective and discussion among team members would minimize the likelihood of errors and promote a more consistent assessment, particularly with respect to unintended effects.

The majority of the issues related to expertise and judgement identified in the concordance study do not reflect "real" conditions under which GM food safety assessment is typically conducted. As noted above, a team approach with a multidisciplinary approach is more likely to be used in regulatory practice. The following elements can be included in further assessment and practical application of the proposal to improve its performance:

1) inclusion of an orientation/training session for the raters;
2) clarification of specific aspects of the scheme raised by raters in the concordance study;
3) automation of aggregation rules;

4) use of an interdisciplinary team comprised of members who are familiar with GM food assessment; and
5) use of reference product profiles that are developed using realistic data and expert input.

Orientation and training with resource materials can help elaborate specific aspects of the scheme that require clarification. The specific aspects include the principles underlying the aggregation of factor ratings into concern levels for each part in the scheme. The interpretation of criteria and implications of the RBCS for further assessment and testing can be illustrated with product examples that include a rationale for the ratings and suggested further assessment or testing (as represented in Chapter 8). The range of examples could be expanded to illustrate concerns regarding questionable criteria raised by the raters in the concordance study (factors A.3, A.5, B.6, C.1, C.3 and C.5 in particular).

In conclusion, based on the range of reference products assessed, the RBCS as proposed can reasonably differentiate GM foods into at least two levels: those that meet baseline requirements, and others that require further assessment and/or testing. Application of an intermediate level for intended gene products with toxic and antinutritional effects (Part A) requires further exploration and the development of additional product examples. The applicability of the scheme for GM crops modified using non-nucleic acid techniques also needs to be further evaluated. The development of the proposed RBCS is contingent on available testing methods as the basis of the criteria for differentiating GM foods that require more scrutiny from those that do not. Therefore, it is not surprising to find those aspects of GM food safety assessment that are less standardized are also the part of the RBCS that received the most discordant concern levels.

The review of the literature presented in the preceding chapters (3 and 6 in particular) would suggest that while uncertainty remains with predicting unintended changes in GM foods in general, the likelihood of associated adverse health effects should be reasonably low in food crops that have a history of safe food use and are developed using well-established breeding practices. More basic research is needed to clarify the extent to which this generalization holds true with crops modified using complex metabolic engineering not previously achieved using more conventional genetic modification techniques. In contrast, given the potential for severe health consequences of allergic reactions, uncertainty in the assessment of allergenic potential of

a GM food would necessarily trigger the need for further assessment. This conclusion is drawn with the knowledge that the allergenic potential of a food, in contrast with other adverse health effects, cannot be predicted entirely based on the characteristics of a novel gene product. An allergic response is an antigen-specific response in genetically susceptible individuals. Effective risk management options (including food labelling and postmarket monitoring), in addition to rigorous premarket assessment, are needed to address concerns related to allergenicity of novel substances in the food supply. For crops that are modified for food use, the likelihood of increasing the range of food sources of exposure to inherent constituents of existing food crops is higher than the introduction of novel substances that are highly toxic to mammals or humans. Results of the concordance study suggest reasonable concordance for the two parts of the proposed RBCS (Parts A and C) that reflect more important population health risks of GM foods. On this basis, and with further refinements suggested above, the proposed RBCS can provide a useful starting point for further discussion on how to differentiate among GM foods that require more scrutiny from those that do not.

Box 9-1.

Databases used in developing reference panel of GM foods for the concordance study

a) Dietary exposure (food consumption)

- CSFII Analysis of Food Intake Distributions. U.S. Environmental Protection Agency, Office of Research and Development, National Center for Environmental Assessment, Washington Office, Washington, DC, EPA/600/R-03/029, 2003.
[http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=56610].

b) Amino acid sequence of proteins

- NCBI Protein Sequence Database [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein].

c) Safety/toxicity information on specific compounds and microorganisms

- TOXNET [http://gateway.nlm.nih.gov/gw/Cmd].
- IPCS Chemical Safety Information [http://www.inchem.org/].
- NIH Guidelines for Research Involving Recombinant DNA Molecules, 2002
[http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html].

d) Allergens (amino acid sequence identity match using FAO/WHO and Codex Alimentarius guidelines)

- Structural Database of Allergenic Proteins [http://fermi.utmb.edu/SDAP/index.html].
- Food Allergy Research and Resource Program [http://www.farrp.org/].
- Allermatch [http://allermatch.org].

e) Information on taxonomy, food uses and composition of food crops and plants

- USDA, ARS, National Genetic Resources Program. *Germplasm Resources Information Network - (GRIN)* [http://www.ars-grin.gov/cgi-bin/npgs/html/index.pl].
- USDA, ARS, Nutrient Data Laboratory [http://www.nal.usda.gov/fnic/foodcomp/index.html].
- Dr. Duke's Phytochemical and Ethnobotanical Databases [http://www.ars-grin.gov/duke/].
- Consensus Documents - Series on the Safety of Novel Foods and Feeds, OECD.
[http://www.oecd.org/document/9/0,2340,en_2649_37437_1812041_1_1_1_37437,00.html].
- Traditional Crop Breeding Practices, OECD, 1993
[http://www.oecd.org/document/39/0,2340,en_2649_37437_16480743_1_1_1_37437,00.html].

The above databases were accessed between June 2005 and December 2005.

Table 9-1. Selected characteristics of GM foods in the reference panel.

I.D.	Trait	Protein-function	Role in metabolism of modified crop	Gene source	Crop	Food Use
Current products						
H	Herbicide tolerance	Enzyme acetolactate synthase (ALS) modified by single point mutation selected following seed mutagenesis by ethyl methanesulfonate	Restores branched-chain amino acid biosynthetic pathway disrupted by herbicide (imidzolinone)	Same as host plant	Rice	Cooked and processed
K	Herbicide tolerance	Enzyme phosphinothricin acetyltransferase (PAT) - Catabolizes herbicide (glufosinate)		Bacterium (*Streptomyces hygroscopicus*)	Maize (field corn)	Ingredients
O	Delayed ripening	Enzyme 1-aminocyclopropane-1-carboxylic acid deaminase (ACCd)	Reduces biosynthesis of ethylene (a plant hormone important in fruit ripening)	Bacterium (*Pseudomonas chlororaphis*)	Tomato	Fresh and processed
S	Insect resistance	Crystal insecticidal protein (Cry 3A) - binds only to receptors on intestinal cells of a narrow range of lepidopteran and coleopteran insects		Bacterium (*Bacillus thuringiensis*)	Potato	Cooked and processed
New products						
A	Modified vine life & fruit ripening	S-adenosylmethionine decarboxylase (ySAMdc)	Catalyzes the conversion of putrescine to spermidine as part of the polyamine biosynthetic pathway, using an aminopropyl-group donor, which is an intermediate metabolite in the synthesis of ethylene	Yeast	Tomato	Fresh and processed
J	Insect resistance	CryIC protein chemically synthesized by a template-directed ligation -PCR method		Bacterium (*Bacillus thuringiensis*)	Alfalfa	Sprouted seeds
M	Insect resistance	GNA lectin protein – an insecticidal protein		Snowdrop (*Galanthus nivalis*)	Potato	Cooked and processed
P	Insect resistance	Crystal insecticidal Cry protein (Cry 3A) GNA lectin protein – an insecticidal protein		Bacterium (*Bacillus thuringiensis*) Snowdrop	Potato	Cooked and processed

I.D.	Trait	Protein-function	Role in metabolism of modified crop	Gene source	Crop	Food Use
Q	Broad spectrum pathogen resistance	NPR1 protein - a regulatory protein (encoded by non-expresser of pathogenesis-related genes 1) Pathogenesis-related (PR) proteins	Nuclear NPR1 activates the expression of pathogenesis-related (*PR*) genes through interaction with a subgroup of the TGA family of transcription factors in systemic acquired resistance	*Arabidopsis thaliana*	Tomato	Fresh and processed
B	Nutritional quality (high lysine)	Target reduction of α-zein proteins (almost completely devoid of the amino acid) by introducing the gene encoding the protein in an antisense orientation		Same as host plant	Maize	Ingredients
F	Nutritional quality (high lysine)	Dihydrodipicolinate synthase (DHPS) modified by site-directed mutagenesis of *dhps* cDNA	Catalyzes biosynthesis of lysine using a modified enzyme that is lysine-feedback-insensitive	Maize	Rice	Cooked and processed
I	Nutritional quality (high lysine)	Chymotrypsin inhibitor-2 (CI-2) engineered with multiple lysine substitutions (10% seed protein)		Barley	Maize	Ingredients
N	Nutritional quality (protein quality)	Amarantin - a seed storage protein (~ 1% total protein)		Amaranth	Maize	Ingredients
C	Nutritional quality (increased carotenoids)	Phytoene synthase (CRTB)	Catalyzes the first committed step in carotenoid biosynthesis	Bacterium (*Erwinia uredovora*)	Oilseed rape	Refined oil only
L	Nutritional quality (increased isoflavones)	Regulatory C1 and R proteins (transcription factors) Suppressed expression of enzyme flavavone 3-hydroxylase (F3H)	C1 and R transcription factors activate phenylpropanoid pathway genes. Cosuppression of F3H blocks a branch pathway that competes for a metabolite in the biosynthesis of flavonoids.	Maize, carrot	Soybean	Primarily for oil production
G	Nutritional quality (high oleic acid)	Expression of fatty acid δ-12 desaturase (FAD) silenced by introduction of second gene copy	Reduced conversion of oleic acid to linoleic acid (18:1 to 18:2) resulting in accumulation of oleic acid	Same as host plant	Soybean	Primarily for oil production
R	Nutritional quality (increased	Ferritin (iron-binding protein)	Iron storage	Soybean	Rice	Cooked and processed

I.D.	Trait	Protein-function	Role in metabolism of modified crop	Gene source	Crop	Food Use
	iron)					
D	Nutritional quality	Lactoferrin (iron-binding glycoprotein present in milk)		Synthetic codon optimized human lactoferrin gene	Rice	Rice-based infant formula and other infant foods. Extraction for pharmaceutical applications
E	Tolerance to abiotic stresses	Bifunctional fusion enzyme trehalose-6-phosphate synthase/ phosphatase (TPSP)	Catalyzes a two-step trehalose biosynthetic process	Bacterium (*Escherichia coli*)	Rice	Cooked and processed
T	Multiple traits	Uncharacterized - introduced by somatic hybridization through protoplast fusion between a cultivated potato variety and a wild species of Solanum		*Solanum commersonii*	Potato	Cooked and processed

Table 9-2. Concordance in concern levels assigned to reference GM foods by two raters.

RBCS	Levels assigned by raters		Corrected levels[a]	
	No. of reference foods[b] (%)	Kappa coefficient (C.L.[c])	No. of reference foods (%)	Kappa coefficient (C.L.)
Part A	16 (80%)	0.59 (0.27 – 0.92)	17 (85%)	0.69 (0.37 -1.00)
Part B	15 (75%)	0.38 (-0.07 – 0.82)	14 (70%)	0.29 (-0.14 – 0.73)
Part C	16 (80%)	0.60 (0.25 – 0.94)	16 (80%)	0.60 (0.25 – 0.94)
3 parts combined	9 (45%)	0.35 (0.12 – 0.59)	8 (40%)	0.29 (0.06 – 0.53)

[a] Levels assigned by raters were corrected for errors in aggregation of factor ratings.
[b] Total number of reference foods = 20.
[c] C.L. = confidence limits.

Table 9-3. Concern levels assigned to reference GM foods with complete concordance.

Product ID	Nature of target gene product	Known function in plant metabolism	Concern levels		
			A[a]	B	C
CURRENT PRODUCTS					
Insect resistance					
S	Crystal insecticidal protein with narrow target range.		I[b]	I	I
Herbicide tolerance					
H	Enzyme modified by single point mutation by chemical mutagenesis.	Restores branched-chain amino acid biosynthesis.	I	I	I
NEW PRODUCTS					
Insect resistance					
M	Novel insecticidal protein.		III	I	II
P	Stacked insecticidal proteins with potential for synergistic effects.		III	I	II
Q	Regulatory protein.	Activates expression of pathogenesis-related genes.	I	I	II
Nutritional quality					
I	Protein engineered with multiple lysine substitutions.		III	I	II
C (refined oil only)	Enzymatic protein.	Catalyzes the first committed step in carotenoid synthesis.	I	II	I
Other					
T	Uncharacterized proteins introduced by somatic hybridization.	Uncharacterized.	I	II	II

[a] Parts in RBCS.
[b] Based on levels corrected for errors in aggregation of factor ratings.

Table 9-4. Concern levels assigned to reference GM foods with partial concordance.

Product ID	Nature of target gene product	Known function in plant metsm	Discordant concern levels (corrected)						Factors contri-buting to discor-dance
RBCS			Part A		Part B		Part C		
			R1	R2	R1	R2	R1	R2	
CURRENT PRODUCTS									
Herbicide tolerance									
K	Enzyme – catabolizes herbicide.		I	I	**II**	**I**	I	I	B.4.1
Product quality									
O	Enzyme – reduces ethylene synthesis.	√	I	I	I	I	**II**	**I**	C.5
NEW PRODUCTS									
Product quality									
A	Enzyme – catalyzes polyamine synthesis, with connection to ethylene synthesis.	√	II	II	I	I	**I**	**II**	C.1
Insect resistance									
J	Insecticidal protein – chemically synthesized.		I	I	I	I	**II**	**I**	C.2
Nutritional quality									
B ↑lysine	Endogenous protein low in lysine – expression reduced by gene silencing.		I	I	**I**	**II**	I	I	B.6
F ↑lysine	Enzyme modified by site-directed mutagenesis – insensitive to lysine-feedback.	√	I	I	**I**	**II**	I	I	B.4.1
N ↑protein quality	Seed storage protein high in lysine.		**II**	**I**	II	II	II	II	A.5
L ↑isofla-vone	Regulatory protein, transcription factors, and suppressed expression of enzyme in a competing branch pathway.	√	III	III	**I**	**II**	II	II	A.3 B.5.3
G ↑oleic acid	Enzyme – expression suppressed to reduce conversion of oleic to linoleic acid.	√	I	I	**I**	**II**	**II**	**I**	B.6 C.5
R ↑iron	Ferritin protein – iron storage protein.	√	**I**	**III**	I	I	II	II	A.3, A.4.1 A.5
D	Lactoferrin		**I**	**II**	I	I	II	II	A.5, C.4.1
Other									
E	Bifunctional fusion enzyme – catalyzes trehalose synthesis.	√	I	I	**II**	**I**	I	I	B.5.3

Table 9-5. Summary of distribution of concern levels by parts of the RBCS.

Level	Part A	Part B	Part C
Number of products with concordant concern levels in one or more parts of the RBCS			
I	12	11	7
II	1	3	9
III	4	-	-
	17	14	16
Number of products with discordant concern levels in one or more parts of the RBCS			
I/II	2	6	4
I/III	1	-	-
	3	6	4

Based on levels corrected for errors in aggregation of factor ratings.

CHAPTER 10 - SUMMARY AND DISCUSSION

10.1. Summary of the research

10.1.1. Objectives

The primary objective of this research is to develop and evaluate a risk-based classification scheme (RBCS) as guidance for the testing of foods that are genetically modified by a range of methods, including conventional and modern techniques. The focus of the proposed scheme is on human health risks from dietary exposure to foods derived from GM crops.

10.1.2. Methods

The research consisted of three iterative phases:

(1) development of conceptual framework;
(2) development of a graded approach to testing GM foods; and
(3) evaluation of selected functional aspects of the proposed scheme.

Phases 1 and 2 of the research were supported by comprehensive literature reviews (Chapters 2, 3, and 6), spanning diverse topic areas that are relevant to the objectives of the thesis. Four expert consultations totalling 45 expert participants provided general guidance on the development of the conceptual framework and the graded approach to testing. Expert participants with affiliations with academic, government, industry/consulting, and public research institutions were drawn from multiple disciplines and experiences relevant to the risk assessment of agricultural biotechnology products from ten countries in North America, Europe, and the Asia-Pacific region. The consultations were conducted through self-administered structured questionnaires (consultations 1, 2, and 3), as well as individual and group interviews supplemented with written comments (consultation 4). Phase 3 of the research consisted of several activities: development of a panel of 20 reference GM foods with a range of characteristics reflective of current and new/expected products; development of evaluation tools (including a combined RBCS-product rating form) and resource materials for raters who participated in the pilot test (nine experts in several disciplines); and the concordance study (two toxicologists evaluated the 20 reference products using the RBCS).

10.1.3. Conceptual framework (Chapters 4 and 5)

The conceptual framework is built on three components: factors, criteria, and aggregation of factors and criteria, which are integrated in determining concern levels for a GM food. The framework is built on basic concepts of health risk and by applying current knowledge about the sources of *potential* hazards in GM foods and the factors influencing their occurrence and level of exposure. The RBCS is divided into three parts, each focusing on one category of potential adverse health effects of relevance to GM foods. The three categories of potential adverse health effects are: toxic and antinutritional effects from intended changes (Part A), toxic and antinutritional effects from unintended changes (Part B), and allergenic effects from intended and unintended changes (Part C). In each part of the scheme, the factors are rated individually using applicable criteria. The ratings of individual factors are then aggregated separately for each part according to predetermined aggregation rules in assigning concern levels for each part. Three concern levels are proposed for Part A and two concern levels are proposed for each of Parts B and C.

10.1.4. Graded approach to testing GM foods (Chapter 7)

The graded testing of GM foods proposed here provides a structured approach to the assessment and testing of this category of products. The suggested graded testing corresponds to the respective concern levels in the proposed RBCS. The graded approach can be broadly divided into two main parts: baseline requirements for Level I classification, and further data acquisition and testing for products in Level II or higher. Baseline requirements for Level I classification are largely based on testing that is generally accepted for premarket GM food safety assessment. Further testing can be tailored to address specific questions about the safety aspects of the product in Levels II or higher. Explicit criteria for the determination of concern levels also provide a transparent, structured approach for tailoring further testing. This contrasts with published guidelines that are typically descriptive and generic in nature.

Principles governing the testing of chemical substances in general also apply to the testing of primary and secondary gene products that are intentionally introduced or modified with respect to toxic and antinutritional effects. In contrast, there is less general agreement on the testing of potential adverse health effects from unintended changes. Here, a tiered approach to expanded biochemical characterization of GM foods in Level II is presented. The proposal includes a discussion of the rationale for and the scope of analytes for a tiered approach. There are two

considerations in determining the scope of analytes. One consideration is that analytes should be prioritized based on their health relevance in the total diet. The other consideration is the likelihood of changes in their levels in the modified crop predicted on the basis of metabolic network connectivity. The proposed approach takes into consideration developments in emerging methods in metabolite fingerprinting and profiling methods, as well as functional genomics and systems biology, and their growing importance in the design and development of GM crops, particularly those involving extensive metabolic engineering. Other dimensions of the proposal emphasize the importance of product design in reducing unintended effects in GM crops. It is also proposed that the biochemical characterization of GM foods for identifying substances with the potential for adverse health effects be better integrated with strategies for toxicity testing of complex mixtures.

The assessment of the allergenic potential of introduced proteins and GM foods presents a different type of challenge. Assessment is limited by the availability of methods with acceptable positive and negative predictive values and the complexity of the interactions between an allergenic protein and the genetically susceptible individual. The proposal suggests that postmarket monitoring among high risk occupational groups may be justified with certain GM foods that do not meet baseline requirements.

The application of the proposed RBCS and testing options is illustrated by a product example (Chapter 8).

10.1.5. Evaluation of selected functional aspects of the RBCS (Chapter 9)

Four functional aspects of the RBCS were evaluated by two independent raters in the concordance study: applicability, discriminating ability, ease of use, and inter-rater agreement.

Applicability - Among the 20 reference products that covered a broad range of product characteristics and that were classified similarly by the two raters, the majority of the products were classified in the lower levels for Parts A and B, compared to more products in the higher concern level for Part C. Only one product differed by more than one level in Part A.

Discriminating ability - The two raters provided mixed responses regarding the ability of some criteria in the proposed scheme to discriminate among GM foods. The reasons provided for the

negative response from one rater were related to ambiguity in the principles underlying the aggregation of factors into concern levels, as well as a lack of clarity on the implications of the concern levels for testing in the supplementary information provided to the raters. However, the rater agreed that products assigned higher concern levels according to the specified criteria should require further assessment.

Ease of use - Both raters considered the scheme to be slightly difficult to use (average rating of 4.7 on a 7-point scale, with 1 being very easy, and 7 being very difficult). However, the scheme became easier to use with practice. It took an average of approximately 30 minutes to complete the rating of one product for both raters.

Inter-rater agreement - Descriptive and kappa statistics indicate reasonable agreement between the two raters for Parts A (85%, kappa coefficient 0.69) and C (80%, kappa coefficient 0.60) of the RBCS. Less agreement was obtained for Part B (70%, kappa coefficient 0.29) and for the three parts combined (40%, kappa coefficient 0.29). Several reasons may account for the disagreement in the assignment of concern levels between the two raters. They include: differences in the application of default decision rules and expert knowledge beyond the information provided in product profiles, and possible raters' errors resulting from the counter-intuitive wording of some criteria in the RBCS.

It is expected that higher concordance would be obtained when the RBCS is applied in the "real world" under the following conditions: (1) specialists in agricultural biotechnology will be involved; (2) the assessment will be done by a team of evaluators; (3) before making a decision, the evaluators will have the opportunity to seek clarification about ambiguities and to explore in depth any product information that may be limited; (4) product decisions may be subject to review by another group of experts; and (5) as evaluators gain experience in using the scheme to assess GM food products, more uniform decisions can be expected.

Issues related to ease of use and limited or ambiguous information identified in the concordance study can be readily addressed. Options include: automation of aggregation rules; addition of orientation/training for users; improvement in the resource materials to be used in conjunction with the RBCS; and the inclusion of product data more similar to those submitted for premarket assessment as part of resource materials and training.

Review of the literature as discussed in Chapters 3 and 6 would support the view that the likelihood of adverse health effects associated with unintended changes in GM foods would be low in food crops with well-established breeding practices and a history of safe food use. However, this postulate cannot be quantified with available empirical data. On the other hand, the allergenic potential for novel proteins cannot be fully assessed using available methods. Given the possibility of severe health consequences of allergenic reactions, a conservative approach to decision-making is required to reduce the likelihood of introducing novel proteins with allergenic potential in GM crops. The most likely health relevant consequences of genetic modification of food crops are altered dietary exposure to the intentional introduction of novel protein substances at relatively low expression levels, and increased or altered intakes of inherent food constituents (from intentional introduction or modification of enzymatic proteins), many of which lack adequate toxicity data, information on long-term effects, or their optimal balance. Therefore, Parts A and C of the RBCS should be accorded more weight relative to Part B with respect to their importance in assessing potential health risks of GM foods. On this basis, despite less concordance in the assignment of concern levels for Part B, the proposed RBCS provides a useful starting point for further discussion on how to differentiate among GM foods those requiring more scrutiny from those that do not.

10.2. Strengths of the research

This research is the first systematic effort toward the development and evaluation of a classification scheme that differentiates GM foods into different concern levels based on potential health risks. Several features of the scheme and output of the research are highlighted.

1) The core factors in the proposed RBCS are founded on basic concepts of health risk assessment. Explicit criteria for differentiating GM foods based on potential for health risks are developed for each factor.

2) The scheme adopts a tailored approach to the aggregation of a comprehensive set of factors that take into account the relative importance of their contribution to potential health risks.

3) The structure of the scheme is flexible in that new factors may be added and that different components of the scheme can be revised independently. This offers the advantage of keeping

the scheme current in response to future developments in genetic modification techniques and testing methods.

4) To distinguish the different sources of uncertainty, intended and unintended changes are classified separately for potential toxic/antinutritional effects in the proposed scheme. Failure to communicate this distinction may have contributed to the confusion that exists in understanding the basis of the comparative (substantial equivalence) concept on which current practices of safety assessment of GM foods are based. In making the distinction in the sources of uncertainty respecting intended and unintended changes, the proposed RBCS can help to reduce this confusion.

5) The core factors in the proposed RBCS are of a generic nature and are expected to be applicable to the majority of GM foods developed for commercialization.

6) Special cases that require more detailed case-by-case assessment are not overlooked and can be assessed under "other considerations". Special cases include foods derived from crops that are modified by uncommon techniques or by using uncommon source organisms, or that are intended for ingredient use in foods targeted for vulnerable population groups. "Other considerations" facilitate an efficient application of the scheme for the majority of cases that do not require additional considerations.

7) As a tool intended to differentiate those GM foods that require more scrutiny from those that do not, the proposed RBCS has been developed to anticipate questionable GM foods that would require further testing beyond Level I, even though this likelihood would be expected to be low for a product at the time of data submission for formal review at the premarket stage.

8) Implications of the RBCS for testing GM foods are illustrated by a proposal for a graded approach to testing that corresponds to the concern levels in the RBCS. A graded approach promotes more efficient testing in proportion to the potential for health risks. At the same time, tailored testing within a concern level is also possible. This can be achieved by taking into account the specific factors that contribute to the classification.

9) The RBCS presented here is the result of original developmental work without any prior template. While classification systems have been developed in various fields related to chemical, environmental, biotechnology and biosafety risk assessment, as well as for microbiological and chemical hazards in foods and for assessing ecological risks of GM crops, the risk-based classification scheme developed in this doctoral thesis is the first systematic approach to classifying potential health risks of foods derived from GM organisms.

10) Four expert consultations using purposive recruitment and selection of participants provided peer-review during successive stages of the development of the scheme. Evaluation of the functional aspects of the proposed scheme using 20 reference products in the concordance study further confirmed the overall soundness of the scheme. While the proposed scheme can be enhanced with refinements and clarification of certain details, in its present form, it provides a useful adjunct to current guidelines in facilitating an efficient approach to the assessment and testing of GM foods. It can be used to support an open process in decision-making regarding product approvals.

11) As guidance for testing, the RBCS can be used in its entirety as part of a formal assessment process. The explicit criteria and the type of evidence on which the criteria are based provide clarity and transparency to how concern levels are determined. The conceptual framework of the RBCS can also be used in an informal process for a quick assessment of where a specific product may fall within the continuum of concerns relevant to GM foods. Other potential uses may include guiding decision-making regarding acceptability for field testing from a health risk perspective. It can also guide decision-making regarding the continuation or termination of GM crop development during different stages of applied research.

12) While the primary purpose of the RBCS is to guide the testing and assessment of GM foods, the conceptual framework can also be adapted as a communication tool to illustrate the critical factors in determining testing requirements in GM food safety assessment. More effective communication would be one step towards facilitating public understanding of how the potential risks of a new technology in food production are assessed and managed.

13) The reference panel of diverse GM foods comprising a wide range of characteristics developed as part of the concordance study and the in-depth literature synthesis can provide a useful resource for others conducting research or training with similar objectives.

10.3. Limitations of the research

Several limitations of the proposed RBCS are recognized. The development of the RBCS is limited by currently available information and testing methods. However, an analysis of current and emerging methods and their applicability and limitations to GM food safety assessment was prepared as part of the comprehensive literature review. It is beyond the scope of this project to develop new testing approaches to fill the gaps that currently exist. There are also no "gold standards" by which the adequacy of a graded approach to testing can be judged. Also excluded was an assessment of risks that are not directly related to health, such as the broader issues of ecological risks and genetic contamination of plant species.

In this research, risk is defined narrowly based on a scientific definition. A broader notion of risk that incorporates social and cultural dimensions of risk was absent. The broader societal notion of risks includes considerations such as ethics, values, and acceptability of risk [Wolt and Peterson, 2000; Jensen and Sandøe, 2002; Finucane, 2005]. However, scientific assessment remains fundamental and can be an integral part of an ethical framework for the introduction of modern food biotechnology [Mepham, 2000].

This research may also be subject to critique because of a lack of risk-benefit assessment of genetic modification in agricultural production. Risk-benefit assessment is typically examined within the domain of risk management and is most meaningful when applied to specific categories of GM products. This differs from the objective of this research in developing a tool for general application as part of risk assessment. Risk-benefit assessment of GM agricultural products is a subject that has already been examined in detail in other studies [e.g., Benbrook, 2003; Wu, 2004, 2006].

The research was also subject to several methodological limitations due to resource and time constraints, including the potential for selection bias in the participation of experts in the consultation process and for the concordance study. However, the responses received were

diverse and were indicative of what might be expected based on the literature. Preparation of product profiles used in the concordance study did not have input from subject experts. The information provided in the product profiles, while sufficient for the purpose of the study, was not indicative of data normally encountered in product review processes. As well, only one type of expertise was consulted in the concordance study whereas in normal situation of GM food assessment, the expertise of a multidisciplinary team would be required. Despite these limitations, reasonable concordance between the two raters was obtained. As explained, higher concordance is expected when the RBCS is applied in "real world" situation.

10.4. Population health perspectives

In order to explain the relevance of this research in the context of population health, the perspectives of population health on which this research is based are described briefly. The field of population health represents an extension to the natural evolution of the fields of public health and health promotion that focus on mitigating disease risks and fostering positive health-related behaviours in population groups. Population health is concerned with a broader concept of health by addressing the entire range of biological, social, and environmental factors and their interactions that influence overall health and well-being [Dunn and Hayes, 1999; Kindig and Stoddart, 2003]. The population health concept was further developed in an integrated framework for risk management and population health [Krewski et al., 2003]. This framework emphasizes three categories of health determinants and their interactions in population health risk assessment: biology and genetics, environment and occupational, and social and behavioural factors.

Fundamental to population health research and practice is the application of a transdisciplinary approach to problem analysis and solution. This transdisciplinary approach transcends disciplinary boundaries inherent in multidisciplinary and interdisciplinary research [Rosenfield, 1992; van Manen, 2001]. In transdisciplinary research, problems are analyzed and knowledge is synthesized at multiple levels and includes all relevant disciplinary insights with the goal of developing common conceptual approaches that are useable by all the disciplines involved.

10.5. Contribution to population health

This thesis research addresses a focused food safety issue that reflects several dimensions pertinent to the concept of population health. As a source of energy and nutrients, food is clearly an important environmental health determinant of population health. Food safety is also a health issue that can invoke a wide spectrum of personal and policy responses shaped by societal factors. Interventions to address food safety concerns require broad multisectoral support based in transdisciplinary science-informed analysis. In this context, the contribution to population health of the proposed RBCS and associated graded approach to testing GM foods can be viewed from both the practical and conceptual perspectives.

- **Practically, ensuring the safety of the food supply is an important objective of population health policy. To this objective, the proposed RBCS provides a scientifically based tool that can be used to guide the assessment and testing of GM foods.**

- **When implemented, a RBCS can provide a more efficient approach to the assessment and testing of GM foods in proportion to their potential for health risks.**

This means that more evaluation resources should be devoted to GM foods that are more likely to pose health risks than those that are less likely to do so. This benefit will not only accrue to regulatory agencies. It is anticipated that a risk-based framework will also facilitate decision-making in the development of GM crops for field-testing and commercialization. The RBCS would also make an important contribution if it could promote safety considerations in product design during early stages of GM crop development.

- **Conceptually, the proposed RBCS is offered as a framework for differentiating foods derived from crops that are genetically modified by a range of conventional and more modern methods.**

Genetic modification of food crops is among a wide range of technology-driven changes to our food supply from production to processing. This includes conventional and more modern genetic modification methods that have been introduced in the past several decades. While there is recognition that the scientific basis of requiring premarket assessment of crops and derived foods based exclusively on a particular method of genetic modification is questionable [Stirn and Beusmann, 2003; Cellini et al., 2004; NRC, 2004a; Wilson A. et al., 2004a], no systematic approach has been developed to review or revise this policy. The proposed RBCS provides the first analytical framework in this direction.

The proposed scheme provides a starting point for adopting an alternate, more scientifically justifiable approach to the assessment of GM foods than the predominate approach in current practice, which is based on the method of genetic modification. While the RBCS focuses on foods derived from GM crops, the principles developed for this specific category of GM foods can be applied to other categories of food-related GM products.

The framework also differentiates the sources of uncertainty in the potential for health risks of GM foods. This should serve to anchor more focused deliberations on the relative importance of their contribution to the overall health risk potential of GM foods in general as a policy matter, as well as to specific GM foods in particular as part of premarket assessment. It is also hoped that more focused deliberations will stimulate support for necessary research in testing underlying assumptions and in bridging information gaps that fuel debates about potential adverse health effects of GM foods.

The framework is the first exploration of the feasibility of developing a systematic approach to the assessment and testing of a category of foods in proportion to their potential for health risks. This should complement a long-recognized principle of proportionality in risk management.

- **The proposed scheme can provide a more transparent and informative framework for communicating regulatory assessments and decisions regarding GM foods in jurisdictions where this form of communication can be improved.**

At present, the detail with which decisions regarding GM food approval are communicated to the public varies greatly among different jurisdictions. The effectiveness of the different formats

of communication has not been evaluated with respect to the underlying principles of GM food assessment, explicitness of the criteria used, and conciseness of information. The proposed RBCS can provide a basis for improving the communication of product decisions.

A population health approach to food safety issues should promote individual accessibility to information that supports personal decision-making regarding food choices. At the policy level, there is also a growing public expectation of participation and transparency in regulatory processes. By making the principles and criteria for evaluating GM foods explicit, the proposed scheme can contribute towards the development of an open regulatory process and the promotion of informed public dialogue on how GM foods should be assessed.

10.6. Implementation

While a variety of tools exist in chemical and ecological risk assessment (as described in sections 4.2.1 and 5.3.2), there does not appear to be a systematic review of their adoption and implementation for regular use. However, examples of actual use of risk-based classifications in prioritizing detailed assessments exist in environmental remediation and evaluation of medical devices and manufacturing practices of therapeutic products [CCME, 1992; Health Canada, 1998a,b, 2000].

For a discussion of implementation, I will rely on concepts of diffusion of innovations and the Ottawa Model of Research Use (OMRU) [Rogers and Shoemaker, 1971; Logan and Graham, 1998]. OMRU is a comprehensive interdisciplinary model of research utilization developed for the health care field, but its principles are equally applicable in other settings. The model consists of six key related elements that are central to the process of implementation of innovations: the practice environment, potential adopters, the innovation, transfer strategies, adoption, and outcome. Here, innovation refers to the proposed RBCS that is new to the potential adopter, even though the underlying principles may have been available for some time [Eveland, 1986; Rogers, 1994]. For brevity, I will touch on a few salient factors relevant to understanding the barriers and supports to implementation.

The environment exerts powerful influences on policymakers and practitioners. With respect to safety assessment of food products, structural, regulatory, institutional, and social influences are

recognized. The culture and belief systems operating within the regulatory setting, domestic industry, the public interest climate, as well as international trade considerations cannot be underestimated.

The interests of potential adopters may be influenced by scientific and extra-scientific considerations. Twelve attributes of an innovation have been identified to be most likely to affect the speed and extent of the diffusion process [Zaltman and Duncan, 1977; Rogers, 1983; Oldenburg et al., 1997]. Several of these attributes have been discussed in this thesis (complexity, communicability, time required, modifiability). Other attributes such as relative advantage, compatibility, and trialability are expected to be key factors influencing acceptance by potential users. Attributes such as social impact (on public confidence in product safety assessment process), perception of risk, and uncertainty of change, as well as commitment required and observability (the extent to which the results can be observed and easily measured) will also need to be considered.

Knowledge transfer strategies can be categorized based on objectives, process, and extent of intervention. Knowledge transfer can take a passive form (diffusion) with an objective merely to inform and to create awareness. It can also take a more active and targeted approach (dissemination), with an additional objective to change attitude with information tailored to a specific audience. Implementation is the most active strategy, where the objective is to promote behavioural change with supports provided to overcome barriers, and to initiate use from decision to continuation with ongoing use.

Based on this brief summary, it is evident that implementing the RBCS must take into account complex factors that can facilitate or deter adoption. It will require a process consisting of diffusion of the tool in peer-reviewed scientific literature in initial stages. This should be followed by interactions with potential users to facilitate trials and modifications of the proposed scheme. To promote a positive environment, it would also be necessary to engage a wider community of stakeholders.

10.7. Implications

Risks to human health from genetic changes in food crops must be placed in the proper context. It has been suggested that "Of all the risks to human health arising from the environment, none presents a greater challenge to our understanding than those associated with the consumption of foods. This sweeping conclusion is based on the adoption of the broadest possible view of health risks, taken to encompass the total burden of morbidity and mortality that can be related to food consumption." [Brock et al., 2003]. This challenge in assessing the health role of foods stems from their chemical complexity, the balance between beneficial and harmful levels of their constituents, and the multiple environmental, social, behavioural, and technological factors that influence food consumption and food composition.

In addition to the specific objectives of the research reported here, several observations and implications of the research also require attention. This discussion elaborates on two determinants of population health relevant to this research: public policy related to health risk assessment and management, and personal coping skills and health practices as affected by science and risk communication.

10.7.1. Imbalance in the assessment of food-related chemical risks

There is a highly uneven approach to the assessment of substances intentionally introduced to foods compared to natural constituents [NRC, 1996; Gold et al., 2001; Brock et al., 2003]. It seems likely that as a class, the impact of natural non-nutritive food constituents on human health – both adverse and beneficial – can be substantial compared to low molecular weight substances added to foods [NRC, 1996; Brock et al., 2003]. This view is based on our knowledge of the chemical structures, the biological properties of the relatively few that have been studied, and the immense numbers of the former class of food substances. This knowledge gap is a source of controversy over the potential for unintended adverse health effects of GM foods and whether the results of an untargeted approach to compositional analysis would be interpretable (discussed in section 6.3.9). Compared to intentional changes at the food processing level such as the addition of food additives, changes to food composition induced by genetic modification at the level of crop production may be less predictable but are not necessarily more risky in terms of health effects. Yet, a reductionistic focus on metabolite changes in a single GM crop or food being assessed is unlikely to enhance our ability to adequately address fundamental

questions related to unintended effects of GM foods from a public health perspective. In addition, food constituents can also be extracted and added back to foods, often at higher levels. Therefore, a holistic approach to prioritizing the relative importance of the vast number or classes of natural food constituents from a total diet perspective is proposed as part of a graded approach to testing GM foods. However, this proposal regarding GM food assessment should not be viewed as a policy option for a specific product category in isolation from other changes to the food supply.

10.7.2. Generic vs. restricted approval

The approval of GM foods commercialized to date is generic in nature in that their distribution as foods or ingredients in the broader food supply is essentially unrestricted. As future GM crops may involve modifying secondary metabolites for nutritional or health benefits, they can expand traditional routes of food fortification. This means that fresh foods can be fortified at the agricultural production level. Further, ingredients processed from foods genetically modified to have higher levels of nutritive or bioactive substances can enter the food supply through additional routes not currently regulated. How the approval of such GM crops should be managed requires further analysis.

10.7.3. Regulatory decision process and support

The current approach to the assessment of GM foods based predominately on the method of genetic modification is perhaps analogous to what appears to be a case of legislative and regulatory prerogatives driving science in the uneven application of resources in assessing food chemical risks. These phenomena may be traced to the reactive nature of regulatory science and the historical origin of food regulations as a response to food adulterations. Fragmentation in the institutional structures now in place to manage food-related risks is also recognized as a contributing factor to our inability to address food-related health issues using a holistic approach [NRC, 1998; Brock et al., 2003;]. This is a complex problem outside the remit of science.

At the same time, there is increasing recognition that the broader societal context of risk should be considered in risk assessment and management, as the determination of risk acceptance has an extra-scientific dimension [Steinhauser, 2001; Klinke and Renn, 2002; Finucane, 2005; Knudesn et al., 2005]. Processes are being developed to promote participatory deliberations [e.g.,

Stirling and Mayer, 2001; Klinke and Renn, 2002]. It remains fundamental that public participation be based on an informed process.

In the case of GM foods, as indicated from the literature review conducted for this thesis, it is difficult to find empirical evidence that supports the respective assertions of either the critics or the proponents of modern agricultural biotechnology regarding the general safety or the hazardous nature of GM foods for human health, and how safety concerns should be addressed through testing. Assertions are largely based on inference or generalization of observations from one level of biological systems to another (e.g. from the molecular to metabolic level), from one field of biological science to another (e.g., from microbial to plant metabolism), or assuming the lack of evidence of adverse effects as having evidence of no adverse effects. In the absence of scientific data that meet generally accepted standards of scientific evidence, expert judgement has been relied upon in the development of current practices in the assessment and testing of GM foods. For the same reasons, some aspects of the proposal forwarded in this thesis remain provisional in nature and require further research for validation. Three issues related to regulatory and individual decision support concerning GM foods deserve emphasis.

a) Evidence building as an evolving process

Where reliable evidence is lacking, it is understood that prior knowledge and experience can serve as a starting point upon which research may be developed. For example, extending prior knowledge of conventional genetic modification to more modern DNA recombinant techniques has been used as the basis for comparative safety assessment. However, more than a decade after the adoption of this strategy, gaps in information remain (as noted in this thesis). Unlike efforts in promoting participatory regulatory decision making, there does not appear to be similar efforts in promoting public dialogue about the type of research that should be conducted to support or refute assumptions underlying current requirements for premarket assessment of GM foods, or how the assessment may be conducted more efficiently.

b) Transdisciplinarity in science and risk communication

A range of expertise in natural and applied sciences is required in the safety assessment of GM foods. However, the extent to which differences in disciplinary perspectives and training affect the assessment and communication of GM food risks by subject experts has not received serious consideration. Even among specialists in biological sciences, it is not uncommon to find

dissonance in the understanding of hazards vs. risks and a tendency to generalize observations across disciplines or across levels within biological systems. As the design of GM crops increases in complexity, greater transdisciplinary understanding and cooperation among scientists across disciplines and sectors will become increasingly important [Edmeades et al., 2004]. This is also an important aspect of risk communication as the lay public generally reported a higher level of confidence in information provided by academic scientists compared to some other information sources [Krewski et al., 2005].

c) Information seeking behaviour of non-experts

Consumers in North America appear to have a higher level of acceptance of technological innovations and of the GM foods marketed to date compared to their European counterparts [Priest, 2002]. However, this generalization cannot be assumed with the third generation of GM crops where food crops are not excluded from modification to produce pharmaceutical or industrial substances [Stewart, 2004].

There has been considerable interest in understanding public perceptions of food-borne risks from the psychometric and affect heuristic perspectives, including differences in risk perceptions between experts and the lay public [e.g., Sparks and Shepherd, 1994; Fife-Schaw and Rowe, 1996; Savadori et al., 2004; Townsend, 2006]. However, the role of subject knowledge in promoting effective communication of socioscientific issues remains unclear and requires further research [e.g., Yim and Vaganov, 2003; Batrinou et al., 2005; Sadler and Zeidler, 2005]. There is a need to better understand audience segmentation and their information seeking behaviour, as well as how information is processed with respect to potential health risks arising from new technologies [Frewer et al., 1997; Wilson C. et al., 2004; Macgill and Siu, 2005; Silk et al., 2005; Verbeke, 2005].

While the RBCS was not developed with a risk communication objective in mind, it has the potential to provide an impetus for enhancing communication about technology-driven changes in the food supply. The current approach in generalizing a heterogeneous group of GM foods as if they were a homogeneous category based on the method of genetic modification hinders effective communication. The proposed RBCS can be adapted to communicate those factors that contribute to potential health risks of GM foods. Research investigating information processing among different population segments may facilitate an understanding of the cognitive

differences and the means to narrow gaps in understanding about potential health risks of GM foods among different groups.

10.8. Future research

Four major areas of future research are proposed based on their direct relevance to the enhancement of the RBCS proposed in this research. The first three areas of research addresses fundamental knowledge gaps that are linked to concerns about potential health risks of GM foods and their assessment. The fourth area is an extension of this research with respect to implementation.

1. Research is required to provide support for the major assumptions underlying factors that are used to indicate the potential for unintended effects in GM crops. As discussed in section 7.3.2.d, based on knowledge of their function, certain types of proteins introduced into food crops can be expected to have minimal interactions with the host plant's metabolic pathways on theoretical grounds. These types of proteins are expected to be less likely to induce pleiotropic effects compared to proteins that mediate global responses through their regulatory or signalling functions. Confirmation of those characteristics would validate their use as predictors of the extent or likelihood of unintended effects in the RBCS.

2. The concepts of and methodologies for priority setting and ranking potential health hazards among naturally occurring food chemicals for toxicity testing need to be further explored and developed. A diet-based strategy that is consistent with a more holistic approach is needed to complement current approach that compares levels of food constituents in individual modified crops to natural variations in their unmodified counterparts; a major limitation of this approach is the lack of data for interpreting the health relevance of statistical deviation from natural variations.

3. As emerging technologies become more established for the purposes of hazard identification and risk assessment, the graded testing approach proposed in parallel with the RBCS should be re-examined. New testing approaches that have the potential to improve testing efficiency should be incorporated into the RBCS.

4. To accomplish the goal of achieving a more efficient use of resources in assessing and testing GM foods for potential health risks, there is the need to invest in further evaluation and enhancement of the scheme proposed in this research. This will involve research on the following issues.

    - In-depth evaluation of the proposed scheme by potential users in more realistic situations involving multidisciplinary teams of experts familiar with GM food safety assessment.
    - Investigation of potential barriers to and supports for acceptance of a risk-based approach to GM food assessment by potential users.
    - Further development of the scheme for other food-related GM products, including food use of GM microorganisms, food derived from GM animals, GM feed, and possibly food risk implications of therapeutic and industrial uses of GM crops.
    - Adaptation of the scheme to communicate product approval decisions to the public.
    - Adaptation of the scheme to communicate to interested non-experts those factors that contribute to the potential for health risks in GM foods.

10.9. Conclusion

The risk-based classification scheme developed from this research is the first systematic effort of its kind applied to the assessment and testing of foods derived from GM crops, with potential for wider application to other categories of food-related GM products. The proposed scheme provides explicit criteria for classification based on factors related to the potential for health risks. The scheme is structurally flexible for modification in keeping with scientific and technical advances. An exploratory evaluation of several functional aspects of the scheme supports the conclusion that in its present form, the proposed scheme offers a useful starting point for further discussion and development. As with any new tool, its acceptance depends on an iterative process of evaluation and modification. The objective is the implementation of a more efficient approach to the testing of GM foods in proportion to their potential for health risks.

The proposed scheme also has the potential to facilitate a more focused public dialogue on the different sources of uncertainty in GM food assessment and their relative importance in contributing to their potential for health risks. It is hoped that in parallel with advances in analytical techniques and knowledge of the molecular and biochemical effects of genetic modification processes, research can be directed to address current information gaps and to reduce uncertainty in how potential health risks of this technology should be evaluated. The ultimate goal is to strengthen GM food assessment practices and public confidence in how GM foods are assessed.

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A Risk-Based Classification Scheme for Genetically Modified Foods: Establishing Levels of Concern to Guide Testing Requirements

VOLUME II

(Appendices A – F)

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements for the
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APPENDIX A

Preface – Volume II

Volume II of the thesis (Appendices A – F) provides detailed supplementary information on the collection and analyses of data not covered in Volume I.

Expert consultations 1 to 4 conducted in Phases 1 and 2 that supported the development of the risk-based classification scheme (RBCS) and graded testing options are described in Appendices A to D, respectively. In Phase 3, selected functional aspects of the proposed RBCS were evaluated in a concordance study. A pilot test of the research instruments used in the concordance study (expert consultation 5) was conducted and is described in Appendix E. The research instruments used in the concordance study (expert consultation 6) are provided in Appendix F.

PHASE 1 (Conceptual Development)

APPENDIX A

EXPERT CONSULTATION 1

Assessment of the importance and appropriateness of selected factors and criteria

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Phase 1 (Conceptual Development)

Phase 1 of the research focused on the conceptual development of the risk-based classification scheme (RBCS). Two expert consultations (1 and 2) were conducted in Phase 1 of the research project. Appendices A and B describe the purpose, methods, and results of these two consultations not discussed in detail in Volume I of the thesis.

1. Expert Consultation 1 - Assessment of the importance and appropriateness of selected factors and criteria

1.1. Purpose

The purpose of expert consultation 1 was to assess initial ideas about the factors and criteria to be considered in developing a RBCS with the guidance of a multidisciplinary group of experts. Specific objectives of this survey were to:

- rate the importance of a list of factors identified from literature review that may be relevant to determining the type and level of testing for assessing the potential health risks of GM foods, and
- assess the appropriateness of the criteria to be used in classifying GM foods into levels of concern based on the factors identified.

1.2. Recruitment and selection of experts

Recruitment and selection of expert participants was based on purposive and snow ball sampling in order to reach a minimum of ten individuals with specified characteristics (relevant expertise, interest in the project, and availability) [Morse and Richards, 2002]. Relevant expertise included:

- expertise in health risk assessment of GM foods, and
- training or active research in one or more of the following disciplines: plant sciences, molecular biology/genetics, microbiology, toxicology, immunology, systems biology, analytical biotechnology, food/nutritional sciences, and health risk assessment.

Individuals were judged to have expertise in health risk assessment of GM foods if they have participated in one or more of the following activities on the topic:

- participation in government advisory committees or publicly funded research,
- author or reviewer of a major guidance document,
- author of a publication in a peer-reviewed scientific journal, or
- safety assessment of GM foods or related biotechnology products in an evaluation or management capacity.

A two-step recruitment process was used, comprising (1) notification and (2) specific recruitment of a selected group of experts. The recruitment process was conducted in accordance with the rules of the Health Sciences and Science Research Ethics Board at the University of Ottawa that specified only one reminder contact should be made with potential participants. The certificate of ethical approval for expert consultation 1 (Phase 1a) is appended as **Annex 1-1**.

(1) Notification

An information/recruitment letter was sent by e-mail to two groups of recipients whose contact information was identified from public sources (such as directories of faculty members of universities on the Internet and contact information of authors in scientific publications). A signed copy of the letter on University letterhead was also attached to assure recipients of the legitimacy of the research project. The two groups of recipients were (a) individuals who met the criteria for expertise described above (n=84), and (b) individuals who served as contact points (n=54). Individuals who met selection criteria were identified from membership on government advisory committees or relevant expert groups, as well as from authorship of selected authoritative reports and citations in peer-reviewed literature. Contact points included coordinators of relevant scientific committees, contact persons for government departments that have responsibility for safety assessment of GM crops and derived foods, and team leaders or directors with a science-related function in research/scientific institutes and industry organizations related to plant biotechnology or plant breeding (**Tables 1-1A and 1-1B**). The specific expertise of individuals in the latter group could not be identified from public sources at the time of mailing.

The information/recruitment letter (**Annex 1-2**) described the objectives of the project, the type of expertise sought, and the scope of participation which included responding to self-administered questionnaires for consultations (surveys) 1 and 2 of the project. Potential participants were given the option to participate in one or both consultations. Recipients of the letter were asked to distribute the letter to colleagues whom they thought would be interested in the topic. They were also invited to respond themselves if the project was of interest to them personally.

(2) Specific recruitment

A targeted group of individuals who met the general criteria for expertise were sent by e-mail a customized letter requesting their consideration for participation in the study (n=24). This recruitment letter outlined why the individual's expertise was valuable to the project. This targeted group of individuals were selected with the view to achieve a balance of expertise in the final composition of the participants.

Fourteen individuals responded by agreeing to participate, including three individuals who were not sent the original information/recruitment letter directly by the researcher. These individuals responded after receiving the letter from a contact point. Participating individuals were asked to return a copy of a signed consent form (**Annex 1-3**) with a completed questionnaire (**Annex 1-4**).

Of the 14 individuals who expressed an interest in participating in the study, four specialized in different domains of plant sciences, five had expertise in food sciences or food-related toxicology and immunology, one in cell biology, and four conducted and coordinated research or evaluation related to the safety of GM crops and derived products as their primary function. Four of these individuals were affiliated with universities, two with government regulatory agencies, and eight with contract laboratories or research institutes associated with government or university. Eight of the participants were from Europe, five from North America, and one from the Asia-Pacific region. In addition, seven other individuals provided information about upcoming major reports and guidance documents, or informed the researcher that the information/recruitment letter was forwarded to colleagues.

1.3. Data collection

A self-administered questionnaire (**Annex 1-4**) was developed to elicit expert judgement using a 4-point scale (0=not important/appropriate, 1=somewhat important/appropriate, 2=important/appropriate, 3=very important/appropriate). Expert judgement was sought on the importance of the list of factors and on the appropriateness of the criteria identified from literature review (described in Chapters 3 and 4 of the thesis, Volume I). Respondents were also asked to provide the reasons for their ratings and to suggest alternate or additional factors and criteria. Open-ended questions included: (1) whether certain hazard factors were relevant to crops modified by all methods of genetic modification; (2) the weighting of unintended effects that should be given, relative to intended effects in assessing potential health risks of GM crops for food use. Space was also provided for general comments.

Pilot testing of questionnaire

The primary objectives of the pilot test were to:

- determine the length of time required to complete the questionnaire,
- identify any ambiguities and redundancies in the questions, and
- identify other changes that should be made to the questionnaire.

Participants of the pilot test were selected from the Institute of Population Health (IPH), Health Canada (Healthy Environment and Consumer Safety Branch - Biotechnology Section), and Canadian Food Inspection Agency (Animal Products Directorate - Feeds Section) based on their knowledge in

molecular biology, microbiology, physiology, animal nutrition, health sciences, or health risk assessment. Three individuals also had experience in evaluating biotechnology products. Out of the seven individuals requested to participate in the pilot test of draft 1 of the questionnaire, six returned completed questionnaires. Draft 2 of the questionnaire was reviewed by two individuals affiliated with the IPH. One individual completed the questionnaire and one provided comments.

The following is a summary of the responses received on draft 1 of the questionnaire.

- Time required: 80-130 minutes, average 96 minutes.
- The full rating scale for both the factors and criteria (0-3, not important/not appropriate – very important/very appropriate) were used by all respondents, although one individual indicated that "somewhat appropriate" could be deleted for criteria and two individuals suggested combining "important" and "very important".
- Some comments suggested a level of confusion with the objective of the survey. Some respondents appeared to have mistaken the factors and criteria to be the risk-based classification scheme. The lack of specific examples was another criticism.
- Hazard factors - The large majority of the hazard factors and criteria included in the questionnaire received ratings of 2-3 (important/very important) for either intended or unintended effects. A few factors and criteria received ratings of 0 for both intended and unintended effects. Accompanying comments suggested ambiguities possibly related to the generalities or the wording of the factors/criteria, or unfamiliarity with specific issues of health risk assessment of plant biotechnology products and conventional methods of genetic modification of plants. Further, asking respondents to rate the same hazard factors and criteria for both intended and unintended effects was confusing to some.
- Exposure and consequence factors – There was recognition of the importance of considering exposure and consequence. However, the comments suggested that some of the criteria included were inappropriate, either because they were too general, not sufficiently discriminating, or they were not set at gradations meaningful for assessing relative potential for health risks for foods.

Based on feedback from the pilot test, changes made to the revised questionnaire (draft 2) included the following:

- Reorganization – Different factors were grouped separately under "primarily intended effects" and "primarily unintended effects". Factors were listed once with the corresponding criteria immediately following each factor. This change was made to reduce confusion with assessing the same factors for both intended and unintended effects.

- Factors and criteria – Criteria were made more specific. Examples were given where they were thought to improve clarity. Exposure and consequence factors were streamlined by integrating several factors and criteria in draft 1. One non-discriminatory factor (stability) essential to the assessment of all GM crops was removed from the questionnaire and was included as part of prerequisite information in the proposed RBCS.
- Open-ended questions – One question related to the criteria for conventional food and testing method for crops modified by conventional method was deleted. Instead, proposed criteria were included in the factors and criteria section of the revised questionnaire, and a general question related to testing method was added in the open-ended section. The question probing the relative weighting of intended and unintended effects was simplified.
- Introduction to the questionnaire was revised to provide background information for the questionnaire in the context of the complete research project in an easy-to-read format.

Responses received on draft 2 of the questionnaire were summarized as follows:

- Time required to complete the revised questionnaire: approximately one hour
- One respondent commented on the lack of specific examples and suggested that the relative importance of the factors and criteria be ranked. Suggestions were also made to re-organize part of the content in the introduction and to place the request for "other factors and criteria to be considered" immediately following each section of the questionnaire

In view of the primary objective of consultation 1 and how the factors and criteria will be used in the development of a draft RBCS, it was not considered necessary to ask respondents to rank factors and criteria. Minor editorial changes were made to draft 2 of the questionnaire for distribution to the expert participants.

1.4. Data analysis

Ten completed questionnaires were returned out of the 14 sent to expert participants. The ten respondents represented a cross section of expertise similar to that of individuals who received the questionnaire: plant sciences (4), food sciences or food-related toxicology and immunology (5), research and evaluation related to the safety of GM crops and derived foods (1). One other questionnaire was returned with only partial completion. The ratings from this questionnaire were excluded from the quantitative analysis but the comments were noted. Respondents' ratings of the importance of factors and the appropriateness of criteria included in the questionnaire are summarized in descending ranked order of importance by factors in (**Table 1-2**). In general, the responses of the participants supported the expected relative importance of the factors identified from the literature review. Most of the criteria

proposed were rated as appropriate or very appropriate by the majority of respondents. Additional factors and criteria suggested by the expert participants are listed in (**Table 1-3**). Comments to open-ended questions are summarized in **Tables 1-4, 1-5, 1-6**.

Importance of factors (Table 1-2)

There was an overall agreement on the importance of hazard factors related to intended effects. Two respondents emphasized the need to assess marker genes. Not surprisingly, a divergence of expert views was evident in some hazard factors related to unintended effects, particularly with respect to the method of gene transfer. The more divergent views may reflect the need for specifying the condition under which certain factors apply. For example, regarding the method of gene transfer, one respondent commented that this factor would be important in the case of random or selective mutation as it relates to the accuracy of the method. However, for gene transfer in the case of rDNA technique, what is important is the nature of the construct, rather than the method of gene transfer. Two hazard factors concerning the donor organism (prior knowledge of biology and composition and history of safe use) were generally considered to be of lesser importance by more respondents compared to the other hazard factors. As expected, among the five exposure factors being rated, the most consistent rating was given to direct dietary exposure (important or very important by all respondents), while the ratings for the other exposure factors were more varied. Judging from the respondents' comments, the varied ratings for these factors could be related to the perceived reliability of methods for controlling expression, which is influenced by many factors, and to the relevance of an exposure factor only when gene products involve allergens or toxins. Of the four consequence factors being rated for potential hazards from intended effects, prior knowledge of the nature of adverse health effects of target protein/enzyme and direct product of target enzyme was unanimously judged to be very important by all respondents. The existence of susceptible subpopulations was also considered by the majority to be very important. In contrast, prior knowledge of the nature of adverse health effects of some components of the host and donor organisms received more varied ratings, ranging from not important or somewhat important (3 respondents), to important or very important (7 respondents).

Appropriateness of criteria (Table 1-2)

Among the criteria proposed for assessing intended effects of three hazard factors (gene product, product of enzyme, and product of marker gene), one criterion, prior knowledge of potential adverse human health effects, was consistently rated as very appropriate by all respondents for assessing the direct product of enzyme. The criteria proposed to assess the graded levels of evidence in establishing prior knowledge about potential adverse human health effects generated varied comments. Another criterion, experience in human diet, was also judged to be very appropriate or appropriate for assessing gene product and the direct product of enzyme. For assessing unintended effects of these hazard factors,

their role in the metabolic processes of the host plant and the specificity of function of the protein/enzyme were generally judged to be appropriate or very appropriate criteria by the majority of respondents. Prior knowledge of stability of a gene product and the direct product of an enzyme under typical processing conditions received mixed ratings as a criterion for assessing intended effects of gene products and products of enzymes. Respondents who rated this criterion as only somewhat appropriate noted that this criterion is only relevant to highly processed food, or if the gene product is expected to be a health risk. One comment questioned the practicality of the criteria for assessing metabolic pathways and another questioned the relevance of focusing on the target pathway since it would likely be the unknown second pathway that would produce unintended effects. A comment was made that the absence of any toxic activity would be more persuasive than destruction by cooking. The criterion for assessing the allergenic and toxic potential of the host and donor organisms was generally rated to be appropriate or very appropriate. However, the other criteria (prior knowledge of the biology and composition and history of safe use) were judged to be less appropriate in assessing donor organism by some respondents. The primary reason given was that it is the gene/protein/metabolite that is of concern primarily, and not the donor organism. In addition, among the criteria proposed for assessing non-plant donor organisms, which is applicable only to rDNA techniques, molecular characterization of the genetic material transferred was also considered by the majority of the respondents to be appropriate or very appropriate. The criterion infectivity/pathogenicity of microbial organisms was not considered appropriate because "pathogenicity can often be plasmid encoded and therefore not dependent on the organism's genome. Very similar strains of Salmonella may, or may not be benign".

A combination of the proposed criteria for assessing direct dietary exposure was generally rated as appropriate or very appropriate. Other criteria had mixed ratings: presence or absence of expression for assessing tissue specificity of expression, the extent of pre-sensitization for assessing non-dietary exposure, the extent of controllability for assessing the timing of expression of gene product, and the extent of accumulation via feed and animal-derived food for assessing indirect dietary exposure. The criterion for assessing indirect dietary exposure was considered by some respondents to be relevant only to chemicals and not applicable to proteins. For assessing the consequence factor related to prior knowledge of adverse health effects of protein/enzyme and product of enzyme, the majority of the respondents also rated the extent of severity to be a very appropriate criterion. The criteria for assessing susceptible subpopulations and the extent of alteration in the potential for significant changes in dietary practices were also rated as appropriate or very appropriate by the majority. Two respondents noted that the criteria for assessing the adverse health consequence of host and donor organisms should be separate as their potential contribution to adverse effects will be different depending on the type of transformation.

Additional factors and criteria suggested by respondents (Table 1-3)

Some respondents suggested additional factors and criteria to be considered. Among the nine new hazard factors identified by the respondents, three were considered somewhat important (site of insertion, stability of insert, and recombination and deletion), two important (gene deletion, molecular detection method), and four very important (nature of the change induced in the protein and/or product of the genetic modification; risk-benefit analysis; gene stacking, and effect of agronomic stress). Three additional exposure and consequence factors included: aggregate exposure, variability in individual consumption, and monitoring capacity for identification of hypothetical, potential adverse effects. The level of importance of another factor (crossing between different modified plants, marker gene) was not specified.

Responses to open-ended questions

1. Relevance of the hazard factors (H1 to H8) to all methods of genetic modification (Table 1-4)

Of the seven responses to this question, three (3) respondents agreed that the factors are all relevant to different methods of genetic modification, while others provided a different view. Reasons for the different views included:

- Inappropriate terminology was used ("target" gene product and metabolic pathway not appropriate for conventional breeding)
- The only relevant factors are direct products of inserted genes (2 respondents)
- Some specific criteria were inappropriate (e.g., breeding histories as a criterion in assessing host or donor plant)

2. Weighting to be given to the assessment of unintended effects relative to intended effects (Table 1-5)

All ten respondents of completed questionnaires responded to this question. One respondent suggested higher weighting for unintended effects relative to intended effects. The other respondents expressed a range of responses: lower weighting from three respondents (0 - 0.2 relative to a weighting of 1 for intended effects), equal weighting from two respondents, and variable weighting from four respondents. The reasons or comments for the respective views are included in Table 1-5.

3. General comments (Table 1-6)

The general comments can be grouped into the following themes: marker gene, testing concepts and methods, allergenic effects, unintended effects, degree of confidence in response, and application to RBCS. A summary of the responses and sample comments are included in Table 1-6.

1.5. Discussion

Consultation 1 provided a basis for developing a RBCS for testing foods derived from GM crops. The methodological approach underlying consultation 1 is based on expert subjective judgement elicitation, which is a common technique used in characterizing the magnitude of and uncertainty in environmental risk assessment and in Bayesian decision analysis [Morgan and Keith, 1995; Walker et al., 2001]. Unlike the quantitative nature of many technical problems tackled in environmental risk assessment and Bayesian decision analysis, the current state of knowledge and the nature of the problem being investigated in this research project do not allow formalization or quantification of the judgement of individual experts. However, the qualitative nature or the diversity of expert opinions does not necessarily negate the usefulness of the results obtained in this phase of the research. Diversity of expert opinions is to be expected when uncertainty is high or when there are fundamentally different views about underlying assumptions and processes affecting the phenomenon being studied. In interpreting the results provided by the expert participants to the self-administered questionnaire, several contextual factors were considered.

- First, respondents were asked to rate the factors and criteria with no specific examples given. Some factors may be important in some conditions but less so in others. For example, as some respondents noted, the importance of donor organism as a hazard factor would depend on the type of transformation involved and whether the donor organism is a source of allergen and toxicant.
- Second, based on the comments provided, without seeing how the criteria would be applied in assessing a specific factor, some respondents appeared to have difficulty in rating the appropriateness of certain criteria, when one of the gradations included "limited or lack of evidence for no effect" or "limited data/unable to evaluate". This difficulty was not eliminated despite providing an explanation on the criteria in the beginning of the questionnaire regarding the illustrative nature of the gradations in the criteria included.
- Third, the regulatory and public focus on crops and derived foods modified by rDNA is likely to influence how respondents rated the factors and criteria. This influence is evident based on respondents' comments, such as "it is the gene/protein/metabolite that is of concern primarily and not the donor organism". This type of comments seems to reflect an emphasis on the rDNA approach.
- Fourth, some respondents may have an understanding of the purpose of the proposed RBCS that is different from what is intended, despite the background information provided in the beginning of the questionnaire. This difference in the perception of the purpose of the proposed RBCS may be inferred from the apparently different interpretations of the intent or use of the graded criteria, as noted above. An expectation that the RBCS is to be used to classify GM

foods based on inherent health hazard, rather than as an indicator of the level of uncertainty and hence the level of testing required, could lead to different ratings of the factors and criteria.

- Fifth, respondents may apply different weightings to different elements of importance and appropriateness. For example, the importance of a hazard factor for assessing unintended effects may depend on how adverse health effects may be mediated, as well as the probability of their occurrence.

Despite these limitations, the results of consultation 1 have provided useful information relative to the purpose of the consultation and to guiding the development of a draft RBCS. The results confirm that:

- the initial ideas about the factors and criteria to be considered in developing a RBCS can be pursued
- major factors and criteria have been considered

In addition, the consultation provided additional factors and criteria to be considered, some of which may be incorporated as part of testing options in a future phase of the project.

Conclusion

In guiding the development of a draft RBCS, several generalizations of the results that are congruent with conclusions of the literature review may be made.

- As expected, there was general agreement about the importance of specific hazard factors related to intended effects.
- Not surprisingly, expert opinions were varied regarding the weighting that should be put on assessing human health implications of unintended effects. Varied opinions were related to different underlying rationales, ranging from the basic premise that unintended effects represent a normal risk that is accepted in traditional breeding, to the need for case-by-case evaluation of the specific factors (such as the nature of the crop and of the metabolic pathway modified).
- The RBCS needs to be comprehensive in order to accommodate the types and range of intended and unintended effects that may be encountered. This will be particularly important for the next generation of GM crops and derived foods for which assumptions about the "acceptability" of unintended effects based on conventional breeding may not be applicable, and the demarcation between conventional and modern GM approaches may become increasingly blurred.
- Given the emphasis on rDNA as the method of genetic modification of food crops that requires scrutiny, it may be useful to keep hazard factors related to unintended effects separate between conventional and modern methods of genetic modification in the RBCS. Analytical methods available or used for molecular characterization of genetic changes in the modified plant may

provide a basis for separating these two general categories of genetic modification as an indicator of the level of uncertainty and further testing required.

- Exposure factors should focus on direct dietary exposure.
- If the gene product is not expressed or metabolized in the edible portion of the modified crop, the GM food does not warrant extensive testing.
- Consequence factors could be integrated into criteria for classifying the hazard potential of GM crops and derived foods.

In addition, a low rating for a factor or criterion obtained in this consultation does not necessarily mean that the factor or criterion should be eliminated from the RBCS; it may mean that the condition of its application should be made more specific (e.g., assessing food processing effect only if the gene product is expected to be a health risk), or that it would be more appropriate to include the parameter as a testing option, rather than as a determinant of concern level (e.g., monitoring capacity, benefit assessment).

1.6. References

Morse JM, Richards L. 2002. Read me first for a user's guide to qualitative methods. Thousand Oaks: Sage Publications. 262p.

Morgan MG, Keith DW. 1995. Subjective judgments by climate experts. Environ Sci Technol 29(10):468-76.

Walker KD, Evans J, Macintosh D, 2001. Use of expert judgment in exposure assessment Part I. Characterization of personal exposure to benzene. J Expo Anal Environ Epidemiol 11:308-22.

Table 1-1A. Distribution of information/recruitment letter - individuals.

Source	Individual[1]
1. Advisory committees, authors and reviewers of major reports	
▪ Royal Society of Canada Expert Panel on the Regulation of Food Biotechnology in Canada, 2001 (14)[2]	4
▪ U.S. NAS-NRC Committee on process to identify hazards and assess the unintended effects of genetically engineered foods on human health, 2004 (13)	7
▪ U.S. NAS-NRC GM Pest-Protected Plants, 2000 (12)	3
▪ FDA Food Advisory Committee - Food Biotechnology Subcommittee (7)	4
▪ UK Royal Society GM Science Review, 2003 (24)	9
▪ UK Royal Society Report - GM plants for food uses and human health, 2002 (10)	4
▪ European Food Safety Authority (EU: 15 current + 10 candidate member countries)	
Panel on genetically modified organisms (21)	4
Panel on plant health, plant protection products and their residues (18)	2
Panel on dietetic products, nutrition and allergies (13)	1
▪ EC Guidance Document for Risk Assessment of Genetically Modified Plants and Derived Food and Feed, 2003	
Scientific Committee on Food (11)	4
Scientific Committee on Plants (8)	4
Experts + Reviewers (2 + 26)	8
▪ Commission on Genetic Modification, Subcommittee on Agriculture, The Netherlands	1
▪ Flanders Interuniversity Institute for Biotechnology Report - Safety of Genetically Engineered Crops, 2001 – editor (1), authors (11) and reviewers (9)	3
▪ The Edmond Institute's Manual for Assessing Ecological and Human Health Effects of Genetically Engineered Organisms, 1998 – Working Group (11)	4
2. Research groups with a focus on agri-biotech safety	
▪ EC-supported research on GMOs	
SAFOTEST - New methods for the safety testing of transgenic food) (5)	3
GMOCARE - New methodologies for assessing the potential of unintended effects in GM food crops (10)	2
3. Governmental organizations	
▪ FAO-WHO Expert Roster - Safety evaluation of GM foods	7
4. Researchers with specific expertise (identified from peer-reviewed publications)	
▪ GMO safety	5
▪ Conventional breeding, mutagenesis	5

[1] Number of individuals in scientific disciplines identified in the recruitment/information letter whose contact information (e-mail address) can be obtained from publicly available sources. The number excludes duplication where an individual belongs to more than one grouping.
[2] Total number.

Table 1-1B. Distribution of information/recruitment letter - contact points.

Organizations	Contact Point
1. Advisory committees, authors and reviewers of major reports	
▪ FDA Food Advisory Committee - Food Biotechnology Subcommittee	Executive Secretary
▪ European Food Safety Authority (EU: 15 current + 10 candidate member countries)	Coordinator of Scientific Committees
▪ EC Guidance Document for Risk Assessment of Genetically Modified Plants and Derived Food and Feed, 2003	
Scientific Committee on Plants	Secretariat
▪ Health Council of the Netherlands Report – Safety Assessment of Novel Foods, 2002 (18)	Gov't Contact Person
2. Research groups with a focus on agricultural biotechnology safety	
▪ UK Food Standards Agency – G01 (Safety of Novel Foods – 13 projects?) & G02 (Safety Assessment of Genetically Modified Foods – 6 projects)	Gov't Contact person
3. Scientific/policy organizations - agricultural biotechnology safety	
▪ ILSI - International Food Biotechnology Committee	
Safety assessment of nutritionally enhanced GM crops	Sr. Science Program Mgr.
▪ European Federation of Biotechnology	
Agri-Biotechnology Section	Section Chairman
Task Group on Safety in Biotechnology	Task Group Chairman
4. Industry organizations - agricultural biotechnology	
▪ CropLife International (network of associations in 85 countries)	Director General
▪ EuropaBio – Plant Biotechnology Unit	Director
▪ Biotechnology Industry Organization (31 countries, approx 200 members including government, universities and industries)	
Food & Agriculture - Science and Regulatory Policy	Vice President
Food & Agriculture - Plant and Animal Biotechnology	Director
5. Governmental organizations – units with responsibility for GM food safety or general food safety	
▪ Codex (33 member countries at Commission meeting July 2003) - Ad Hoc Intergovernmental Task Force on Foods Derived from Modern Biotechnology, 2000-2003	
Australia-New Zealand	2
Asia (Japan-2, Singapore, S. Korea)	4
Europe (France, Switzerland, UK, Denmark)	4
North America (Canada, U.S.)	2
▪ OECD (30 member countries)	
International Coordination Group for Biotechnology	Secretary
▪ European Food Safety Authority	Head of Science
6. Research institutes - agricultural biotechnology general	
International	
▪ CAMBIA – Centre for the Application of Molecular Biology to International Agriculture (based in Australia)	Chairman/CEO Chief Scientist Principal Scientist

Organizations	Contact Point
▪ Plant Biology Institute for Developing Countries	Director Scientific advisors (2)
Regional	
▪ APEC Agricultural Technical Cooperation Working Group, Subgroup on Research, Development and Extension of Agricultural Biotechnology	
National	
▪ Canada - NRC Plant Biotechnology Institute	Research Director
- Agriculture Agri-Food Canada	Science Director - Genomics and Biotechnology
▪ The New Zealand Institute of Crop and Food Research	Research team leaders (4)
▪ Australia - CSIRO Plant Industry	President
▪ UK - Scottish Crop Research Institute	Research/program leaders (2)
- Institute of Arable Crops Research	Director
▪ Belgium - Agricultural Research Centre	Director
7. Scientific organizations - agricultural biotechnology - general	
▪ International Society for Horticultural Science	
Board	President + VP/Scientific Coordinator
Commission Biotechnology and Molecular Biology	Contact person
Commission Plant Protection	Contact person
▪ International Society for Plant Molecular Biology	President
▪ Bio-Scope, Europe (expert pool on variety of subjects related to agricultural biotechnology)	Contact Person
▪ Inter-University Research Center for Technology, Work, and Culture, Graz, Austria (developing documents for Austrian government and organizing conference: Scrutinizing GMO risk assessment in Europe)	Contact Person
8. Research - general food safety (toxicology)	
▪ EC-supported research on food safety: FOSIE (Food Safety in Europe, ILSI Europe)	Scientific Supervisor/ ILSI Europe contact person
9. Research - ecological risks	Contact Persons (2)

Table 1-2. Ranking of factors and criteria (Total number of respondents = 10).

Factors	% Max. score[1]	Mean ± S.D.	No. of respondents[2]				Criteria	% Max. score	Mean ± S.D.	No. of respondents			
			0[3]	1	2	3				0	1	2	3
Hazard factors													
H1. Target gene product	100	3.0 ± 0.0	-	-	-	10	b) Prior knowledge of potential adverse human health effects	97	2.9 ± 0.3	-	-	1	9
							a) Experience in human diet	90	2.7 ± 0.5	-	-	3	7
							c) Prior knowledge of stability	83	2.5 ± 0.9	-	2	1	7
H2. Direct product of target enzyme	93	2.8 ± 0.4	-	-	2	8	b) Prior knowledge of potential adverse human health effects	100	3.0 ± 0.0	-	-	-	10
							a) Experience in human diet	90	2.7 ± 0.5	-	-	3	7
							c) Prior knowledge of stability under typical conditions of processing and consumption of the derived GM food	87	2.6 ± 0.7	-	1	2	7
H4. Direct product of target enzyme (unintended)	83	2.5 ± 0.7	-	1	3	6	a) Role in metabolic processes of host plant	87	2.6 ± 0.5	-	-	4	6
							b) Novelty in host plant	77	2.3 ± 0.8	-	2	3	5
H10. Direct product of marker gene	81	2.4 ± 0.9	-	2	1	6	b) Prior knowledge of potential adverse human health effects	90	2.7 ± 0.7	-	1	1	8
							a) Experience in human diet	80	2.4 ± 0.8	-	2	2	6
H3. Target gene product (unintended)	78	2.3 ± 0.9	-	2	2	5	b) Novelty in host plant	90	2.7 ± 0.5	-	-	3	7
							a) Specificity of function of protein/enzyme	83	2.5 ± 0.8	-	1	2	5
H5. Nature of target metabolic pathway in host plant	77	2.3 ± 1.0	1	-	4	5	b) Linkage to non-target/competitive pathways	78	2.1 ± 1.0	1	-	3	5
							a) Complexity	63	1.9 ± 1.0	1	2	4	3
H6. Host plant	73	2.2 ± 0.9	-	3	2	5	b) Source of food allergen and toxicant	83	2.5 ± 0.9	-	2	1	7
							a) History of safe use as food	73	2.2 ± 0.8	-	2	4	4

Factors	% Max. score[1]	Mean ± S.D.	No. of respondents[2]				Criteria	% Max. score	Mean ± S.D.	No. of respondents			
			0[3]	1	2	3				0	1	2	3
H8. Method of gene transfer/modification	60	1.8 ± 1.4	3	1	1	5	a) Characterization of genetic material transferred or modified	70	2.1 ± 1.0	1	1	4	4
							d) Extent of genomic modification	60		2	2	2	4
							b) Directedness of gene modification (including introduction/deletion) in host plant	57	1.7 ± 1.1	2	1	5	2
							c) Precision	50	1.5 ± 1.1	2	3	3	2
							e) Molecular characterization or *mitigation of likely disruptions* of* transformation on host genome	50	1.5 ± 1.2	2	4	1	3
H7. Donor plant	57	1.7 ± 0.8	-	5	3	2	c) Source of allergen and toxicant	77	2.3 ± 0.8	-	2	3	5
							b) Prior knowledge of the biology and composition of the donor plant and taxonomically related plants	53	1.6 ± 0.8	-	6	2	2
							a) History of safe use in relation to food	50	1.5 ± 0.7	-	6	3	1
H9. Non-plant donor organism	52	1.6 ± 0.7	-	5	3	1	e) Genetic material transferred (molecular characterization)	83	2.5 ± 0.9	-	2	1	7
							c) Source of food allergen or toxin (*Animal donor organism*)	73	2.2 ± 0.9	-	3	2	5
							b) Prior knowledge of the biology of the donor organism and taxonomically related organisms	63	1.9 ± 0.9	-	4	3	3
							d) Infectivity/pathogenicity to humans (*Microbial donor organism*)	60	1.8 ± 1.1	1	4	1	4
							a) History of safe use in relation to food	57	1.7 ± 0.7	-	4	5	1
Exposure factors													
E3. Direct dietary exposure	90	2.7 ± 0.5	-	-	3	7	Combination of intended use, quantity, and frequency of consumption	87	2.6 ± 0.5	-	-	4	6

Factors	% Max. score[1]	Mean ± S.D.	No. of respondents[2]				Criteria	% Max. score	Mean ± S.D.	No. of respondents			
			0[3]	1	2	3				0	1	2	3
	80	2.4 ± 0.8	-	2	2	6	Expressed, not expressed	83	2.5 ± 0.9	-	2	1	7
E1. Tissue specificity of expression, metabolism and storage of target gene product													
E4. Indirect dietary exposure	70	2.1 ± 1.0	1	1	4	4	Extent of accumulation via feed and animal-derived food	67	2.0 ± 0.9	1	1	5	3
E2. Controlled timing of expression, metabolism and storage of target gene product	67	2.0 ± 0.9	-	4	2	4	Can be controlled, not readily controlled	77	2.3 ± 0.8	-	2	3	5
E5. Non-dietary exposure	67	2.0 ± 0.9	-	4	2	4	Extent of pre-sensitization (e.g., via inhalation and dermal routes)	80	2.4 ± 0.8	-	2	2	6
Consequence factors													
C1. Prior knowledge of nature of adverse health effects of target protein/enzyme and direct product of target enzyme	100	3.0 ± 0.0	-	-	-	10	Extent of severity (none, minor, mild, moderate, severe)	90	2.7 ± 0.9	1	-	-	9
C3. Existence of susceptible subpopulations	87	2.6 ± 0.7	-	1	2	7	Extent of susceptibility (none, susceptible, hypersusceptible)	87	2.6 ± 0.7	-	1	2	7
C4. Potential for significant alteration of dietary practices	73	2.2 ± 0.6	-	1	6	3	Extent of alteration (limited, moderate, major)	73	2.2 ± 0.4	-	-	8	2
C2. Prior knowledge of the nature of adverse health effects of some components of the host and donor organisms	63	1.9 ± 1.0	1	2	4	3	Extent of severity (none, minor, mild, moderate, severe)	70	2.1 ± 1.1	1	2	2	5

[1] Maximum score = number of respondents x maximum score of 3. For example, maximum score for 10 respondents = 10 x 3 = 30.
[2] Total number of respondents = 10. The number of respondents for individual factors and criteria varied between 8 to 10.
[3] Ratings for factors/criteria: 0=Not important/Not appropriate 1=Somewhat important/Somewhat appropriate 2=Important/Appropriate 3=Very important/Very appropriate

Table 1-3. Additional factors and criteria suggested by experts (Number of respondents = 9).

Level of importance	Hazard factors			Exposure factors	Consequence factors
	Assessment of primarily intended effects	**Assessment of primarily unintended effects**	**Specific to genetic engineering**		
Very important	▪ Nature of the change induced in the protein and/or product of the genetic modification ▪ Known beneficial effects where the introduced substance may exert both beneficial and adverse effects (to allow for risk-benefit analysis)	▪ Gene stacking (interactions between gene products from separate events after crossing genetically modified plant lines) ▪ Effect of agronomic stress in variability of gene expression ▪ Criteria for assessing antibiotic resistance marker gene: potential for horizontal gene transfer		▪ Aggregate exposure (i.e., contribution to exposure to the same substance from other GM foods with the same modification that have already been introduced into the market) – this factor was noted by 3 respondents ▪ Variability in individual consumption	▪ Monitoring capacity for identification of hypothetical, potential adverse effects
Important		▪ Gene deletion ▪ Molecular detection method			
Somewhat important			▪ Site of insertion ▪ Stability of insert ▪ Recombination and deletion		
Not specified	▪ Crossing between different modified plants – e.g., plants modified in related pathways ▪ Marker gene				

One respondent also provided recommendations for the criteria to be used in assessing the factor suggested. One recurring theme from one respondent was that no other factors are important since we have accepted the effects of traditional breeding and derived foods already in our diet.

Table 1-4. Relevance of listed factors and criteria to all methods of genetic modification (Total number of respondents = 7).

Comments (number of respondents)
▪ All are relevant (3)
▪ Donor plant might not be applicable in mutation breeding. As well, target gene product and target metabolic pathway might not be relevant in all cases, e.g., in conventional breeding (1)
▪ The only relevant factors are direct gene products of inserted genes (2)
▪ The factors and criteria apply to GM using molecular genetics but do not apply to conventional plant breeding, such as introgression and mutagenesis (1)
▪ The relevance of breeding histories as a criterion in assessing host or donor plants was questioned, noting that breeding histories provide no information about the impact of a transgene (1)

Table 1-5. Weighting of unintended effects relative to intended effects (Number of respondents = 10).

	No. respondents	Relative weighting[1]	Reasons / Comments
Lower weight	3	0 - 0.2	▪ No different than existing food from traditional breeding; we have "unintended" effects in conventional breeding which are more widespread and less defined than those induced by GM technology.
Equal weight	2		
Higher weight	1	3	▪ It is likely that hazard will arise from coupled, downstream effects on minor metabolites which may have a major impact on health (and by implication, which are minor parts of the diet that we know less about).
Variable weight	4		▪ Unintended effects would be given lower weight because in many cases they can be considered as the normal (traditional) risk that is accepted in traditional breeding without a general test for these. Unintended effects may or may not be present. However, the weight to be given to unintended effects relative to intended effects should also be considered on a case-by-case basis. Higher weighting would be appropriate where new pathways are involved, whereas lower weighting may be given to specific and well-known modification not involving pathways. Unintended effects should focus on health relevant effects of substances known to be related to health risk (e.g., solanine in potato). ▪ Higher weighting should be given where (1) primary/secondary metabolism has been modified (regulation of metabolic networks not always known), (2) where pathways may lead to modified toxin production, (3) new pathways are inserted. Lower weighting may be given where other components of risk assessment process have been rigorously carried out (e.g., detailed animal feeding trials for toxicity, nutrition and wholesomeness testing, allergenicity tests, detailed proximate analysis completed). Equal weighting might be given where genes are inserted into regulatory sequences of host genome and so could have unknown consequences on the expression of other genes. ▪ Compared to genetic modifications currently on the market that are relatively simple ones, future genetic modifications will alter basic plant metabolism to achieve more complex desired traits and will have a higher probability of producing unintended effects. ▪ The weighting given to unintended effects would depend on the crop in which the genetic modification is conducted and the level of knowledge of the transgene integration. Higher weighting should be considered. The likelihood of interfering with metabolic pathways known to be related to the production of toxic substances cannot be excluded when a gene is introduced.

[1] Relative to a weighting of 1.0 for intended effects

Table 1-6. General comments (Number of respondents = 7).

Topic	Summary of responses
Marker gene	▪ When marker genes are used, the consequence of this application should also be included in the safety assessment of GM food.
Testing concepts and methods	▪ The concept of "threshold of toxicological concern" and its potential application to the assessment of GM food was explained. A reference was provided. ▪ A reference was made to a new publication relevant to the assessment of unintended effects and the development/application of broad based "profiling" approaches.
Allergenic effects	▪ "Regarding food consumption patterns and its relationship with allergy, this is important but we know little about it, let alone how it may change in future."
Unintended effects	▪ Unintended effects may be perceived as low in conventional breeding (including crossing with related species and mutagenesis) despite minimal toxicity testing and reliance on improvement of a field trait. In cases where a health risk was identified, invariably there was an unexpected disturbance of secondary metabolism. Knowledge already exists in the nature of toxic substances present in many food stuffs. This, when coupled with the availability of new molecular approaches, should advance our ability in the measure and control of hazards in foods, regardless of the type of gene manipulation. ▪ Conventional breeding (including mutagenesis) has always introduced unintended effects, but since crossing is done with related species, risk is perceived as low.
Degree of confidence in response	▪ "It is very difficult to be as confident in responding to this questionnaire as one would be if it were associated with an old established practice in toxicology" ▪ "The brief comments are based on well reasoned and fundamental arguments presented in our published papers."
Application to RBCS	▪ Not sure if the questions were meant to address only modern genetic modification. ▪ The factors in this survey can be used in an interrelated fashion across categories for classifying GM plants into a certain risk category.

Three respondents also provided references relevant to the safety assessment of GM foods.

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Cabinet du vice-recteur à la recherche | Office of the Vice-Rector, Research

Annex 1-1

HEALTH SCIENCES AND SCIENCE RESEARCH ETHICS BOARD

CERTIFICATE OF ETHICAL APPROVAL

This is to certify that the University of Ottawa Health Sciences and Science Research Ethics Board has examined the application for ethical approval for PHASE 1a of the research project entitled **A Risk-Based Classification Scheme for Testing Genetically Modified Foods (file H 11-03-06)** submitted by Eunice Chao, who is supervised by Daniel Krewski, both of the Institute of Population Health. The Board found that this research project met appropriate ethical standards as outlined in the Tri-Council Policy Statement and in the Procedures of the University of Ottawa Research Ethics Boards, and accordingly gave it a Category 1a (approval). This certification is valid for one year from the date indicated below.

Rita D'Alessandro
Protocol Officer for Ethics in Research
For the Chair of the Health Sciences and Science REB

March 12, 2004
Date

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Université d'Ottawa • University of Ottawa

Centre R.-Samuel-McLaughlin d'évaluation du risque sur la santé des populations
R. Samuel McLaughlin Centre for Population Health Risk Assessment

Annex 1-2. Information/recruitment letter

March 18, 2004

Dear Sir or Madame:

We are writing to inform you of a research project on *a risk-based classification scheme for testing genetically modified foods* that is currently underway at the University of Ottawa, Canada. We are seeking your assistance in distributing this letter to any colleagues you think would be interested in this topic, preferably by electronic mail. Please feel free to respond yourself if this project is of interest to you personally.

Included below are a description of the project and information on how to contact the investigator responsible for the project. The original signed letter is also attached as a pdf file.

Thank you for your consideration and cooperation. We apologize in advance for any cross-postings.

Project Description

The primary objective of the proposed research is to develop and evaluate a risk-based classification scheme for testing foods that are genetically modified by a range of methods, including conventional (e.g., induced mutation, tissue culture) and more modern (e.g., recombinant DNA) techniques. The proposed scheme aims to (1) differentiate and classify genetically modified (GM) foods based on levels of concern about potential health risks and attendant uncertainty, and (2) guide the application of testing methods for GM foods in proportion to their potential to produce human health risks. The research will focus on potential human health risks from dietary exposure to foods derived from GM plants.

Phase 1 of the research will focus on the development of a risk-based classification scheme under the guidance of a multidisciplinary expert panel who have expertise in health risk assessment of GM foods. We are seeking experts knowledgeable in relevant disciplines, including plant sciences, molecular biology/genetics, microbiology, toxicology, immunology, systems biology, analytical biotechnology, food/nutritional sciences, and health risk assessment. The panel will be selected to reflect a balance of expertise in these areas.

The expert panel will be asked to participate in two surveys by completing two self-administered questionnaires. A panel member may elect to complete one of the two surveys.

The questionnaire in survey 1 is designed to

- rate the importance of a list of factors that may be relevant to determining the nature and level of testing required for assessing the potential health risks of GM foods,
- assess the appropriateness of the criteria to be used in classifying GM foods into levels of concern for potential health risks based on the factors identified.

.../2

1, rue Stewart, Ottawa (Ontario) K1N 6N5 Canada
1 Stewart St., Ottawa, Ontario K1N 6N5 Canada
(613) 562-5381 • Télé./Fax (613) 562-5380

Expert panel members may also choose to provide additional comments, including

- alternate factors or criteria that should be considered,
- the reasons for their ratings,
- responses to open-ended questions on specific issues related to the factors and criteria.

In survey 2, the expert panel will be asked to review a draft classification scheme developed in part from responses to survey 1. This will involve reviewing the logical soundness of the draft scheme using specific product examples provided by the researcher. The expert panel will also be asked to provide comments on specific issues related to the draft scheme.

We estimate that each questionnaire will take approximately one hour to complete. Individuals who provide detailed comments may spend more time. Normally, a respondent will have 3-4 weeks to return a completed questionnaire.

All data collection and dissemination will be conducted in accordance with confidentiality and ethics guidelines for research involving human subjects.

The research is being conducted as part of a doctoral thesis at the University of Ottawa Population Health PhD Program. The principal researcher is Eunice Chao, doctoral candidate, who is working with Dr. Daniel Krewski, Professor and Director, McLaughlin Centre for Population Health Risk Assessment, Institute of Population Health, University of Ottawa. The research project is supported by the McLaughlin Centre through a grant from the R. Samuel McLaughlin Foundation. No industry funding is involved in this project.

Contact Information

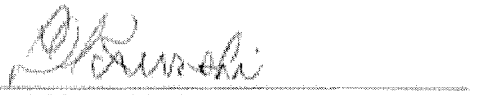
Individuals interested in participating on the expert panel or in obtaining further information on this research project can contact Eunice Chao by:

e-mail: echao097@uottawa.ca
phone: 1-613-562-5800, ext 2367
fax: 1-613-562-5380
mail: McLaughlin Centre for Population Health Risk Assessment
University of Ottawa
1 Stewart Street, Room 318B
Ottawa, Ontario
CANADA K1N 6N5

Any information on your scientific background that you are willing to provide (a short bio, full CV, or a simple statement of interests) would be helpful. We will follow-up with individuals who appear to have specific expertise relevant to this project, including those who express an interest in participating, by e-mail or phone to set up a telephone interview.



Eunice Chao, MSc
Doctoral candidate and project researcher
Population Health PhD Program
University of Ottawa



Daniel Krewski, PhD, MHA
Professor and Director
McLaughlin Centre for Population Health
Risk Assessment
University of Ottawa

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Annex 1-3. Consent Form - Participation in Surveys

Name of researcher(s): Eunice Chao

Institution, Faculty, Department: University of Ottawa, Population Health PhD Program

Telephone number: 1-613-562-5800 ext 2367 **Fax number:** 1-613-562-5380
E-mail address: echao097@uottawa.ca
Address: McLaughlin Centre for Population Health Risk Assessment
University of Ottawa
1 Stewart Street, Room 318B, Ottawa, Ontario, CANADA K1N 6N5

Name of supervisor: Dr. Daniel Krewski

Institution, Faculty, Department: University of Ottawa, Faculty of Medicine, Department of Epidemiology and Community Medicine, Institute of Population Health

Telephone number: 1-613- 562-5800 ext 8261 **Fax number:** 1-613-562-5380
E-mail address: dkrewski@uottawa.ca
Address: see McLaughlin Centre for Population Health Risk Assessment address above

I, (please print) _____ (*Name of research participant*), agree to participate in the research project "A risk-based classification scheme for testing genetically modified foods" conducted by Eunice Chao of the Department of Population Health PhD Program at the University of Ottawa. The project is under the supervision of Dr. Daniel Krewski. The purpose of the research is to develop and evaluate a risk-based classification scheme for the testing of foods that are genetically modified by a range of methods. The proposed scheme aims to (1) differentiate and classify GM foods based on levels of concern about potential health risks and attendant uncertainty, and (2) guide the application of testing methods for GM foods in proportion to their potential to produce human health risks. The research will focus on potential human health risks from dietary exposure to foods derived from GM plants.

My Participation

I will be asked to participate in two surveys by completing two self-administered questionnaires. If I prefer, I may elect to complete only one of the two surveys.

The questionnaire in survey 1 will be designed to: (1) rate the importance of a list of factors that may be relevant to determining the nature and level of testing required for assessing the potential health risks of GM foods, and (2) assess the appropriateness of the criteria to be used in classifying GM foods into levels of concern based on the factors identified.

I may also choose to provide additional comments, including: other factors and/or criteria that should be considered, the reasons for my ratings, and responses to open-ended questions on specific issues related to the factors and criteria.

In survey 2, I will be asked to comment on a draft classification scheme developed in part from responses to survey 1. This will involve reviewing the logical soundness of the draft scheme using several examples prepared by the researcher. I will also be asked to comment on specific issues related to the draft scheme.

.../2

Each questionnaire will take approximately one hour to complete. I may spend more time if I choose to provide detailed comments. Normally, I have about 3-4 weeks to return each completed questionnaire.

Confidentiality and Anonymity
I understand that the contents of the completed questionnaires will be used only for the purposes of developing a risk-based classification scheme for testing genetically modified foods and that my confidentiality will be respected through the following actions taken by the researcher.

All of the data collected will be tabulated and maintained by the researcher in accordance with confidentiality standards for research involving human subjects. The data will be accessible only to the researcher in a locked filing cabinet and a password protected personal computer on the premises of the Institute of Population Health, University of Ottawa.

Anonymity of my individual views and responses will be protected. Questionnaires will be coded for follow-up purposes and my name will not appear on the completed questionnaires. While group results of the surveys at the aggregate level will be published, and discussed within the research team and at conferences, no names will be linked to individual views or responses in data analysis, reports or in presentations.

I also understand that in order to acknowledge participants' contribution to the research project and to make the expert consultation process transparent and credible from a research perspective, my name and my affiliation (the organization where I work or which I represent) will be included in a list as an appendix to the doctoral thesis. Only one combined list of participants for all phases of the research will be published without identifying the specific phase to which they made a contribution.

I agree not to disclose or publish any aspects of the risk-based classification scheme developed by the researcher except with the express consent of the researcher.

Voluntary Participation
There is no direct individual benefit from participating as a member of the expert panel and from answering the questionnaires. I understand that all costs associated with participation in the project (Canadian postage and long-distance phone calls) will be born by the McLaughlin Centre for Population Health Risk Assessment. Return postage for participants from countries outside Canada will be paid in the form of international postal reply coupons, if requested.

Participation is completely voluntary. I am free to withdraw from the project at any time, before or during a survey, refuse to participate and refuse to answer certain questions.

I agree to participate in: □ surveys 1 and 2 □ survey 1 □ survey 2

I do not wish my name to appear in the researcher's thesis □ (check here only if you choose this option)

Any information about my rights as a research participant may be addressed to **Protocol Officer for Ethics in Research, University of Ottawa, 550 Cumberland Street, Tabaret Hall, Room 159, Ottawa, Ontario, Canada, K1N 6N5, tel.: (613) 562-5387, e-mail**: ethics@uottawa.ca .

There are two copies of the consent form, one of which I may keep.

If I have any questions about the conduct of the research project, I may contact the researcher or his or her supervisor at the coordinates listed in the beginning of this consent form.

Researcher's signature: _____ Date: _____

Research participant's signature: _____ Date: _____

Annex 1-4. Questionnaire

Annex 1-4. Questionnaire

Risk-Based Classification Scheme for Testing Genetically Modified Foods

Survey 1

Risk-based classification scheme for testing GM foods: A risk-based classification scheme (RBCS) as envisioned in this research is intended to be a tool to efficiently differentiate genetically modified (GM) foods that require more extensive testing and regulatory oversight from those that do not.

- **Focus**: potential human health risks from dietary exposure to foods derived from GM crops
- **Scope**: include a range of conventional (e.g., induced mutation, tissue culture, interspecific and intergeneric crosses) and modern (e.g., cell fusion, in *vitro* and recombinant deoxyribonucleic acid - DNA) techniques of genetic modification to intentionally change the heritable traits of food crops
- **Assumptions**:
  - stable integration and inheritance of modified phenotype is a basic requirement for the commercialization of GM crops
  - criteria for differentiating GM foods can be based on information required in developing the modified crop or information that can be rapidly assessed in order to make the RBCS practical
- **Intended uses**:
  - classify GM foods based on levels of concern about potential health risks and attendant uncertainty
  - guide the application of testing methods in proportion to their potential to produce human health risks
- **NOT intended** to be used as a health risk assessment tool *per se*, nor for decision-making regarding the approval or disapproval of a GM food

This survey is the first step of Phase 1 in the development and evaluation of a risk-based classification scheme for testing GM foods to ensure that all relevant factors and criteria are considered. **Specific objectives of this survey** are to:

- rate the importance of a list of factors that may be relevant to determining the type and level of testing for assessing the potential health risks of GM foods,
- assess the appropriateness of the criteria to be used in classifying GM foods into levels of concern based on the factors identified.

There are **2 parts to this questionnaire**

- **Part I - rating of factors and criteria**. Space is provided for suggesting alternate or additional factors and criteria. Legible writing would be appreciated. Please use additional paper if necessary.
- **Part II - open-ended questions**. Space is also available for general comments.

Development of this questionnaire: The factors and criteria included in this questionnaire were identified from a review of the scientific literature on major approaches to genetic modification of plants and their effects on plant genome structure and gene expression and function, as well as national and international guidelines on safety assessment of GM foods, and well-established concepts of health risk assessment.

Next steps: A combination of approaches will be explored in aggregating the factors and criteria confirmed in this survey into a manageable number of overall concern levels (draft RBCS). Minimum information on a GM food required in using the RBCS will also be determined. Preliminary assessment of the logical soundness of the draft RBCS will be conducted using specific product examples.

Future phases: Detailed evaluation of the utility of the scheme will be conducted using a panel of reference products with a range of characteristics, as well as the development of graded testing options.

I.D. Number:

Part I - Factors and Criteria

There are 3 categories of factors in this questionnaire: hazard, exposure, and consequence factors. One or more criteria are listed under each factor. Each criterion is consisted of specific attributes for discriminating GM crops and derived foods according to the factor identified. The attributes are arranged generally in an order that reflects an increasing level of concern and uncertainty. **Please consider the attributes collectively when rating the appropriateness of each criterion.**

A. Hazard factors

For the purposes of developing a risk-based classification scheme for testing GM foods, hazard factors are those factors

- that can affect or are associated with the production of harmful substances in GM crops for food use, and
- that are relevant to determining the type and level of testing required.

Hazard factors are divided into 3 groups in this questionnaire. Factors in groups 1 and 2 are to be considered for both conventional and modern methods of genetic modification used to change the heritable traits of plants by intentional manipulation. Group 1 factors are to be assessed primarily for their ***intended*** effects, and group 2 factors, primarily for their ***unintended*** effects. Group 3 factors are specific to genetic engineering (*in vitro* nucleic acid techniques). Please consider conventional and modern methods of genetic modification that have been applied to-date and will likely be applied to commercial food crops in the next 5-20 years.

Intended effects are those effects mediated by the expression product of the gene of interest (e.g., insecticidal effect of a protein produced by the target gene, or detoxification of a herbicide by an enzyme produced by the target gene in the modified plant). Potential hazards of concern to human health may result from the toxicity of the insecticidal protein and of the metabolite of the plant detoxification process. ***Unintended*** effects are those effects arising from alterations in metabolic pathways, modification or disruption in the expression of native genes (silencing, activation, or interactions). These alterations may lead to the generation of new toxic/allergenic compounds, or modified expression of endogenous toxic substances in different tissues or at different levels.

Please rate the importance of the factors and the appropriateness of the criteria using the scale provided.

Factors	**0=Not important**	**1=Somewhat important**	**2=Important**	**3=Very important**
Criteria	**0=Not appropriate**	**1=Somewhat appropriate**	**2=Appropriate**	**3=Very appropriate**

	Hazard Factors	Rating	Alternate Factors and Criteria/ Comments (e.g. reasons for low ratings)
	1. Assessment of primarily *intended* effects		
Factor	**H1. Target gene product (protein/enzyme)**	0 1 2 3	
Criteria	a) Experience in human diet ▪ An existing component of plants and plant-derived foods - modified in levels ▪ An existing component of plants and plant-derived foods - limited and well-characterized modification of function (e.g. point mutation) ▪ Novel component of plants and plant-derived foods	0 1 2 3	

Factors	0=Not important	1=Somewhat important	2=Important	3=Very important
Criteria	0=Not appropriate	1= Somewhat appropriate	2=Appropriate	3=Very appropriate

	Hazard Factors	Rating	Alternate Factors and Criteria/ Comments (e.g. reasons for low ratings)
	b) Prior knowledge of potential adverse human health effects (toxicity, allergenicity, antinutritional and other biological effects with relevance to human health) ▪ Extensive evidence for no effect ▪ Limited evidence for an adverse effect ▪ Limited or lack of evidence for no effect	0 1 2 3	
	c) Prior knowledge of stability under typical conditions of processing and consumption of the derived GM food ▪ No change in level and nature ▪ Toxic activity destroyed by cooking or processing ▪ Concentration in level or transformation into toxic metabolite ▪ Limited data/unable to evaluate	0 1 2 3	
Factor	**H2. Direct product of target enzyme**	0 1 2 3	
Criteria	a) Experience in human diet ▪ An existing component of plants and plant-derived foods - modified in levels ▪ Novel component of plants and plant-derived foods	0 1 2 3	
	b) Prior knowledge of potential adverse human health effects (toxicity, allergenicity, antinutritional and other biological effects with relevance to human health) ▪ Extensive evidence for no effect ▪ Limited evidence for an adverse effect ▪ Limited or lack of evidence for no effect	0 1 2 3	
	c) Prior knowledge of stability under typical conditions of processing and consumption of the derived GM food ▪ No change in level and nature ▪ Toxic activity destroyed by cooking or processing ▪ Concentration in level or transformation into toxic metabolite ▪ Limited data/unable to evaluate	0 1 2 3	
	Other hazard factors and criteria to be considered for *intended* effects	0 1 2 3	

Factors	**0=Not important**	**1=Somewhat important**	**2=Important**	**3=Very important**
Criteria	**0=Not appropriate**	**1=Somewhat appropriate**	**2=Appropriate**	**3=Very appropriate**

	Hazard Factors	**Rating**	**Alternate Factors and Criteria/ Comments** (e.g. reasons for low ratings)
	2. Assessment of primarily *unintended* effects		
Factor	**H3. Target gene product (protein/enzyme)**	0 1 2 3	
Criteria	a) Specificity of function of protein/enzyme ▪ Highly specific (e.g. an enzyme that metabolizes one substrate or is sensitive to one metabolite) ▪ Broad range function (e.g. transcription factor) ▪ Limited data/unable to characterize	0 1 2 3	
	b) Novelty in host plant ▪ Native protein/enzyme - unmodified in nature ▪ Native protein/enzyme with limited and well-characterized modification ▪ New or significantly modified protein/enzyme	0 1 2 3	
Factor	**H4. Direct product of target enzyme**	0 1 2 3	
Criteria	a) Role in metabolic processes of host plant ▪ Terminal metabolite - not metabolized further ▪ Precursor or intermediate metabolite of other pathways ▪ Modulator of enzyme activity via feedback mechanism ▪ Signal for one or more regulatory genes ▪ Limited data/unable to evaluate	0 1 2 3	
	b) Novelty in host plant ▪ Native to host plant ▪ New or significantly modified	0 1 2 3	
Factor	**H5. Nature of target metabolic pathway in host plant**	0 1 2 3	
Criteria	a) Complexity ▪ Involves single or limited number of enzymes ▪ Involves multiple enzymes and branch points, regulated by multiple genes	0 1 2 3	
	b) Linkage to non-target/competitive pathways ▪ Not linked to other pathways ▪ Linked to primary pathways ▪ Linked to secondary pathways, with the potential for modified biosynthesis of toxic or bioactive secondary metabolites ▪ Limited data/unable to evaluate	0 1 2 3	
Factor	**H6. Host plant (including the transformant and parental lines in backcrossing) from which food is derived**	0 1 2 3	
Criteria	a) History of safe use as food ▪ Established breeding history, commercialized and widely consumed for several generations ▪ Lacking in one or more of above elements	0 1 2 3	

Factors	0=Not important	1=Somewhat important	2=Important	3=Very important
Criteria	0=Not appropriate	1=Somewhat appropriate	2=Appropriate	3=Very appropriate

	Hazard Factors	Rating	Alternate Factors and Criteria/ Comments (e.g. reasons for low ratings)
	b) Source of food allergen and toxicant ▪ Not known to be allergenic or a source of toxicant ▪ Known to be allergenic or a source of toxicant	0 1 2 3	
Factor	**H7. Donor plant (Non-plant donor organisms - see H9)**	0 1 2 3	
Criteria	a) History of safe use in relation to food ▪ Edible plant with established breeding history, commercialized and widely consumed for several generations ▪ Edible plant lacking in one or more of above elements ▪ No history as an edible plant	0 1 2 3	
	b) Prior knowledge of the biology and composition of the donor plant and taxonomically related plants ▪ Extensive published scientific literature ▪ Limited/no published scientific literature	0 1 2 3	
	c) Source of allergen and toxicant ▪ Not known to be allergenic or a source of toxicant ▪ Known to be allergenic or a source of toxicant ▪ Limited data/unable to evaluate	0 1 2 3	
Factor	**H8. Method of gene transfer/modification**	0 1 2 3	
Criteria	a) Characterization of genetic material transferred or modified ▪ Well-characterized molecular structure and function ▪ Insufficient or provisional characterization of molecular structure and function	0 1 2 3	
	b) Directedness of gene modification (including introduction/deletion) in host plant ▪ Site-specific modification ▪ Partial control or predictability of modification ▪ Completely random modification	0 1 2 3	
	c) Precision (in terms of number and nature of new/target genes; one or more other non-target genes can be affected) ▪ No new genes (e.g. induced mutation) ▪ One or a few genetically related genes introduced (e.g. *in vitro* DNA technique) ▪ One or a few genetically unrelated genes introduced (e.g. *in vitro* DNA technique) ▪ Many unknown, but genetically related genes introduced (e.g. intraspecific crosses) ▪ Many unknown, genetically unrelated genes introduced (e.g. interspecific crosses)	0 1 2 3	

Factors	0=Not important	1=Somewhat important	2=Important	3=Very important
Criteria	0=Not appropriate	1=Somewhat appropriate	2=Appropriate	3=Very appropriate

	Hazard Factors	Rating	Alternate Factors and Criteria/ Comments (e.g. reasons for low ratings)
	d) Extent of genomic modification ▪ Random insertion in host genome, with other parts of the genome essentially intact (e.g. *in vitro* DNA technique) ▪ Recombinations and rearrangement generally rare (e.g. intraspecific crosses) ▪ Many changes possible, ranging from point mutations to large rearrangements (e.g. induced mutation) ▪ Rearrangements necessary to stabilize host genome (e.g. interspecific crosses) ▪ Limited data/unable to characterize	0 1 2 3	
	e) Molecular characterization or mitigation of likely disruptions* of transformation on host genome ▪ Methods available ▪ Methods being developed/can be developed ▪ Not methodologically feasible * Anticipated disruptions include expression of new (fusion) protein, transcriptional/post-transcriptional silencing of native genes, or interference from genes in adjacent regions	0 1 2 3	
	Other factors and criteria to be considered for *unintended* effects	0 1 2 3	
	3. *Factors specific to genetic engineering (in vitro* nucleic acid techniques)		
Factor	**H9. Non-plant donor organism**	0 1 2 3	
Criteria	a) History of safe use in relation to food ▪ Ubiquitous/adventitious component of food supply ▪ Novel to food supply	0 1 2 3	
	b) Prior knowledge of the biology of the donor organism and taxonomically related organisms ▪ Extensive published scientific literature ▪ Limited/no published scientific literature	0 1 2 3	

Factors	0=Not important	1=Somewhat important	2=Important	3=Very important
Criteria	0=Not appropriate	1=Somewhat appropriate	2=Appropriate	3=Very appropriate

	Hazard Factors	**Rating**	**Alternate Factors and Criteria/ Comments** (e.g. reasons for low ratings)
	Animal donor organism: c) Source of food allergen or toxin ▪ Not known to be allergenic or a source of toxin ▪ Known to be allergenic or a source of toxin ▪ Limited data/unable to evaluate	0 1 2 3	
	Microbial donor organism: d) Infectivity/pathogenicity to humans ▪ Not known to be pathogenic ▪ Known to be pathogenic ▪ Limited data/unable to evaluate	0 1 2 3	
	e) Genetic material transferred ▪ Well-characterized non-coding sequences (e.g. promoter or other known control sequences) ▪ Non pathogenic bacteria, fungi, plant viruses*: well-characterized genes of known function ▪ Pathogenic organisms: well-characterized genes of known function unrelated to pathogenicity or infectivity ▪ Pathogenic organisms: any sequences for which pathogenic or infectious function cannot be excluded * No evidence is known that plant viral genes can be transferred to the genomes of microorganisms, plants, or animals. However, from a plant health and ecological risk perspective, insertion into plants of sense noncapsid protein, non-coding sequences containing replicase recognition, and genes encoding replicase or helper components are considered high risk and should be avoided.	0 1 2 3	
Factor	**H10. Direct product of marker gene**	0 1 2 3	
Criteria	a) Experience in human diet ▪ An existing component of plants and plant-derived foods - modified in levels ▪ Novel component of plants and plant-derived foods	0 1 2 3	
	b) Prior knowledge of potential adverse human health effects (toxicity, allergenicity, antinutritional and other biological effects with relevance to human health) ▪ Extensive evidence for no effect ▪ Limited evidence for an adverse effect ▪ Limited or lack of evidence for no effect	0 1 2 3	

Factors	**0=Not important**	**1=Somewhat important**	**2=Important**	**3=Very important**
Criteria	**0=Not appropriate**	**1=Somewhat appropriate**	**2=Appropriate**	**3=Very appropriate**

	Hazard Factors	**Rating**	**Alternate Factors and Criteria/ Comments** (e.g. reasons for low ratings)
	Other hazard factors and criteria *specific to genetic engineering* to be considered	0 1 2 3	

B. Exposure factors

Exposure factors include direct and indirect routes of exposure to the GM crop being evaluated and will focus on the *intended* effects of genetic modification.

- Direct exposure includes all food uses and consumption of edible parts of the GM crop (e.g., various food ingredients derived from the crop - oils, syrups, flour etc., as well as unprocessed or minimally processed edible portion of the crop for human consumption - e.g., fresh fruits and vegetables).
- Indirect exposure includes uses of the GM crop as animal feed and non-dietary exposure to the GM crop that may elicit pre-sensitization to a new allergenic component in the GM food in exposed individuals.

Please rate the importance of the factors and the appropriateness of the criteria using the scale provided.

Factors	**0=Not important**	**1=Somewhat important**	**2=Important**	**3=Very important**
Criteria	**0=Not appropriate**	**1=Somewhat appropriate**	**2=Appropriate**	**3=Very appropriate**

	Exposure Factors	**Rating**	**Alternate Factors and Criteria/ Comments** (e.g. reasons for low ratings)
Factor	**E1. Tissue specificity** of expression, metabolism and storage of target protein/enzyme and direct product of target enzyme in the GM crop (e.g., use of tissue-specific promoter and organelle targeting of recombinant protein)	0 1 2 3	
Criteria	▪ Not expressed, metabolized, or stored in edible parts ▪ Expressed, metabolized or stored in edible parts	0 1 2 3	
Factor	**E2. Controlled timing** of expression and metabolism of target protein/enzyme and direct product of target enzyme in the GM crop (e.g., use of inducible system based on an acceptable chemical)	0 1 2 3	
Criteria	▪ Timing can be controlled ▪ Timing cannot be readily controlled	0 1 2 3	

Factors	0=Not important	1=Somewhat important	2=Important	3=Very important
Criteria	0=Not appropriate	1=Somewhat appropriate	2=Appropriate	3=Very appropriate

	Exposure Factors	Rating	Alternate Factors and Criteria/ Comments (e.g. reasons for low ratings)
Factor	**E3. Direct dietary exposure** to food derived from GM crops	0 1 2 3	
Criteria	▪ Rare food, infrequently consumed whole (e.g. exotic fruits), or in very small amounts (e.g. spices, seasonings) ▪ Common food regularly consumed in limited form as highly refined ingredient (e.g. oils from some oilseeds) ▪ Common food, variable consumption (e.g. seeds, nuts) ▪ Common food, regularly consumed whole with minimal processing (e.g. many fruits and vegetables) ▪ Common food, regularly consumed whole, or as ingredients in wide spectrum of applications (e.g. corn, soybean, wheat) ▪ Food for individuals with special medical needs ▪ Primary source of nutrition for subpopulations (e.g. infant formulas and infant foods)	0 1 2 3	
Factor	**E4. Indirect dietary exposure** via animal feed to target protein/enzyme and direct product of target enzyme	0 1 2 3	
Criteria	▪ Not applicable (modified plant not used as feed) ▪ Not known/unlikely to accumulate in animal tissues ▪ Potential to accumulate in animal tissues ▪ Has pharmaceutical properties relevant to human health ▪ Limited data/unable to evaluate	0 1 2 3	
Factor	**E5. Non-dietary exposure** (e.g., contact, inhalation) to new allergenic component of GM crop (target protein/enzyme)	0 1 2 3	
Criteria	▪ Not known/unlikely to pre-sensitize susceptible individuals via non-dietary routes ▪ Potential to pre-sensitize susceptible individuals via non-dietary routes ▪ Limited data/unable to evaluate	0 1 2 3	
	Other exposure factors and criteria to be considered	0 1 2 3	

C. Consequence factors

Consequence factors will focus on adverse health-related impact of *intended* effects of genetic modification of food crops or effects that may be anticipated.

Please rate the importance of the factors and the appropriateness of the criteria using the scale provided.

Factors	**0=Not important**	**1=Somewhat important**	**2=Important**	**3=Very important**
Criteria	**0=Not appropriate**	**1=Somewhat appropriate**	**2=Appropriate**	**3=Very appropriate**

	Consequence Factors	Rating	Alternate Factors and Criteria/ Comments (e.g. reasons for low ratings)
Factor	**C1. Prior knowledge of the nature of adverse health effects of the target protein/enzyme and direct product of target enzyme**	0 1 2 3	
Criteria	▪ None ▪ Minor: individual rarely seeks medical attention ▪ Mild: sometimes requires medical attention ▪ Moderate: requires medical attention in most cases ▪ Severe: life threatening in some individuals ▪ Limited data/unable to evaluate	0 1 2 3	
Factor	**C2. Prior knowledge of the nature of adverse health effects of some components of the host and donor organisms**	0 1 2 3	
Criteria	▪ None ▪ Minor: individual rarely seeks medical attention ▪ Mild: sometimes requires medical attention ▪ Moderate: requires medical attention in most cases ▪ Severe: life threatening in some individuals ▪ Limited data/unable to evaluate	0 1 2 3	
Factor	**C3. Existence of susceptible subpopulations**	0 1 2 3	
Criteria	▪ None or not applicable ▪ Susceptible ▪ Hypersusceptible ▪ Limited data/unable to evaluate	0 1 2 3	
Factor	**C4. Potential for significant alteration of dietary practices** (e.g. food modified for health benefits)	0 1 2 3	
Criteria	▪ Limited ▪ Moderate ▪ Major ▪ Limited data/unable to evaluate	0 1 2 3	
	Other consequence factors and criteria to be considered	0 1 2 3	

Part II - Open-ended questions

1. In your judgment, which hazard factors or criteria listed in Part I (H1 to H8) are not relevant to crops modified by all methods of genetic modification?

2. Given current plant breeding practices and future developments in plant biotechnology, how much weight[1] should be given to unintended effects, relative to intended effects in health risk assessment of GM crops for food use?

Relative to intended effects, unintended effects should be given

□ Higher weight □ Equal weight □ Lower weight

If a different weight should be given, please suggest appropriate weighting.

Relative to a weight of 1.0 for intended effects, unintended effects should be given a weight of :

_____

Reasons:

If a weight for unintended effects relative to intended effects cannot be generalized, please provide examples for each of the following and the reasons.

Higher weighting

Equal weighting

Lower weighting

[1] The question of weighting is intended to incorporate the notion of likelihood of adverse health effects resulting from unintended effects in modified crops.

3. General comments (You may comment on the premise and assumptions of the project, emerging technologies and approaches in genetic modification of food crops, testing methods for enhancing GM food safety assessment, the degree of confidence you attach to your response, and other points you may wish to include. Relevant references and resources would also be helpful).

THANK YOU FOR YOUR PARTICIPATION IN THE SURVEY. Please return the completed questionnaire to:

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Ottawa, Ontario
K1N 6N5 CANADA

echao097@uottawa.ca
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APPENDIX B

APPENDIX B

EXPERT CONSULTATION 2

Assessment of draft RBCS

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2. Expert Consultation 2 - Assessment of draft RBCS

2.1. Purpose

The purpose of expert consultation 2 was to obtain an assessment from experts on the logical soundness of a draft RBCS. The specific aspects of the draft scheme assessed were:

- relevance of prerequisite and core information required to use the scheme,
- acceptability of the criteria for assessing the concern levels for the component categories of the scheme,
- reasonableness of the method of aggregation of the component categories, and
- appropriateness of the component and aggregate concern levels assigned to sample products.

2.2. Recruitment and selection of experts

Public sources of information and participant selection criteria similar to those used for consultation 1 were used in the selection of potential participants for this phase of the research. Four groups of individuals with relevant expertise or who could serve as contact points because of their professional role were contacted with the aim of recruiting 10-20 participants. They included four groups of individuals:

1) Respondents in expert consultation 1 who agreed to participate in consultation 2, and others who did not decline to participate in a second consultation,

2) Individuals who indicated an interest in the study, but were not able to participate in consultation 1 at that time,

3) Selected individuals who did not respond to a request to participate in consultation 1, and

4) New contacts not included in the recruitment process for consultation 1, selected using criteria comparable to those for consultation 1.

The recruitment process was reviewed by the Health Sciences and Science Research Ethics Board (HSSREB). The certificate of ethical approval is appended as (**Annex 2-1**). Included in **Annex 2-2** is a letter informing potential participants in group four of this phase of the research project. Modified versions of the letter were prepared for potential participants in the other three groups.

Table 2-1 summarizes the distribution by the above four groups of experts who were contacted and who agreed to participate. Individuals were sent an information letter about consultation 2 and were invited to participate, or to nominate other experts. Peer nomination was included as part of the

recruitment process in this phase of the research. It is an acceptable approach to targeted selection of potential participants in research involving expert judgement [Balter et al., 1993; Walker et al., 2001]. Peer nomination is also routinely used by organizations such as the U.S. National Research Council and the Royal Society of Canada in selecting experts to serve on their technical committees. In all cases, potential participants were first contacted by e-mail and then followed by a phone call. If an individual could not be reached directly by phone after several attempts following the initial e-mail, only one follow-up contact was made either by e-mail or by a faxed letter, a condition of receiving ethics approval by the HSSREB.

A total of 36 individuals were sent an information letter about the research project. Twenty-one individuals from eight countries in Europe, North America and Asia Pacific provided a positive response to participate. Expertise of these 21 individuals included plant sciences and biotechnology (7), molecular biology and genetics (3), toxicology and immunology (8), and food science and microbiology (2) (Table 2-1). Affiliations included universities (10), research institutes (3), and government or policy advisory committee (8). Two additional individuals were willing to receive the questionnaire, but were unable to commit to participation. The reasons given were concerns about time constraints, or about the relevance of an individual's expertise to providing a scientific response to all aspects of the questionnaire. Similar reasons for non-participation were given by another six individuals who responded, including resource constraints and changed responsibility. Direct contact could not be made with the remaining seven individuals during follow-up within the specified time frame for the recruitment process. The former two categories of individuals received a project information sheet that described the required elements of the conduct of the study in accordance with guidelines for research involving human participants at the University of Ottawa (**Annex 2-3**). They were randomly assigned one of five versions of a self-administered questionnaire that differed in the product examples to be reviewed (**Annex 2-4**).

2.3. Data collection

A four-part self-administered questionnaire was developed to assess specific aspects of the draft scheme. The four parts of the questionnaire were:

Part I - prerequisite and core information required to use the scheme

Part II - component categories comprising the scheme

Part III - aggregate concern levels of the scheme

Part IV - product examples with assigned concern levels using the scheme

In Part I, respondents were given a proposed list of prerequisite and core information and were asked to:

- rate the relevance of the individual components of the prerequisite and core information (yes/no),

- provide comments if the information was judged not relevant,
- suggest the type of data that should be included in assessing potential health risks of plants with modification involving complex or new metabolic pathways, and
- identify other information that should be included as prerequisite or core information.

In Part II, a 3-point scale (0=not appropriate, 1=acceptable with modification, 2=acceptable as is) was used in rating the acceptability of the criteria proposed for assessing each level of concern for the three component categories comprising the scheme (dietary exposure, intended effects, and unintended effects). Three concern levels (low, medium, high) were proposed for each component category. Comments and suggestions were requested for ratings 0 and 1.

In Part III, respondents indicated whether the proposed method of aggregation of component categories was reasonable (yes/no) and to comment and provide suggestions for modification if the answer was 'no'. Space was also provided for general comments.

In Part IV, respondents reviewed one product example randomly selected among five examples (products A-E) prepared by the researcher, and to indicate whether they agreed with the assignment of component and aggregate concern levels (yes/no). If the response was 'no', respondents were asked to provide comments. Respondents also indicated if the product information provided was sufficient to assess the appropriateness of the concern levels assigned using the criteria proposed for the draft RBCS.

Only one product example was reviewed by each participant in order to keep the completion of the questionnaire within a reasonable timeframe. Product examples were chosen to illustrate how the criteria for the concern levels of the draft RBCS can be applied. The examples included products that have been approved as food in at least one country based on publicly available product information. Information on experimental products was obtained using the scientific literature. The product information was kept brief to provide only essential core information.

2.4. Data analysis

A total of 14 completed questionnaires were returned from the 21 questionnaires sent. The range of expertise required was represented by the respondents (**Table 2-1**). Overall, the comments suggest support for the general direction in which the scheme was being developed. However, the need to refine specific aspects of the draft scheme was clearly indicated. An interpretation of support was based on the majority of ratings that were in general agreement with the core information, specific criteria for the component categories, and the trend of positive responses for the product examples. Certain aspects of

the draft scheme also received low ratings (not relevant, unacceptable, or acceptable with modification) from almost half of the respondents. The low ratings were notably associated with intended benefit (prerequisite information), dietary exposure, medium concern level (intended and unintended effects), and high concern level for multiple gene modifications (unintended effects). In general, the criteria for unintended effects generated more ratings of "acceptable with modification" than those for intended effects. The inclusion of molecular characterization as a marker for differentiating crops modified by rDNA and conventional methods was contentious. While only four respondents rated the method of aggregating component categories to be reasonable, seven "no" responses were related to specific aspects of the aggregation with suggestions for modification. The need and suggestions for detailed testing (the focus of another phase of the project) was evident in the responses throughout different sections of the questionnaire. In many responses, similar comments were made despite different numeric ratings. Therefore, the comments provided the context for the numeric ratings and were more informative in identifying *how* the draft scheme may be revised, while the numeric ratings were indicative of *where* improvement would be most needed.

Summaries of the numeric ratings and respondents' comments corresponding to the different sections of the questionnaire are included in **Tables 2-2 (A-E). and Table 2-3**. Supporting detailed comments provided by the respondents are also included in **Tables 2-4 (A-E)**. Summary of ratings and comments for the product examples are included in **Tables 2-3 and 2-5**, respectively. Comments on emerging analytical techniques and general comments are included in **Tables 2-6 and 2-7**, respectively. An overview and discussion of the results are presented below.

Prerequisite and core information (Tables 2-2A and 2-4A)

In general, with respect to relevance, there were fewer "no" responses for core information than for prerequisite information. "No" responses for both prerequisite and core information were expressed by respondents who would like to see the scope of the information better defined (e.g., stability, molecular characterization, history of safe use), or the level of detail required (e.g., structure and physico-chemical properties). Of the prerequisite information proposed, intended benefit was the most contentious. This information was considered irrelevant by some respondents because it was deemed a value judgement, or because it is not part of scientific risk assessment. Similar views were expressed regarding the substantiation of efficacy as part of prerequisite information. Reasons for concerns were that this information was unrelated to risk assessment and that it may be difficult to establish "efficacy" in relation to need. Some respondents also noted that a distinction should be made between the application of "good laboratory practices" for animal testing and "best practices" for other aspects of testing and plant genetic modification and breeding. Of the core information proposed, the criteria for gene function and known or suspected adverse human effects were the most consistently rated to be relevant by all

respondents. There appears to be an emphasis on rDNA technique as a means of genetic modification of crops, judging from the focus on the gene of the donor in responses provided to donor organism as part of core information.

Dietary exposure (Tables 2-2B and 2-4B)

Several comments questioned the appropriateness of separating dietary exposure at the food level (the focus of this component of the draft scheme) from dietary exposure at the level of the food constituent (included as part of the component of intended effects). The criteria for "low" concern level was judged to require modification because of practical difficulty in separating edible and non-edible parts in post-harvest handling and food processing. The inclusion of most common foods in the "high" concern level was also questioned because this was considered as scrutinizing a very large group of foods that generally lack safety concerns with little gain in protection. Other questions raised by respondents included the need to consider changes in food consumption and use patterns, intake distribution among population groups, and the need for reliable exposure data. A lack of clarity on how the special case would work was noted by some respondents, as were issues generally considered at the level of specific chemical substances or food constituents (e.g., dose-effect relationships, concentration of the level of food constituents by processing, and health concerns of very low exposure and of individual susceptibility to substances like allergens).

Intended effects (Tables 2-2C and 2-4C)

Compared to the other concern levels, criteria for "medium" concern level appear to generate the most "acceptable with modification" rating. In general, comments for the component category of intended effects were related to the generic nature of the criteria, the need for evidence-based criteria supported by analytical data or actual testing, and the need to consider at risk groups, food uses, and substitution effects. Some comments raised concerns that could be considered unintended effects. It was also suggested that the difference between "existing" and "novel" component of edible plants and plant-derived foods should be clarified.

Unintended effects (Tables 2-2D and 2-4D)

The criterion related to the unavailability of molecular characterization methods was considered unacceptable or acceptable with modification because molecular characterization is generally considered essential in assessing GM crops modified by rDNA techniques. While one respondent recognized the difference in analytical requirements that may exist in assessing GM crops modified by conventional methods, few comments provided alternative suggestions for testing. With respect to the criteria proposed for "high" concern level, half of the respondents generally preferred more specific criteria or more analytical data, particularly concerning multiple gene modifications. Regarding a modified trait that

has a negative health consequence, one respondent questioned whether products with such characteristics should be permitted.

Aggregation (Tables 2-2E and 2-4E)

Seven respondents did not consider the method of aggregating component categories as presented in the questionnaire to be reasonable. A review of their comments suggests concerns with specific aspects of the aggregation, rather than a rejection of the approach in its entirety. In addition, a concern about the loss of differentiation by aggregation was raised by one respondent. The need for testing and case-by-case approach was reiterated. Other comments included difficulty in following the aggregation and how it may be applied in terms of testing, particularly with respect to the special case. The inclusion of a "reject" category in the scheme was considered unreasonable because it is a risk management option.

Product examples (Tables 2-3 and 2-5)

Of the 14 respondents who returned the questionnaire, 11 completed the section on product examples. Four products were each reviewed by at least two individuals (products C and E by two individuals, products A and D by three individuals). Product B was reviewed by one individual. Out of a total of 11 assessments for all five products (Table 2-3), there were ten "yes" responses for (agreements with) the concern levels assigned by the researcher for exposure and intended effects, and nine "yes" responses for unintended effects and the aggregate concern level. Several responses provided for "no" ratings appear to be related to the comments on further testing of the product included in the questionnaire. Seven respondents viewed the information provided on the product to be sufficient to assess the appropriateness of the concern levels assigned by the researcher. A breakdown of the ratings and comments by individual products is included in Table 2-5. These results support the feasibility of assigning concern levels using only essential core information and the criteria proposed for the draft scheme, although some respondents would prefer more detail for certain types of information (e.g., quality of the molecular data, toxicology and allergenicity data on a novel gene product in a food crop).

Comments on emerging analytical techniques (Table 2-6)

Comments provided by eight respondents addressed four specific aspects of testing and assessment (profiling techniques, toxicology and allergenicity, food composition, and monitoring after release). Concerning profiling techniques or "omics" technologies, the issues of harmonization, validation and natural variability were raised. It was also suggested that their complexity may render them useful only in specific cases (e.g., bioengineered crops with transcription factors or crops modified by interspecific/intergeneric breeding or induced mutation where the specific targets are unknown). The use of molecular markers to improve the sensitivity of animal testing, the development of integrated testing systems for whole food testing, as well as new animal models for the assessment of allergenic potential

were noted. Additional comments included the issue of the extent of testing, the use of bioinformatics approaches, and the challenges of testing crops modified by conventional methods.

General comments (Table 2-7)

The general comments provided by eight respondents may be grouped into seven themes (essentiality of testing options, product scope, preference for case-by-case approach, applicability of the scheme for research and development purposes, suggestions for further product examples, and comments about specific component categories, aggregation, and specific terms) and other (nothing to add/RBCS useful in providing context for GM crops). Detailed comments are included in Table 2-7.

2.5. Discussion

Three general observations and six specific aspects of the draft scheme that emerged from the responses of expert consultation 2 warrant a discussion of their implications on the revision of the draft scheme before proceeding to the next phase of the project.

General observations

Risk-based classification scheme (RBCS) as guidance for testing. The need for detailed testing was reiterated by several respondents in response to different aspects of the draft scheme. These comments may be interpreted in two ways. First, the comments on testing provided a basis for developing testing options, the focus of another phase of the project. Second, the testing suggestions were intended by the respondents to be included as part of the criteria for discerning the different concern levels. The second interpretation could challenge a basic premise of RBCS as guidance for testing foods derived from GM crops. One basic premise was that GM crops and derived foods can be discriminated for their health risk potential using core information that is (1) based on information required or generated as part of developing the modified crop, and (2) amenable to be easily assessed. If the second interpretation was accepted, it would mean that core information would need to incorporate more product-specific testing. Alternatively, the second interpretation may mean that the respondents have an understanding of "concern level" that is different from what is intended for the RBCS. It is possible that respondents regarded concern level as an indication of the intrinsic toxicological and allergenic properties of a modified food, while concern level in the RBCS refers to a level of testing that is warranted in proportion to the potential for risk using information on a modified food that is readily available. Despite a precedent that already exists in the usage of the term "concern level" to indicate the type and extent of testing of food additives [FDA, 2003], it may be necessary to use more precise wording to describe "concern level" in order to promote a common understanding.

Case-by-case approach. One view that was expressed or implied in the responses is that it may be unrealistic for generic criteria such as those proposed in the draft scheme to capture the wide range of possible scenarios of GM crops. Therefore, a case-by-case approach is necessary. In consultation 2, the likely combinations of the principal criteria underlying the different concern levels within component categories (dietary exposure, intended effects, unintended effects) were organized in a format that would facilitate response within a reasonable timeframe using a questionnaire format. To make the RBCS applicable to a broader range of GM crops and derived foods than what was presented in the questionnaire, an alternative, more flexible approach to combining criteria within component categories will be included in the revised scheme. Despite its limitations, the approach to combining criteria within component categories in the draft scheme has demonstrated that the concept of graded concern levels can be realized using the criteria included. However, some of the criteria need to be refined or made more specific. It is also not clear whether the comments regarding a case-by-case approach were opposed to the concept of having different (minimum) levels of testing based on product characteristics. The concept of graded levels of minimum testing is not incongruent with the need for further testing where warranted, and the need to make risk management decision on a case-by-case basis regarding a specific product using all available data.

Applicability of the scheme. The RBCS is intended to be used principally for determining testing requirements for regulatory review. An extended use of RBCS in guiding the research and development of GM crops for food use would be desirable. Whether an RBCS can serve both purposes will be assessed in the next phase of the project. It will depend on the type of core information to be included in parallel with the development of testing options. Prioritization of objectives may become necessary.

Specific aspects of the draft scheme

Prerequisite information. In the context of an RBCS, data quality will be included as part of "prerequisite" information, which should be attained by all products and will not provide a basis for differentiation. Application of good laboratory practices and other best practices relevant to the testing and production of GM crops and derived foods would be one indication of data quality. Another example of prerequisite information is stability data. In addition, the inclusion of information on the intended purpose of the modified trait in terms of efficacy and benefit as part of prerequisite information is consistent with the need to assessing the potential impact of a modified crop. The requirement for environmental impact assessment is consistent with current practices of premarket assessment of bioengineered crops in a regulatory context [e.g., EFSA, 2004]. However, it is also recognized that such information is not directly relevant to determining the nature and extent of testing in relation to health risks and that complete information in this regard may not be available during early stages of the development of a GM crop. However, an integrated approach to health and environmental assessment has been advocated

[Haslberger, 2006]. Further, the extent of such information required as part of decision making may vary depending on the trait and the regulatory regime.

Core information. In contrast to prerequisite information, core information is expected to have discriminating value in classifying foods derived from GM crops on the basis of their health risk potential using information that can be obtained as part of developing the modified crop and that can be easily assessed. The purpose of core information is to classify GM foods so that testing for the purpose of risk assessment can be determined based on criteria that have been developed *a priori* and that can be applied in a systematic and transparent manner. Judging from the ratings and the comments on this aspect of the draft scheme, important core information for the RBCS would appear to have been identified. However, clarity is needed on the scope of information related to history of safe use and physico-chemical properties of primary and secondary gene products. The level of detail of core information on donor organism may need to vary depending on whether or not the gene modified is targeted.

Dietary exposure at the food level. Given the expected low frequency of modified crops for which modified gene expression will occur only in the non-edible parts of the plant, it may be more appropriate to set this category of modified crops outside of the RBCS. This category of products can be identified using a screening step that will channel them into an appropriate level of testing. Similarly, foods that are consumed as the sole or primary source of energy in the diet are also likely to occupy a minor proportion of the food supply, except for the targeted population groups. This category of foods is best dealt with as a special case, for which testing options will be examined in detail in a separate phase of the project. Relative to specific food substances that are generally the subject of risk assessment, most common foods can be considered to have "high" level of consumption with few characteristics for differentiation. However, these foods may be differentiated from other foods or ingredients that generally play a minor dietary role (e.g., spices and seasonings). It would be reasonable to apply different levels of scrutiny between these two types of foods.

Medium concern level. It appears from the results of consultation 2 that the criteria proposed for "medium" concern level for both intended and unintended effects were less acceptable than those for "low" and "high" concern levels. It is not surprising to find that the criteria for "medium" concern level would be more difficult to define or to gain acceptance, particularly for unintended effects. Given the current status of knowledge concerning the genesis of unintended effects with health risk potential in GM crops and their prediction and detection, it may be necessary to have only two concern levels for unintended effects in the revised scheme.

Role of molecular characterization. Because of their method of production, transgenic crops are more amenable to extensive molecular characterization than comparable plants produced using other breeding methods. Molecular characterization has become an essential component in the assessment of transgenic crops, yet it is not a sufficient means of predicting possible unanticipated changes nor is it a replacement for direct measurements of gene expression or changes in the levels of specific food constituents that are more directly related to their health risk potential. In contrast, crops modified by conventional methods are generally not scrutinized to a comparable extent. However, their inclusion in the RBCS is supported by growing discussion that questions the scientific justification of assessing GM crops based exclusively on the method of genetic engineering [e.g., NRC, 2004]. Their inclusion for assessment may require the development of standards of analysis that are not yet broadly accepted.

Aggregation. The apparent loss of information of the component categories and their differentiation by aggregation was noted as an item of concern. The value of aggregation was further questioned by the large number of aggregate concern levels (or labels) that remain after aggregation. At the time of the development of the draft scheme, four levels of testing of intended effects and two levels of testing of unintended effects were envisioned. It was expected that GM crops and derived foods would have different levels of concern for intended and unintended effects. Therefore, independent allocation of concern levels for intended and unintended effects would provide flexibility in testing. The practicality of this assumption was assessed in Phase 2 of the project.

Limitations

For a subject as complex as the one for this research project, the limitations of the use of a self-administered questionnaire in eliciting expert views are recognized. This medium was considered appropriate given the practical and financial constraints in reaching the targeted number of geographically diverse participants. However, a questionnaire format does not permit instantaneous interactive feedback. As well, to keep the questionnaire to a reasonable length, only minimal background information and the rationale of specific aspects of the draft scheme were provided. The number of experts available for consultation was also limited because of the nature of the subject matter. With these limitations in mind, consultation 2 was instructive in identifying those aspects of the draft scheme that warrant close attention.

Conclusion

In summary, the objectives of consultation 2 were met in verifying the relevance and acceptability of different aspects of the draft scheme. The results supported the general direction in which the gradation of concern levels was structured. However, the results also identified areas for clarification and refinement in the revision of the RBCS for the next phase of the project.

2.6. References

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FDA (U.S. Food and Drug Administration). 2003. Toxicological principles for the safety assessment of direct food additives and color additives used in food. Washington (DC): Center for Food Safety and Applied Nutrition.

Haslberger, AG. 2006. Need for an "integrated safety assessment" of GMOs, linking food safety and environmental considerations. J Agric Food Chem 54:3173-80.

Walker KD, Evans J, Macintosh D, 2001. Use of expert judgment in exposure assessment Part I. Characterization of personal exposure to benzene. J Expo Anal Environ Epidemiol 11:308-22.

Table 2-1. Participants.

Distribution by prior and new contacts	**Number contacted**	**Number of participants**
Group 1 - Participants of consultation 1	11	10
Group 2 - Individuals interested but unavailable for consultation 1	5	1
Group 3 - Individuals contacted but did not respond in consultation 1	17	8
Group 4 - New contacts	3	1
Total	36	20
Distribution by expertise	**Number of questionnaires sent**	**Number pf questionnaires returned**
Plant sciences and biotechnology	7	3
Molecular biology and genetics	3	2
Toxicology and immunology	9	8
Food science and microbiology	2	1
Total	21	14

Table 2-2. Summary of ratings and comments.

A. Prerequisite and core information - number of respondents rating the relevance of listed information and summary of responses (Number of respondents=14).

Prerequisite and core information	Relevant				Summary of responses
	Yes	No	Yes & No	Not rated	
Prerequisite information					
1. Stable expression of new trait for several generations, confirmed by biochemical and molecular analysis, where applicable	12	2	-	-	Both "yes" and "no" responses expressed the need to be more specific about the type of stability information required in relation to risk.
2. Application of good laboratory practices and best practices in all aspects of testing, transformation and selection procedures	11	3	-	-	Both "yes" and "no" responses suggest that a distinction should be made between good laboratory practices and other types of best practices and where they are applied.
3. For crops modified by rDNA techniques: well-characterized sequences for transfer before and after transformation	11	2	1	-	Responses are primarily related to the conditions where characterization of some types of sequences may not be possible. There is the need to specify whether characterization is required for endogenous or inserted DNA; and for inserted DNA, before or after transformation.
4. Substantiation for the efficacy of the new trait	9	3	-	2	Concerns included: - ambiguity of and difficulty in demonstrating "efficacy" - should not be required if efficacy is not related to risk assessment.
5. Indication of the intended benefit and for whom	8	6	-	-	Respondents were divided on the need for this information. Reasons for disagreeing with this requirement varied, including concerns about benefit as a value judgement (best left to market forces to decide), not a regulatory requirement, not related to risk, and the difference between "nice to know" vs. "need to know". Comments also raised the question that benefit consideration is a policy decision and should not be included as part of scientific safety assessment.
Core information					
6. Unmodified host plant					Comments suggest the need to clarify how "history of safe use" is defined and measured.

Prerequisite and core information	Relevant				Summary of responses
	Yes	No	Yes & No	Not rated	
a) History of safe use as food, or intended use if different from historical use	12	1	-	1	
b) Well-established knowledge of the biology and composition of the plant, including toxicants, allergens, nutrients, and antinutrients	13	-	1	-	
7. Donor organism, where applicable					Comments similar to those for host organism were expressed. In addition, views were expressed about the need to distinguish between knowledge of the donor organism at the organism level vs. the gene level. This suggests a focus on gene modification by the rDNA approach.
a) Use or presence in food supply	12	1	1	-	
b) Knowledge of the biology and composition of the donor organism, including toxicants, allergens, nutrients, and antinutrients	12	1 [1]*	1	-	*Equivocal response.
c) Known or suspected adverse human health effects	11	1 [1]*	1	-	*Equivocal response.
8. Gene function and related reactions or pathways that are the target of the modification, or that result in the selected modified trait					Agreement on the relevance of this information was unanimous.
a) Prior knowledge or experimental data	14	-	-	-	

Prerequisite and core information	Relevant				Summary of responses
	Yes	No	Yes & No	Not rated	
9. Primary gene product (protein/enzyme)					The importance of focusing on the gene product is generally accepted. However, questions were also raised about where this requirement would be most relevant (e.g., gene product related to toxicity, the intended use of the modified crop). Most comments were related to the need for specifics or the level of detail required, particularly that required for physico-chemical properties. Similar comments were made in the both the "yes" and "no" responses, emphasizing that the information required should be essential, and not just useful.
a) Tissues and levels of expression in modified and unmodified crop	13	1	-	-	
b) Known or suspected adverse human effects	14	-	-	-	
c) Structure and physico-chemical properties	12	1	-	1	
10. Where applicable, secondary gene products (direct product and metabolite of modified enzyme)					In general, the comments are similar to those for primary gene product in #9.
a) Tissues and levels of expression in modified and unmodified crop	14	-	-	-	
b) Where the primary or secondary gene product is a novel component in the host plant: metabolic fate in the modified crop	14	-	-	-	
c) Known or suspected adverse human effects	14	-	-	-	
d) Structure and physico-chemical properties	11	1	-	2	

Table 2-2. Summary of ratings and comments.
B. Dietary exposure - number of respondents rating the acceptability of listed criteria and summary of responses.
(0=not acceptable, 1=acceptable with modification, 2=acceptable as is; number of respondents=14).

Level	Criteria	Criteria acceptability 0 1 2	Summary of responses
Low - 1	□ Site/tissue-specific modification [a] - food consumed in limited form as highly refined ingredient and well-defined chemical entity	0 5 9	Comments pointed to the difficulty in separating edible and non-edible parts due to outcrossing, mixing in processing, and possible uses of plant by-products in animal feed. There is also the need for analysis as evidence of absence of the noted substances. It was also noted that while intake at the food level may be the primary concern for unintended effects, intake levels of the expressed substance is also important for intended effects, in addition to intake at the food level.
Low - 2	□ Site/tissue-specific modification - food consumed fresh, processed, or as ingredient in wide spectrum of applications	0 6 8	Comments were similar to those for Low-1. Additional comments included a suggestion that non-edible parts may be consumed by subgroups.
Medium	□ Food consumed in limited form as highly refined ingredient and well-defined chemical entity, or □ Common food or food ingredient consumed in very small amounts, or □ Rare food, infrequently consumed	0 6 8	Several factors related to dietary exposure at the food level to be considered were suggested, including changes in intake level, concentration of specific substances during food processing, health concerns related to low level of exposure to some allergens and to novel proteins, as well as distribution of intakes and dose-effect relationships.
High	□ Common food, regularly or variably consumed in appreciable amounts	3 2 9	Comments included concerns about the need for evidence of exposure levels and for experimental evidence, as well as the need to consider prior history of safe use of most foods.
Special case	□ GM food or GM ingredient in a food that is the sole or primary source of nutrition for extended period for vulnerable subpopulations (e.g., infants, patients unable to consume a varied diet) - excludes food and ingredient classified as low-1 for exposure	2 2 8 Not rated: 2	Comments suggest a lack of clarity on how the "special case" would work.

[a] Primary and secondary gene products expressed, metabolized, and stored only in non-edible part of modified crop.

Table 2-2. Summary of ratings and comments.
C. Intended effects - number of respondents rating the acceptability of listed criteria and summary of responses.
(0=not acceptable, 1=acceptable with modification, 2=acceptable as is; number of respondents=14).

Level	Criteria	Criteria acceptability 0	1	2	Summary of responses
Low	□ No new primary or secondary gene product	2	2	10	Concerns were raised regarding the generic nature of this criterion and the need to consider unintended effects.
Low	□ Existing component of edible plants and plant-derived foods	1	3	10	Comments included the need to assess changes in the structure/function of proteins regardless of the extent of modification and changes in exposure level. A suggestion was also made to consider unintended effects.
Low	□ Novel component of edible plants and plant-derived foods	1	1	12	Comments suggest that the criterion could be made clearer.
Medium	□ Existing component of edible plants and plant-derived foods	2	6	6	Several suggestions for modifying the criterion were provided, such as distinguishing between concern about exposure vs. hazard. The need to consider other factors was also noted, such as processing effect (e.g., stability), changes in the intended use of the modified food, and changes in intake levels of the expressed compounds, as well their effects on other food constituents (e.g., bioavailability) and on sensitive subpopulations. The need for toxicological testing and exposure assessment was also mentioned.
Medium	□ Novel component of edible plants and plant-derived foods	1	6	7	Comments similar to those for "medium" above were also expressed here, including comments related to the need to consider at risk groups, intended use of the modified crop, and substitution effects. Other comments previously made were also repeated (e.g., unclear distinction between novel vs. existing component of edible plants and plant-derived foods).
High	□ Existing component of edible plants and plant-derived foods	1	3	10	Some comments previously made were also expressed here, including processing effects and the need for testing.
High	□ Novel component of edible plants and plant-derived foods	2	2	10	Again, the comments emphasize the need for testing.

Table 2-2. Summary of ratings and comments.
D. Unintended effects - number of respondents rating the acceptability of listed criteria and summary of responses.
(0=not acceptable, 1=acceptable with modification, 2=acceptable as is; number of respondents=14).

Level	Criteria	Criteria acceptability 0	1	2	Summary of responses
Low	□ Molecular methods are available and used to localize and characterize changes in genetic elements	1	3	10	The need for analysis was evident in the comments as were suggestions for the type of analysis required.
Low	□ Molecular methods are not available or used to localize or characterize changes in genetic elements	4	3	7	Respondents who rated this criterion unacceptable considered molecular characterization to be essential in assessing GM crops. One respondent noted the difference that may exist in assessing GM crops modified by rDNA vs. conventional techniques. Suggestions for analysis were offered by respondents who rated this criterion acceptable with modification.
Medium	□ Molecular methods are available and used to localize and characterize changes in genetic elements	1	5	8	Similar to previous comments for "low" concern level, the need for analysis was emphasized and the types of analysis deemed necessary were suggested.
Medium	□ Molecular methods are not available or used to localize or characterize changes in genetic elements	3	4	7	Comments were similar to those provided for "low" concern level under the same heading. Principally, respondents who rated this criterion unacceptable considered molecular characterization to be essential in assessing GM crops. One respondent noted the difference that may exist in assessing GM crops modified by rDNA vs. conventional techniques. Suggestions for analysis were offered by respondents who rated this criterion acceptable with modification.
High	□ Host plant or donor organism - source of toxicity, allergenicity, or pathogenicity	1	5	8	One common theme that emerged from the comments is that the listed criteria (except for the criterion regarding a modified trait with negative health consequence) might not be sufficient for assigning concern level. Most of the comments focused on the host plant as a source of known toxicity or allergenicity. These comments suggest that distinctions among these sources of allergen and toxicity should be made based on analysis. One respondent comment ed on the donor organism and noted the need to focus on the gene rather than the organism.
High	□ Complex metabolic pathway	2	3	9	Similar to previous comments, respondents emphasized the need for analysis.
High	□ Multiple gene modifications	4	2	8	The comments suggested that the listed criteria are too generic. This again implies a more specific (case-by-case) approach is preferred.
High	□ Modified trait that has a negative health consequence if stability is not maintained during the commercial lifespan	4	2	8	Two comments for "unacceptable" and "acceptable with modification" would suggest the need for more rigorous testing. One respondent questioned whether such products should be permitted.

Table 2-2. **Summary of ratings and comments.**
E. Method of aggregation of component categories (Number of respondents=14).

Reasonableness of method of aggregating component categories	Number of responses	Summary of responses
No	7	The "no" responses were related to specific aspects of the aggregation, including the "special case" and "reject". There appears to be support for the general direction of the proposed scheme with suggestions for modification. However, there was also a concern for the loss of differentiation by aggregation. The need for testing was reiterated,
Not rated	3	Reasons for not rating included difficulty in following the rationale of some aspects of the aggregation.
Yes	4	One comment was provided with a suggestion for change for "low-1" (exposure) due to concerns about unintended effects.

Table 2-3. Product examples - summary of ratings.

Product	Number returned[a]	Number completed[a]	Concern levels assigned by researcher				Agreement with assigned concern levels (Number of "yes" responses)				Sufficient information ("Yes")
			Exposure	Intended	Unintended	Aggregate	Exposure	Intended	Unintended	Aggregate	
A	3	3	H	L	M	II-A	3	3	1	2	2
B	2	1	H	L	M	II-A	1	1	1	1	1
C	4	2	H	M	L	III-A	1	1	2	1	1
D	3	3	H	M	H	III-B	3	3	3	3	2
E	2	2	H	H	M	IV-A	2	2	2[b]	2	1
Total	14	11					10	10	9	9	7

[a] Number of questionnaires returned and number of respondents who completed the section of the questionnaire on product examples.

[b] A "yes and no" response was provided by the respondent.

Table 2-4. Summary and detailed responses.

A. Prerequisite and core information - number of respondents rating the relevance of listed information and comments (Number of respondents=14).

Prerequisite and core information	Relevant				Summary and detailed comments
	Yes	No	Yes & No	Not rated	
Prerequisite information					
1. Stable expression of new trait for several generations, confirmed by biochemical and molecular analysis, where applicable	12	2	-	-	Both "yes" and "no" responses expressed the need to be more specific about the type of stability information required in relation to risk. No: ▪ Requirement for this information depends on - the question one would like to answer - the crop (whether it should be sexual or vegetative generations) - which and how many generations are required (e.g., is it necessary to test the first few generations if generation 5 proves to be stable). ▪ Stability related to safety (with respect to variable expression) most likely needs to be a protein-based technique (a biochemical method). Yes: ▪ This criterion should be qualified: "Several" should be defined and be reasonable (e.g., at least 3 generations). ▪ Analytical data on the product have to be representative. ▪ This is important information for plants produced through rDNA methods to show that the integrated DNA is no less stable than the rest of the genome. ▪ This information is not really essential to risk assessment but is generally obligatory to document commercial reliability.

Prerequisite and core information	Relevant				Summary and detailed comments
	Yes	No	Yes & No	Not rated	
2. Application of good laboratory practices and best practices in all aspects of testing, transformation and selection procedures	11	3	-	-	Both "yes" and "no" responses suggest that a distinction should be made between good laboratory practices and other types of best practices and where they are applied. No: ▪ GLP's are very strictly defined (most relevant for e.g., animal tests) and are not applicable for many practices (e.g., results of transformation and selection are measured afterwards). Case-by-case approach should be taken. ▪ Change "and" to "or", or state that the principles of GLPs should be followed. Other means for (internal) control can be used. ▪ The end result is more important, not the method. Yes: ▪ Difference between Good Laboratory Practices and Best Practices should be distinguished. ▪ Good laboratory practices may not be applicable for all types of work, such as field testing, for which other practices, such as good agricultural practice, may apply.

Prerequisite and core information	Relevant				Summary and detailed comments
	Yes	No	Yes & No	Not rated	
3. For crops modified by rDNA techniques: well-characterized sequences for transfer before and after transformation	11	2	1	-	Responses are primarily related to the conditions where characterization of some types of sequences may not be possible. There is the need to specify whether characterization is required for endogenous or inserted DNA; and for inserted DNA, before or after transformation. No: ▪ Endogenous (host) DNA sequence at the insertion site may not be known before the transformation event. Most of the crops do not have a full genomic sequence, and even if they do, there is a lot of ambiguity. ▪ This information is normally required (in principle). Even for some transformants after a transformation event, it might not be possible to characterize the inserted sequence (e.g., rearranged and many copies). ▪ It is reasonable to ask for characterization of the plant genomic DNA for ~ 1,000 bp on each boarder of the insert, although this can be difficult to achieve in some cases. Only limited data on boarder sequences can be achieved, unless it is clear that a gene has been interrupted, or that the necessary flanking regions have been provided to evaluate insert stability. Yes and No: ▪ Yes for the sequences before. It might be no for the sequences after. If the site of integration and the flanking DNA should be known, it is important to set specifics (e.g., 500 bp of the flanking DNA on either site). Yes: ▪ "After" transformation should apply to the sequence inserted into the target cells, including regulatory elements.

Prerequisite and core information	Relevant				Summary and detailed comments
	Yes	No	Yes & No	Not rated	
4. Substantiation for the efficacy of the new trait	9	3	-	2	Concerns included: - ambiguity of and difficulty in demonstrating "efficacy" - should not be required if efficacy is not related to risk assessment. No: ▪ Not part of risk assessment; relevant if related to increase in risk; may deter the pursuit of technology. ▪ Sometimes very difficult if there are other means of achieving "efficacy" and there may not be a health-related need (e.g., carotene-enriched rice). Not rated: ▪ This depends on the trait: (e.g., efficacy of herbicide tolerance may have no effect on food safety). For nutritionally changed products this point is relevant. Yes: ▪ Demonstration of probable efficacy, or demonstration of proof of principle, would be a better description, as it may take 2 years after commercial release to "substantiate" efficacy.

Prerequisite and core information	Relevant				Summary and detailed comments
	Yes	No	Yes & No	Not rated	
5. Indication of the intended benefit and for whom	8	6	-	-	Respondents were divided on the need for this information. Reasons for disagreeing with this requirement varied, including concerns about benefit as a value judgement (best left to market forces to decide), not a regulatory requirement, not related to risk, and the difference between "nice to know" vs. "need to know". Comments also raised the question that benefit consideration is a policy decision and should not be included as part of scientific safety assessment. No: ▪ Presents the need for a value judgement. Market forces should dictate value/extent of benefits to end users. If accepted as a criterion it should also apply to other breeding approaches. ▪ This is not required by regulation. The market place will demand it if it is important. ▪ Benefit is opposite of risk. ▪ Since risk assessment focuses on safety this information is 'nice to know' but not 'need to know'. ▪ It is not clear why "for whom" is included. It is basically for man, even if it is a GM feed for animals. The ultimate purpose would be to improve that animal as a food source. Yes: ▪ Benefits should be part of the risk-benefit analysis. This would particularly apply to nutritionally improved crops. However, such an analysis is carried out by risk managers and policy makers at the policy level, not at the level of scientific safety assessment. ▪ This is interesting information and puts the novel food into context, but when we assess GM foods for safety we don't weigh risk versus benefit, we just need evidence that the food is as safe as its conventional counterpart.

Prerequisite and core information	Relevant				Summary and detailed comments
	Yes	No	Yes & No	Not rated	
Core information					
6. Unmodified host plant					Comments suggest the need to clarify how "history of safe use" is defined and measured.
a) History of safe use as food, or intended use if different from historical use	12	1	-	1	No: ▪ No plant could ever become a food plant under this concept. Not rated: ▪ Not sure what is meant by non-historical food use. Does it relate to foods eaten in one part of the world introduced somewhere else? Yes: ▪ It is not clear how the extent of safety should be defined. Some crops have history of safe use but with "blips" (e.g., potatoes with high glycoalkaloids, allergenic crops, peanuts etc.).
b) Well-established knowledge of the biology and composition of the plant, including toxicants, allergens, nutrients, and antinutrients	13	-	1	-	Yes and No: ▪ Some of these can be done by monitoring the population after the plant is used. Yes: ▪ Would not need to know every single component; this information may not yet exist for any food.

Prerequisite and core information	Relevant				Summary and detailed comments
	Yes	No	Yes & No	Not rated	
7. Donor organism, where applicable					Comments similar to those for host organism were expressed. In addition, views were expressed about the need to distinguish between knowledge of the donor organism at the organism level vs. the gene level. This suggests a focus on gene modification by the rDNA approach.
a) Use or presence in food supply	12	1	1	-	No: ▪ Most foods contain toxins; this requirement could prevent the use of most toxic plants that contain useful genes. Yes and No: ▪ Concern about a safe or harmful donor organism should be distinguished from the safe or harmful use of one (or more) of its genes. Yes: ▪ Helpful but not required.
b) Knowledge of the biology and composition of the donor organism, including toxicants, allergens, nutrients, and antinutrients	12	1 [1]*	1	-	No: ▪ Focus should be on the gene of interest (e.g., proof of donor gene product is not the allergen in the donor)*. Yes and No: ▪ Will depend upon the perceived risk level. Yes: ▪ Similar to comments for 6b) above about crops having "blips" of adverse effects despite history of safe use, and whether there is the need to know every single component. This information may not yet exist for any food. *Equivocal response.

Prerequisite and core information	Relevant				Summary and detailed comments
	Yes	No	Yes & No	Not rated	
c) Known or suspected adverse human health effects	11	1 [1]*	1	-	No: ▪ Focus should be on the donor gene, as in b)*. Yes: ▪ Depends on the gene transferred; may not be relevant if not associated with known hazard. *Equivocal response.
8. Gene function and related reactions or pathways that are the target of the modification, or that result in the selected modified trait					Agreement on the relevance of this information was unanimous.
a) Prior knowledge or experimental data	14	-	-	-	Yes: ▪ Vital information, to avoid "side effects".

Prerequisite and core information	Relevant				Summary and detailed comments
	Yes	No	Yes & No	Not rated	
9. Primary gene product (protein/enzyme)					The importance of focusing on the gene product is generally accepted. However, questions were also raised about where this requirement would be most relevant (e.g., gene product related to toxicity, the intended use of the modified crop). Most comments were related to the need for specifics or the level of detail required, particularly that required for physico-chemical properties. Similar comments were made in both the "yes" and "no" responses, emphasizing that the information required should be essential, and not just useful.
a) Tissues and levels of expression in modified and unmodified crop	13	1	-	-	<u>No</u>: ▪ Need to focus on the intrinsic toxicity (if known and if not it should be investigated), rather than the tissues in which the primary gene product is found, or the levels at which it is found. <u>Yes</u>: ▪ This will be dependent upon how the product is used. If the protein is extracted, the level in the crop is meaningless except for estimating the cost of extraction. ▪ Not clear about measuring the new gene product in the unmodified crop, except as a control for background. ▪ This is important for food produced using rDNA techniques. Perhaps less relevant for (or how to assess in) conventionally produced crops when there may not be a targeted change in one gene or it may be difficult to identify the change at a genomic level. ▪ Case by case depending on the protein /enzyme.

Prerequisite and core information	Relevant				Summary and detailed comments
	Yes	No	Yes & No	Not rated	
b) Known or suspected adverse human effects	14	-	-	-	Yes: ▪ See comment related to intrinsic toxicity above. ▪ Evidence of no adverse effects may be more relevant. This would include toxicological studies on the novel protein (e.g., acute oral toxicity testing in animals).
c) Structure and physico-chemical properties	12	1	-	1	No: ▪ Depends on how much detail is required. Not rated: ▪ It would be unrealistic to expect knowledge on the structure and physico-chemical composition of proteins during the R&D process. This would mean that there is not much left to work on. Yes: ▪ The amount of information needed will be case by case. If the protein is not a known toxicant/allergen, information requested may be minimal? ▪ Need to qualify: at what level. Some information may be useful but not essential. For protein, need to know sequence and glycosylation pattern but not 3-D structure, NMR etc. (3)[a]. ▪ Biochemical function of the new gene/protein and how it affects the phenotype of the plant. ▪ Very important if a substitute protein is used for acute toxicity studies.

Prerequisite and core information	Relevant				Summary and detailed comments
	Yes	No	Yes & No	Not rated	
10. Where applicable, secondary gene products (direct product and metabolite of modified enzyme)					In general, the comments are similar to those for primary gene product in #9.
a) Tissues and levels of expression in modified and unmodified crop	14	-	-	-	Yes: ▪ This will be dependent upon how the product is used. If the protein is extracted, the level in the crop is meaningless except for estimating the cost of extraction.
b) Where the primary or secondary gene product is a novel component in the host plant: metabolic fate in the modified crop	14	-	-	-	Yes: ▪ Clarification is needed here. Metabolic fate would not be as relevant for a protein, unless there is a reasonable health concern about a potential stable fragment. Proteins are broken down to amino acids, which are safe. Measurement of accumulation or tissue levels of protein would tell all you need to know in many cases.
c) Known or suspected adverse human effects	14	-	-	-	Yes: ▪ See comment for 9b) about evidence of no adverse effects and toxicological studies.

Prerequisite and core information	Relevant				Summary and detailed comments
	Yes	No	Yes & No	Not rated	
d) Structure and physico-chemical properties	11	1	-	2	No: ▪ Not sure how much detail is required. Not rated: ▪ See above about unrealistic expectation on knowledge about the structure and physico-chemical composition of proteins. Not even of known polymers like starch is the exact structure identified. The question here is how much detail is needed. ▪ Case by case. Yes: ▪ Information on biochemical function of the new gene/protein and how it affects the phenotype of the plant would be more relevant than structure and physico-chemical properties. ▪ Useful, but not necessarily essential.
Other information to be included					Seven individuals completed this section of the questionnaire.
a) Prerequisite information					Molecular characterization and method of genetic modification were suggested. ▪ Characterization of insertion site(s), stability of insertion(s), and analysis of flanking regions regarding open reading frames, transcripts and fusion proteins. ▪ Method of genetic modification (e.g., Agrobacterium or biolistics, or techniques used in conventional modifications) (2). ▪ Nothing additional/I think you have everything (2).

Prerequisite and core information	Relevant				Summary and detailed comments
	Yes	No	Yes & No	Not rated	
b) Core information					Suggestions were provided for detailed testing, the need for context and for benefit-risk consideration. Comments related to specific, detailed testing, such as toxicological testing and exposure assessment suggest that the role of core information as intended for a risk-based classification scheme in guiding testing may not have been well communicated or received. ▪ Detailed testing - toxicity testing of novel/recombinant protein(s) with no history of safe consumption (2) - mode of action and target specificity of recombinant proteins - allergenic potential of novel protein with no history of safe consumption (2); in vitro and in silico studies - documented human exposure to recombinant proteins, if available - edible parts of the changed plant - estimate of exposure to the GM food ▪ Context:: It is important to place the extent of natural variation in compositional characteristics in the proper risk context. GM plants are designed to be different from the parents so some unintentional changes may well occur. ▪ Benefit-risk: Known or claimed beneficial aspects (not only adverse) of a donor organism, host organism, gene product (e.g., the introduction of micronutrients in the case of nutritional modifications where the micronutrient has both known beneficial and adverse effects).

[a] Number of respondents with similar comments.

Table 2-4. Summary and detailed responses.
B. Dietary exposure - number of respondents rating the acceptability of listed criteria and comments.
(0=not acceptable, 1=acceptable with modification, 2=acceptable as is; number of respondents=14)

Level	Criteria	Criteria acceptability 0 1 2	Summary and detailed comments
Low - 1	□ Site/tissue-specific modification - primary and secondary gene products expressed, metabolized, and stored only in non-edible part of modified crop, and - food consumed in limited form as highly refined ingredient and well-defined chemical entity Examples: Refined oils from oilseed rape or cottonseed that expresses a novel protein only in the non-edible parts of the plant	0 5 9	Comments pointed to the difficulty in separating edible and non-edible parts due to outcrossing, mixing in processing, and possible uses of plant by-products in animal feed. There is also the need for analysis as evidence of absence of the noted substances. It was also noted that while intake at the food level may be the primary concern for unintended effects, intake levels of the expressed substance is also important for intended effects, in addition to intake at the food level. 1: ▪ Outcrossing may alter pattern of gene expression. ▪ Should ensure that the non-edible parts are not mixed up with the edible parts in the primary production phase (e.g., fruit juice processing). ▪ Possible uses of plant by-products as animal feed should be considered (if this is part of the safety assessment). ▪ EVIDENCE of absence in ingredient is required (poor purification techniques could allow gene/gene product transfer). ▪ It is the expression level of the new protein and/or metabolite in combination with the intake levels that are of importance for intended effects. Intake levels of the food are important when considering potential unintended effects.
Low – 2	□ Site/tissue-specific modification - primary and secondary gene products expressed, metabolized, and stored only in non-edible part of modified crop, and - food consumed fresh, processed, or as ingredient in wide spectrum of applications	0 6 8	Comments were similar to those for Low-1. Additional comments included a suggestion that non-edible parts may be consumed by subgroups. 1: ▪ Depends on the trait. It might be that non-edible parts could be eaten by sub-groups. ▪ Comments about outcrossing, mixing, use in animal feed were also raised.

Level	Criteria	Criteria acceptability 0 1 2	Summary and detailed comments
Low - 2	Examples: Fruit or kernels of a modified crop that expresses a novel protein only in the non-edible part of the plant		2: ▪ The difference between Low-1 and Low-2 is not obvious. Is it because the foods in Low-2 are consumed in greater amounts, therefore, posing a higher probability of mixing and consumption of non-edible portions?
Medium	□ Food consumed in limited form as highly refined ingredient and well-defined chemical entity, or □ Common food or food ingredient consumed in very small amounts, or □ Rare food, infrequently consumed Examples: Refined oils from oilseed rape or cottonseed; spices, seasonings; exotic fruits	0 6 8	Several factors related to dietary exposure at the food level to be considered were suggested, including changes in intake level, concentration of specific substances during food processing, health concerns related to low level of exposure to some allergens and to novel proteins, as well as distribution of intakes and dose-effect relationships. 1: ▪ Depends on the nature of the modification and whether dietary intake patterns could be modified after marketing. ▪ Novel, highly allergenic proteins can cause problems even with low or infrequent exposure (e.g., cottonseed protein or nau-gai nut). ▪ Small amount globally can mean high intake for some people. ▪ The exposure variation among the population, as well as among subpopulations should be considered. In addition, expression level and magnitude of other changes in the genetically modified crop (e.g., high levels in a minor food may still have impact), as well as dose-effect relationships of the altered compounds (e.g.,. some compounds may exert physiological effects at low doses), should be taken into account. ▪ Evidence of exposure level is required. 2: ▪ Dietary exposure at the food level may be medium but exposure to the novel protein might depend on the nature of the refined food (e.g., the novel protein may be concentrated in the refined food during processing). ▪ Re-evaluation of changes in exposure levels may be required if there was a change of use in products that were marketed to increase consumption (e.g. oil being used to

Level	Criteria	Criteria acceptability 0 1 2	Summary and detailed comments
			make margarine, new fruit, e.g., kiwi).
High	□ Common food, regularly or variably consumed in appreciable amounts Examples: Seeds, nuts; many fruits and vegetables; many cereal grains, and legumes, including corn, rice, soybean, and wheat	3 2 9	Comments included concerns about the need for evidence of exposure levels and for experimental evidence, as well as the need to consider prior history of safe use of most foods. 0: ▪ Should not be decided without evidence of safety. ▪ Not clear who is setting current criteria when regulatory agencies (FDA and EU) don't agree. At a minimum animal feeding trials for any GM food. 1: ▪ Prior history should be considered. Otherwise nothing could be generally regarded as safe (GRAS). A lot of money could be spent with little gain. 2: ▪ Exposure to novel protein would depend on the level of expression of the novel protein, e.g., low expression or high expression. However, for this section exposure refers to the whole food, not to the novel protein/s. Perhaps this point could be emphasized in the introduction so there is less confusion.
Special case	□ GM food or GM ingredient in a food that is the sole or primary source of nutrition for extended period for vulnerable subpopulations (e.g., infants, patients unable to consume a varied diet) - excludes food and ingredient classified as low-1 for exposure Examples: Infant formulas, ingredients used in formulated tube-feeding products	2 2 8 Not rated: 2	Comments suggest a lack of clarity on how the "special case" would work. 0: ▪ For highest risk category, safety assessment is essential and must be rigorous. 1: ▪ It is hard to respond to this question without knowing how the special case process works. It could be a very great hindrance (barrier to technology use) if mishandled (e.g., irradiated foods). Not rated: ▪ I am not sure how this should be interpreted. There are no connections between GM and risk in general.

Table 2-4. Summary and detailed responses.
C. Intended effects - number of respondents rating the acceptability of listed criteria and comments.
(0=not acceptable, 1=acceptable with modification, 2=acceptable as is; number of respondents=14).

Level	Criteria	Criteria acceptability 0	1	2	Summary and detailed comments
Low	□ No new primary or secondary gene product* Examples: Silenced expression of an endogenous gene *The intended silencing of a gene encoding a substance beneficial to health is possible, but unlikely in practice. It is more likely to occur as an unintended effect of gene silencing, which will be assessed separately under considerations for metabolic pathways and multiple gene modifications (Part II-C)	2	2	10	Concerns were raised regarding the generic nature of this criterion and the need to consider unintended effects. 0: Case-by-case determination is necessary. 1: ▪ This criterion is too generic. Consider the case of silencing a gene that processes a toxic compound to a non-toxic compound. ▪ The impact of gene silencing on the composition and nutritional/physiological /safety aspects of the genetically modified crops should be taken into account (e.g., gene-silenced delayed-ripening tomatoes contained altered levels of glutamic- and citric- acids although in this case, changes in the levels of these compounds would not raise any safety concerns).
Low	□ Existing component of edible plants and plant-derived foods 1- Not known to be a toxicant, allergen or antinutrient at the level expressed in crop plants with a history of safe use as food a/ Expressed at levels comparable to those in other edible crop plants, AND b/ Limited and well-characterized modification in structure/function (e.g., point mutation or site-directed mutagenesis) Examples: Alteration of the levels of some plant-derived fatty acids that are common dietary components	1	3	10	Comments included the need to assess changes in the structure/function of proteins regardless of the extent of modification and changes in exposure level. A suggestion was also made to consider unintended effects. 1: ▪ The impact of the consumption of the genetically modified crop on nutrition, in particular on the exposure to the altered compounds, should also be considered (e.g., the genetically modified crop may serve as an additional source of the compounds, thereby increasing its consumption beyond levels of historic safe use). ▪ As long as the new gene/gene product does not interfere with the metabolic machinery in a qualitatively different way. ▪ b/ Adequate studies should be required even in the case of limited and well characterized modification of structure/function of a protein, which could change the toxic or allergenic potential or create new ones.

Level	Criteria	Criteria acceptability 0 1 2	Summary and detailed comments
Low	□ Novel component of edible plants and plant-derived foods 1- Well-characterized modification of target gene (e.g., site-directed mutagenesis), AND 2- No toxicological concern based on prior knowledge: <u>one or more</u> of the following applies a/ Known mechanism for toxicity not applicable to humans, or extensive evidence for no adverse human health effects, OR b/ Established tolerance limit, such as maximum residue level (MRL) or equivalent, and level expressed is below MRL, OR c/Level expressed is below threshold of toxicological concern (TTC)[1] based on aggregate exposure estimates, OR d/ History of safe use in relation to food at the level expressed in the modified crop[2] Examples: Cry protein from Bt (a common soil bacterium) as a pesticide; active compound of a herbicide and its metabolite in a herbicide-tolerant plant that is modified to catabolize the herbicide; coat protein of common plant viruses for virus resistance	1 1 12	Comments suggest that the criterion could be made clearer. <u>1</u>: ▪ This criterion suggests that the intended product is toxic at higher expression levels. <u>2</u>: ▪ This criterion needs clarification: does novel component here refer to novel in the plant and plant-derived foods but not novel in the food supply as a whole AND entirely novel in the food supply?
Medium	□ Existing component of edible plants and plant-derived foods 1- Not known to be a toxicant, allergen or antinutrient at the level expressed in crop plants with a history of safe use as food a/ Expressed at levels higher than those in crop plants, AND b/ Limited and well-characterized modification in structure/function	2 6 6	Several suggestions for modifying the criterion were provided, such as distinguishing between concern about exposure vs. hazard. The need to consider other factors was also noted, such as processing effect (e.g., stability), changes in the intended use of the modified food, and changes in intake levels of the expressed compounds, as well their effects on other food constituents (e.g., bioavailability) and on sensitive subpopulations. The need for toxicological testing and exposure assessment was also mentioned. <u>0</u>: ▪ Case-by-case assessment is necessary. In some cases "medium" would be

Level	Criteria	Criteria acceptability 0 1 2	Summary and detailed comments
Medium	OR 2- Known adverse health effect in some animals or humans, with an established reference dose (RfD) or acceptable daily intake (ADI) or equivalent a/ Expressed at higher level in the modified crop than in the native crop, or at comparable or higher levels than in other edible crops, AND b/ Limited and well-characterized modification in structure/function Examples: Increased level of lycopene in tomatoes for health benefit (lycopene is a naturally occurring constituent in tomato); increased levels of some naturally occurring alkaloids from crop plants as pesticides		acceptable. 1: ▪ Must assess nutritional/toxicological impact of higher levels of exposure, including some beneficial substances (2), as well as some assessment of how increases in nutrients may affect their bioavailability and stability to processing. ▪ Safety must also be demonstrated for highly sensitive sub-populations. ▪ The intended use of the modified crop should also be taken into account, i.e. is it used in the same way as the native crop (with potential effect of processing and potential changes on dietary intake)? In addition, the effect on other beneficial/adverse compounds should be taken into account (e.g., the effect on other carotenoids of increased levels of lycopene in the example mentioned). ▪ 1/ It is not clear why this would be a concern – "low" would be more appropriate. There appears to be a mixing of exposure concern with hazard concern. ▪ 2a/ Data on human exposure to the component from conventional sources (e.g., does current dietary exposure approach the ADI?) and data on the levels of the component in the novel products would need to be assessed. Maybe point 2 should be a higher level of concern than point 1. ▪ 2- Limited structure/function knowledge is not a problem as such.
Medium	□ Novel component of edible plants and plant-derived foods 1- Not known to be a toxicant, allergen or antinutrient at the level present in the food supply a/ Expressed at higher level in the modified crop than other sources in the food supply, AND b/ Limited and well-characterized modification in structure/function	1 6 7	Comments similar to those for "medium" above were also expressed here, including comments related to the need to consider at risk groups, intended use of the modified crop, and substitution effects. Other comments previously made were also repeated (e.g., unclear distinction between novel vs. existing component of edible plants and plant-derived foods). 0: ▪ Case-by-case assessment is necessary. In some cases "medium" would be acceptable. 1: ▪ Extent of potential hazard for at risk groups where intake is likely to increase

Level	Criteria	Criteria acceptability 0 1 2	Summary and detailed comments
			(e.g., specific antioxidants and smokers).
Medium	OR		▪ The intended use of the modified crop should also be taken into account, i.e. is it used in the same way as the native crop (with potential effect of processing and potential changes on dietary intake)? In addition, the substitution effect on the intake of other beneficial/adverse compounds should be taken into account (e.g., high oleic sunflower oil and olive oil are both rich sources of oleic acid. However, secondary compounds differ between both oil products, with sunflower oil being rich in tocopherols and olive oil in flavonoids).
	2- Known adverse effect in some animals or humans, with an established reference dose (RfD) or ADI or equivalent a/ Expressed in the modified crop at comparable or higher level than in other sources in the food supply, AND b/ Limited and well-characterized modification in structure/function Examples: Expression of omega-3 fatty acids in edible crops (dietary omega-3 fatty acids are primarily derived from marine food sources)		▪ Again, this criterion needs clarification. Does this point apply only to novel food components already present in the food supply but not previously found in the plant product under assessment (e.g., omega fatty acids)? If it also includes novel components that have never been present in food before, this may need a higher level of concern. ▪ b/ There may be the need for some knowledge of physicochemical properties and biological effects of compounds like omega-3 fatty acids. Not sure if that is what is meant here. ▪ 1/ It is not clear why this would represent a hazard. There would not appear to be data suggesting a hazard. Therefore it should be Low. 2: ▪ Having good assays for the identified concerns would be excellent.
High	□ Existing component of edible plants and plant-derived foods 1- Evidence of an adverse effect in some animals or humans, without an established ADI or equivalent, OR 2- Limited or lack of evidence for no adverse effect in animals or limited knowledge of mechanism of toxicity (limited data/unable to evaluate), OR 3- Extensive or inadequately characterized	1 3 10	Some comments previously made were also expressed here, including processing effects and the need for testing. 1: ▪ Must assess safety at expected levels of exposure. ▪ Feeding trial data should be mandatory.

Level	Criteria	Criteria acceptability 0 1 2	Summary and detailed comments
High	modification in structure/function Examples: Use of some phytoalexins as pesticides (phytoalexins are chemically diverse low-molecular plant secondary metabolites believed to have a plant protectant role but their mechanisms of action are not well-understood)		2: ▪ Processing effect needs to be considered (e.g., cashews belong to the poison ivy family, but are edible due to roasting). Processing can change very high risk food to an acceptable food.
High	□ Novel component of edible plants and plant-derived foods 1- Evidence of an adverse effect in some animals or humans, without an established ADI or equivalent, OR 2- Limited or lack of evidence for no adverse effect in animals, or limited knowledge of mechanism of toxicity (limited data/unable to evaluate), OR 3- Extensive or inadequately characterized modification in structure/function, OR 4- Primary product of a completely new gene or a completely novel protein in the host plant or in the diet[3] Examples: Expression in edible crop of conjugated linoleic acid (naturally occurring in mammalian or algal sources; structurally could be classified as trans fatty acids for which there is limited information on the potential for adverse health effects of increased intakes); chimeric gene to produce human milk protein in cereal; protein kinase from a mammalian source for pathogen resistance in the modified crop	2 2 10	Again, the comments emphasize the need for testing. 0: ▪ More information should become available. Sufficient testing should be done before release is allowed. 1: ▪ Safety must be thoroughly assessed at expected levels of exposure. ▪ Feeding trial data should be mandatory. 2: ▪ Assessment should include good assays prior to release and monitoring after release. How processing might affect the food should also be considered.

Table 2-4. Summary and detailed responses.
D. Unintended effects - number of respondents rating the acceptability of listed criteria and comments.
(0=not acceptable, 1=acceptable with modification, 2=acceptable as is; number of respondents=14)

Level	Criteria	Criteria acceptability 0 1 2	Summary and detailed comments
Low	□ Molecular methods are available and used to localize and characterize changes in genetic elements 1- Host plant: not known to be a source of allergen or toxicant, AND 2- Donor organism: not pathogenic (microbial donor organism), and unrelated to toxicity and allergenicity (microbial and other donor organisms not known to be a source of allergen or toxicant), AND 3- Modified trait[3] well-defined mechanisms with <u>all</u> of the following characteristics a/ Single or limited number of enzymes b/ High specificity of target enzyme in terms of substrate and function c/ Limited number of metabolites that are not precursors or intermediates in other pathways in the modified crop Examples: Introduction of Cry protein in corn by rDNA techniques	1 3 10	The need for analysis was evident in the comments as were suggestions for the type of analysis required. <u>1</u>: ▪ Data are required on the changes brought about in the intrinsic DNA (e.g., possible effects of insertion of foreign DNA on an intrinsic gene). ▪ This would be acceptable with respect to intended effects. ▪ 3d/ Add criteria: no fusion proteins detectable or evidence that fusion proteins have neither toxic nor allergenic potential. <u>2</u>: ▪ This technology can best be controlled by monitoring before and after it enters the food chain. ▪ This doesn't mean that standard analyses (e.g. composition analysis and comparison between conventional and GM plant) are unnecessary. ▪ 3c/ "Limited number of metabolites that are not precursors or intermediates in other pathways in the modified crop" is not very clear. Suggestion: Limited number of metabolites and none of theses are precursors or intermediates in other pathways in the modified crop.
Low	□ Molecular methods are not available or used to localize or characterize changes in genetic elements 1- Host plant: not known to be a source of allergen or toxicant, AND 2- Donor plant (same as host plant in mutation): has <u>all</u> of the following characteristics	4 3 7	Respondents who rated this criterion unacceptable considered molecular characterization to be essential in assessing GM crops. One respondent noted the difference that may exist in assessing GM crops modified by rDNA vs. conventional techniques. Suggestions for analysis were offered by respondents who rated this criterion acceptable with modification. <u>0</u>: ▪ The result of modification should always be checked at the molecular level to

Level	Criteria	Criteria acceptability 0 1 2	Summary and detailed comments
	a/ Not known to be a source of allergen or toxicant b/ Edible plants with an established breeding history commercialized and widely consumed for several generations c/ Extensive published scientific literature on the biology and composition, AND 3- Modified trait: well-defined mechanisms with <u>all</u> of the following characteristics a/ Single or limited number of enzymes b/ High specificity of target enzyme in terms of substrate and function c/ Limited number of metabolites that are not precursors or intermediates in other pathways in the modified crop Examples: Point mutation of an endogenous gene encoding an enzyme such as ALS (acetolactate synthase) for herbicide tolerance in rice		reach a low level of uncertainty. ▪ Safety assessments of GMOs necessarily include a molecular characterisation of the transformation event. ▪ For the safety assessment of genetically modified crops, molecular characterisation is one of the basic elements that cannot be done without, although it is realised that in some nations, the regulatory requirements for mutation breeding may not be as rigorous. ▪ 1 and 2a/ "Not known to be" should be changed to "known not to be". <u>1</u>: ▪ It should at least be established that the introduced genetic element is not creating a novel protein or the like. ▪ Molecular analysis for assessing the impact on open reading frames and the potential for novel fusion proteins is typically the first prerequisite. ▪ It is not clear why this is separated from the previous example of 'low'. (Simply on the basis of availability/use of molecular methods?) 3/ How can you tell this in induced mutagenesis? Point mutation should be characterized if the gene of interest is known. <u>2</u>: ▪ Again monitor for effects. ▪ Likelihood of unintended effects is low.
Medium	□ **Molecular methods are available and used to localize and characterize changes in genetic elements** 1- Host plant: a less common source of allergen or toxicant not associated with severe adverse health consequences (requiring immediate medical attention	1 5 8	Similar to previous comments for "low" concern level, the need for analysis was emphasized and the types of analysis deemed necessary were suggested. <u>1</u>: ▪ Analysis of other changes in secondary metabolites is required (using available

Level	Criteria	Criteria acceptability 0 1 2	Summary and detailed comments
Medium	or life threatening in some individuals), OR 2- Donor organism: not pathogenic (microbial donor organism), and a less common source of allergen or toxicant not associated with severe adverse health consequences (microbial and other donor organisms), OR 3- Modified trait: well-defined mechanisms with <u>one or more</u> of the following characteristics a/ Multiple enzymes b/ Low specificity of target enzyme in terms of substrate and function c/ Secondary gene product is precursor or intermediate metabolite in other pathways in the modified crop Examples: Alteration of plant-derived fatty acids involving complex pathways of central metabolism using rDNA technique		techniques). ▪ Level of concern should be <u>high</u> given potential impact of modification (not the status quo) of the properties of the host crop on safety. ▪ This could also be high concern level. Case-by-case assessment is required. ▪ 1 and 2/ should be linked by AND instead of OR. ▪ 3b/ Recombinant proteins with low specificity of target in terms of substrate and function might generate a high level of concern. <u>2</u>: ▪ Acceptable with evaluation of possibility of altered composition.
Medium	□ Molecular methods are not available or used to localize or characterize changes in genetic elements 1- Host or donor plant (host plant in mutation) - a less common source of allergen or toxicant not associated with severe adverse health consequences, OR 2- Modified trait: mechanisms not well-established, or with <u>one or more</u> of the following characteristics a/ Multiple enzymes b/ Low specificity of target enzyme in terms of substrate and function c/ Secondary gene product is precursor or intermediate metabolite in other pathways in the modified crop	3 4 7	Comments were similar to those provided for "low" concern level under the same heading. Principally, respondents who rated this criterion unacceptable considered molecular characterization to be essential in assessing GM crops. One respondent noted the difference that may exist in assessing GM crops modified by rDNA vs. conventional techniques. Suggestions for analysis were offered by respondents who rated this criterion acceptable with modification. <u>0</u>: ▪ Safety assessments of GMOs necessarily include a molecular characterization of the transformation event. ▪ For the safety assessment of genetically modified crops, molecular characterisation is one of the basic elements that cannot be done without, although it is realised that in some nations, the regulatory requirements for mutation breeding may not be as rigorous.

Level	Criteria	Criteria acceptability 0 1 2	Summary and detailed comments
Medium	Examples: Alteration of plant-derived fatty acids involving complex pathways of central metabolism using chemical mutagenesis		1: ▪ Insufficient molecular characterisation is an issue. Increasing complexity of transgenic approach needs careful checking particularly where host/donor is known to have a potential adverse effect in some groups. ▪ Chemical mutagenesis is much more likely to generate unintended effects. Scrutiny for such effects must be intense. ▪ Analysis of other changes in secondary metabolites is required (using available techniques). 2: ▪ Again, it is not clear why the two 'medium' groups were separated.
High	□ Host plant or donor organism 1- Host or donor plant: a source of allergen with severe health consequences, OR 2- Donor organism: known pathogenicity/infectivity (microbial donor organism), or known toxicity/allergenicity (microbial and other donor organisms) and characterization insufficient to eliminate transfer of potentially harmful genetic elements, OR 3- Donor organism: unknown or insufficient knowledge regarding pathogenicity, infectivity, allergenicity, or toxicity Examples: Modification of peanuts (known to elicit severe allergic reactions in susceptible individuals); breeding with a wild variety of a crop species that contains some potentially toxic constituents and that has no history of safe use as food	1 5 8	One common theme that emerged from the comments is that the listed criteria (except for the criterion regarding a modified trait with negative health consequence) might not be sufficient for assigning concern level. Most of the comments focused on the host plant as a source of known toxicity or allergenicity. These comments suggest that distinctions among these sources of allergen and toxicity should be made based on analysis. One respondent comment ed on the donor organism and noted the need to focus on the gene rather than the organism. 1: ▪ The inherent toxic/allergenic substances known in the host merely help to decide the key substances to be analysed (potential risks) rather than saying anything about expected risk level The fact that the host is a source of an allergen means that the focus will be on this allergen. The expectation is not that using this host gives more uncertainties compared to a non-allergen host. ▪ One could argue that since the host is already allergenic the result of modification could be that now it is not allergenic (reduced level of the allergen). ▪ This seems too stringent. If there were more knowledge concerning the traits, the concern level could be reduced.

Level	Criteria	Criteria acceptability 0 1 2	Summary and detailed comments
High			▪ Could be safe but careful evaluation is needed. Evaluation should include effect on levels of allergens. ▪ Example: It would not be necessary to have every gene from peanuts in the high risk category, especially when you discover that the gene has 100% homology to a gene that is from a non-allergenic plant species. ▪ 2/ does not apply if the recombinant protein is neither a toxin nor an allergen although derived from a donor expressing known toxins or allergens. 2: ▪ Obvious.
High	□ Metabolic pathway - complex or new in host plant, with one or more of the following characteristics 1- Multiple enzymes and branch points, regulated by multiple genes, OR 2- Secondary gene product is a modulator of enzyme activity via feedback mechanism, or is a signal for one or more regulatory genes, OR 3- Linked to secondary pathways with the potential for modified biosynthesis of toxic or bioactive secondary metabolite, OR 4- Involves redirection of one or more enzymatic reactions to alter the production of existing compounds, produce new compounds, or mediate the degradation of compounds Examples: Limiting production of ethylene (a plant hormone affecting many metabolic processes) for delayed fruit ripening	2 3 9	Similar to previous comments, respondents emphasized the need for analysis. 0: ▪ The described characteristics do not necessarily create a high level of concern but require a case-by-case assessment. 1: ▪ Assignment of concern level should depend more on the trait under investigation and established evidence rather than using a general rule. ▪ Careful evaluation for unintended effects would be necessary. ▪ Although these factors may increase the chance of unintended effects, other than point 3, they may not necessarily have greater potential for hazard from unintended changes. 2: ▪ Obvious.
High	□ Multiple gene modifications, with the potential for one or more of the following 1- Interactions among the modified genes or gene	4 2 8	The comments suggested that the listed criteria are too generic. This again implies a more specific (case-by-case) approach is preferred.

Level	Criteria	Criteria acceptability 0 1 2	Summary and detailed comments
High	products, OR 2- Gene products sharing common or synergistic mechanisms of toxicity or pathways related to toxicant production Examples: Stacking of genes encoding ribosome-inactivating protein and lectin (a protein that normally cannot penetrate the brush-border membranes of the gut lumen may be transported across the gut membrane through increased membrane permeability or co-endocytosis mediated by lectin). Gene stacking may occur in the same or separate transformation events, including crossing two GM crops using conventional techniques		1: ▪ Stacking effects must be carefully evaluated when interactions are possible. ▪ Gene stacking does not necessarily increase risk. It will be more complex to analyse than simple events, but are less complex than conventional methods of genetic modification like EMS mutagenesis. ▪ 1/ does not necessarily cause high level of concern. 2: ▪ Interactions between new genes/gene products may be the desired outcome of the modification (e.g., to give the desired phenotype). Multiple gene modifications may be of well-understood pathways and therefore may not necessarily be more likely to give rise to hazardous unintended effects. ▪ Obvious.
High	□ Modified trait that has a negative health consequence if stability is not maintained during the commercial lifespan - modified trait not readily assessed Examples: Crops modified to reduce allergenic protein, or to express antigen protein for immunoprotection - changes in levels of antigen proteins are not readily assessed at the field level based on current practices	4 2 8	Two comments for "unacceptable" and "acceptable with modification" would suggest the need for more rigorous testing. One respondent questioned whether such products should be permitted. 0: ▪ This type of effect must be carefully assessed for multiple generations to document stability. Additionally, peanut contains several allergens; so reduction of one might increase the others; thus must be evaluated. 1: ▪ Questionable whether one should work on this at all in a commercial setting. ▪ This seems more like a problem for processing than regulation. For these traits, every food batch will need to be assessed. Something that can not be assessed should not be sold. 2: ▪ Statement acceptable but examples perhaps not the best.

Table 2-4. Summary and detailed responses.
E. Method of aggregation of component categories (Number of respondents=14).

Reasonableness of method of aggregating component categories	Number of responses	Summary and detailed comments
No	7	The "no" responses were related to specific aspects of the aggregation, including the "special case" and "reject". There appears to be support for the general direction of the proposed scheme with suggestions for modification. However, there was also a concern for the loss of differentiation by aggregation. The need for testing was reiterated,
Reasons		
Generally reasonable - modifications needed		▪ In general the categories are very reasonable. Indications where thinking may be required are provided. ▪ It seems reasonable to expect that the level of testing would increase from I toV and from A to B in the order indicated.
Testing essential		▪ Testing should be the basis of rejection, rather than theoretical risk. Practically speaking, the possibilities indicated in the scheme would likely not be expected commercially because business risk is too high. ▪ Products should always be assessed rigorously, even in a case of "low" concern. The classification scheme should not be a "fast lane" for product approval.
Differentiation lost in aggregation		▪ The aggregation step has no added value, in the sense that there are still nine to ten different labels, while all relevant content information of the components is lost. ▪ Aggregation of concern levels cannot cover all possible cases. Guidance on type and level of testing for each concern level might be more adequate.
Case-by-case approach		▪ Risk assessment is a case-by-case issue. It may, on occasions, be surprising what can arise especially in the light of new and emerging evidence. ▪ The suggested scheme seems very idealistic. In reality, it might be very difficult to allocate the individual components into either low, medium, or high. Therefore, a case-by-case approach, founded on a weight of evidence base should work better. However, it is possible that the aggregate level could be used as an indicator index of concern.
Special case		▪ Disagree that special case should be level V for all cases. This removes the differentiation based on the classification scheme. A special case may involve a food ingredient that has very low exposure. There should be an aggregate differentiation within this group as there is within the other groups.

Reasonableness of method of aggregating component categories	Number of responses	Summary and detailed comments
Reject		▪ "Reject" is a risk management option. Apparently, the classification scheme is about the outcomes of scientific risk assessment. Therefore, "reject" should not be made at this stage.
Not rated	3	Reasons for not rating included difficulty in following the rationale of some aspects of the aggregation.
Reasons		
Difficult to follow		▪ The aggregation section is a bit difficult to follow. There is concern about combining low and medium unintended effects together (II-A, III-A) especially where intended effects are low (II-A). Low (intended) and low (unintended) infers less risk than low (intended) and medium (unintended). This is because unintended effects are related to the unexpected. ▪ Don't feel qualified to respond.
Yes	4	One comment was provided with a suggestion for change for "low-1" (exposure) due to concerns about unintended effects.
Reasons		
Generally reasonable, with one exception		▪ Reasonable on the whole, except for exposure level (low-1). Unintended effects in the food cannot be ruled out even if novel proteins are not expressed in the food.

Table 2-5. **Product examples - comments by product.**

Product A

Rice - herbicide (imidzolinone) tolerance - endogenous enzyme made insensitive to herbicide by chemical mutagenesis.

Number of respondents receiving this product example[a]: 3
Number of respondents completing this part of the questionnaire: 3

	Agreement[b]		Detailed comments
	Yes	No	
Exposure Level assigned[c]: H	3	-	
Potential hazards from intended effects Level assigned: L	3	-	
Potential hazards from unintended effects Level assigned: M	1	2	**No**: ▪ The difference between an induced and a spontaneous mutation is largely the frequency of occurrence. There is no cost benefit ratio as part of this assessment (e.g., reduced vitamin level in wheat made resistant to Fusarium head blight with reduced exposure to vomitoxin). ▪ It is highly likely that the trait introduced into rice by EMS mutagenesis would only reach the market place through introgression into an elite variety. This will involve extensive backcrossing. Any breeder undertaking such work will be able to map the new trait and will be able to give an indication of the extent of modification of genetic material surrounding the new trait (unintended effects). This is more appropriate than checking the actual composition of the grain.
Aggregate concern level Level assigned: II-A	2	1	**No**: ▪ The rating is too severe. One should take into account the track record of the technology. **Yes**: ▪ The question remains: since natural mutants exist and they are used, how would substantial equivalence be applied? Does it mean that every new variety (irrespective of its origin) will have to be tested like this?
Is information provided sufficient?	2	1	**No**: ▪ See comments above - no cost benefit rationale.

[a] Total number of questionnaires returned=14. [b] Agreement with levels assigned by researcher. [c] Level assigned by researcher.

Product B

Soybean - high oleic acid by silencing an endogenous gene encoding an enzyme that converts oleic acid to linoleic acid via biolistic transformation.

Number of respondents receiving this product example[a]: 2
Number of respondents completing this part of the questionnaire: 1

	Agreement[b]		Detailed comments
	Yes	No	
Exposure Level assigned[c]: H	1	-	
Potential hazards from intended effects Level assigned: L	1	-	
Potential hazards from unintended effects Level assigned: M	1	-	
Aggregate concern level Level assigned: II-A	1	-	
Is information provided sufficient	1	-	▪ Key issue in this case is exposure levels in commonly eaten products. Evidence is needed on the effect of exposure on specific general population or at risk groups. In terms of unintended effects non polar fractions analysis should cover most eventualities. In risk assessment, overall pathway integration will be examined, as well as whether changes in other compounds could influence intake levels.

[a] Total number of questionnaires returned=14. [b] Agreement with levels assigned by researcher. [c] Level assigned by researcher.

Product C

Maize - Bt and herbicide (glufosinate) resistance by introducing bacterium-derived protein (Cry1Ac) and enzyme that inactivates the active compound in the herbicide via biolistic transformation.

Number of respondents receiving this product example[a]: 3
Number of respondents completing this part of the questionnaire: 2

	Agreement[b]		Detailed comments
	Yes	No	
Exposure Level assigned[c]: H	1	1	**No**: ▪ Exposure assessment should include assessing the level of the expressed proteins, possibly as a category separate from the exposure to the food.
Potential hazards from intended effects Level assigned: M	1	1	**No**: ▪ Potential hazard of the individual traits should be separated because testing would be specific for the trait. The potential hazard of Cry1Ac, based on current evidence, is low. Having tissue levels of Cry1Ac, especially in multiple sites/growing seasons does demonstrate the "fate" of the protein sufficiently to estimate potential hazard (when taken together with information of the lack of hazard). The potential hazard from glufosinate ammonium and metabolites may be medium.
Potential hazards from unintended effects Level assigned: L	2		**Yes**: ▪ Localizing and characterizing changes cannot be done 100% because one can never know for sure what has happened in the whole genome. This is comparable to traditional breeding.
Aggregate concern level Level assigned: III-A	1	1	**Comments related to further testing** **No**: ▪ It is not sure how biochemical/functional equivalence of novel protein expressed in modified crop to proteins in toxicity testing would be used to establish an ADI. No prior animal toxicity data on proteins was presented (although it is in the range of 2-5 g/kg in mice). Need more explanation on testing. ▪ Maize has very low allergenicity, and it is primarily due to one or two proteins (based on current evidence). To assess allergenicity, the most direct way is to measure the levels of the most likely allergen (LTP). ▪ The use of proteomics to assess expression of new proteins has not been done before, and is much more difficult than one would assume, unless the level of detection, or expression, is set pretty high.

	Agreement[b]		Detailed comments
	Yes	No	
			Alternatively, one evaluates the molecular insert (or mRNA) to indicate whether there are any potential fusion proteins. **Yes**: ▪ Assessment of unintended effects should follow the OECD guidelines (consensus document on maize). In EU the assessment of using the herbicide is not connected to the GM approval. This should be a separate issue and left out of this scheme. Otherwise one should consider shift in cropping, management etc. as a consequence of the modification as well.
Is information provided sufficient?	1	1	**No**: ▪ A bit more known information would have helped in the example.

[a] Total number of questionnaires returned=14. [b] Agreement with levels assigned by researcher. [c] Level assigned by researcher.

Product D

Tomato - delayed ripening (by limiting ethylene production with the introduction of a bacterium-derived enzyme) via Agrobacterium-mediated transformation.

Number of respondents receiving this product example[a]: 3
Number of respondents completing this part of the questionnaire: 3

	Agreement[b]		Detailed comments
	Yes	No	
Exposure Level assigned[c]: H	3	-	
Potential hazards from intended effects Level assigned: M	3	-	
Potential hazards from unintended effects Level assigned: H	3	-	
Aggregate concern level Level assigned: III-B	3	-	
Is information provided sufficient?	2	1	**Comments related to further testing** **No**: ▪ Animal testing with the whole product should be considered if additional safety assurance would be needed to account for unintended effects (although the sensitivity of animal models may not be as high as analytical methods to detect such effects). The use of the whole product (genetically modified tomato) and its altered characteristics should also be taken into account (e.g., long-ripening tomatoes have been used for tomato processing because of improved viscosity characteristics). **Yes**: ▪ It is unclear if recommendations on the testing of each aggregate concern level have already been developed or if they are yet to be developed. The current best international methods for assessing GM food safety include toxicological testing of novel proteins and compositional analysis as routine to determine if there are any unintended changes on the levels of key nutrients and toxicants (i.e. even on a product like Bt cotton).

[a] Total number of questionnaires returned=14. [b] Agreement with levels assigned by researcher. [c] Level assigned by researcher.

Product E

Potato - introduction of Galanthus nivalis (snowdrop)-derived lectin for pest resistance via Agrobacterium-mediated transformation.

Number of respondents receiving this product example[a]: 2
Number of respondents completing this part of the questionnaire: 2

	Agreement[b]		Detailed comments
	Yes	No	
Exposure Level assigned[c]: H	2	-	
Potential hazards from intended effects Level assigned: H	2	-	
Potential hazards from unintended effects Level assigned: M	1	-	**Yes and No**: 1 ▪ Depends on the quality and rigour of the molecular data. If the insert and the insertion site both are sequenced and no open reading frames or gene fragments are detected, then the assigned levels are acceptable.
Aggregate concern level Level assigned: IV-A	1	1	**No**: ▪ See comment above.
Is information provided sufficient?	1	1	**No**: ▪ Besides the quality of the molecular data (see above), more details of toxicology and allergenic potential of GNA should be provided.

[a] Total number of questionnaires returned=14. [b] Agreement with levels assigned by researcher. [c] Level assigned by researcher.

Table 2-6. Comments on emerging analytical techniques (Number of respondents=8).

Theme	Detailed comments
Profiling techniques	▪ It is not clear to me at this time that proteomics or metabolomics will be useful in assessing most GM crops, where specific genes, coding for enzymes, storage proteins etc., will help in assessing potential risk. The degree of variation in crops, in different environments or years is sufficiently great that proteomics and metabolomics are far too complex and results ill-defined to be useful. Those methods may be reasonable for assessing GM crops with added transcription factors or interspecific/intergeneric or induced mutational crops, where the specific targets are unknown. Otherwise the science should be quite directed.
	▪ I am not aware of novel emerging analytical technologies, but compositional analysis now is done with HPLC and GC techniques and these will be still relevant and improved in future. Also the techniques used now to isolate and characterize DNA and proteins like PCR, ELISA and SDS-PAGE will be further improved.
	▪ Transcriptomics, *i.e.* gene expression analysis (if not yet considered part of "functional" genomics).
	▪ There is a need for harmonization and validation of the "omics" technologies, such as, sampling and analysis. In addition, there is a need for databases on background variations in order to be able to assess the relevance of differences observed in the comparison of a genetically modified crop with its appropriate counterpart
Toxicology and allergenicity	▪ Sensitive animal models for detecting toxic and anti-nutritive effects.
	▪ Sensitive biomarkers using DNA microarrays to identify early biomarkers for specific toxicological, nutritional, or disease endpoints to increase the sensitivity of both in vivo and in vitro test systems. Validation of these biomarkers as indicators of toxicity and clinical outcome (3).
	▪ Testing strategy for whole foods by combining in vivo animal models, in vitro toxicological systems, the use of specific cell line cultures and ex vivo systems, linked to the use of selected profiling methods (2)[a].
	▪ Animal models for characterization of allergenic potential and methods of targeted serum screening. Validated model systems are needed to more reliably assess allergenic potential (2).
Composition database and analysis	▪ A much richer database of the range of typical composition of conventional varieties of crops (and animals) must be created for comparative purposes.
Monitoring	▪ Monitoring system should be in place for after release (e.g., Kiwi fruit was a new food when first introduced. Its allergenicity to some people was known after introduction but it was not regulated. Upfront testing was not needed).

Theme	Detailed comments
Testing of crops modified by conventional methods	▪ For conventional breeding, this is a complex question. Whilst there may be no a priori reason to extend any new approach, (including "omics" approaches) to conventional breeding (the approach of exploring mutation breeding or conventional breeding which exploits germplasm diversity in wild relatives to introgress new genes for quality /disease resistance etc.), one may introduce undesirable effects, particularly where secondary metabolites are concerned. This is a policy which would be based on the belief that such approaches are inherently more dangerous than (merely) crossing one variety with another. How would such a policy be introduced and would it be evidence based? ▪ With regard to GM, it can be argued that the key issues are the genes inserted (entirely novel to the crop?), the trait established (e.g., whether the changes would require a risk assessment under a (non GM) novel food, or that the plant would be designated a new variety (under UPOV rules) due to its distinctiveness).
Extent of testing	▪ The question is how far one should analyse. The crops and the food from it should be safe. With new techniques the abilities to zoom in will improve and one is likely to find (traces of) substances which might prove to be a hazard. The next question is: are those levels a danger and how far one should analyse. The key is to identify the problem to be addressed and then develop better tools to find problem foods (e.g., use the best assays to identify allergenic foods if one is concerned about allergies).
Other	▪ Bioinformatic approaches. ▪ No further comment/ cannot think of any (2).

[a] Number of respondents with similar comments.

Table 2-7. General comments (Number of respondents=8).

Theme	Detailed comments
Testing options essential	▪ It is hard to judge the scheme until there is a testing scheme that goes with the grading scheme that would be applied in various scenarios. One has to be realistic about what can be tested and the conclusions that may be drawn from such tests. Be cautious about developing a scheme that is based on precautionary principle that blocks the development of new products. ▪ It is not clear that examples help, unless the examples would be used to help define alternative testing schemes (which is reasonable). ▪ Concerns are possible with several of these scenarios but often these concerns can be carefully evaluated pre-market. If done well, the seriousness of the concern can be addressed and appropriate decisions can be made.
Component categories and aggregation	▪ Exposure: it appears that you have mixed some consideration of exposure with the concern for hazards, especially in the "low" case. This seems counter to your classification scheme. An exposure assessment should not only take into consideration the consumption of the whole food, with the presence or absence of the protein (or metabolite) in the food, but should also evaluate the level of expression of the protein. Is it less than a few ppm? If so, and if it does not have any known adverse effects or likelihood based on evidence (characterization), then the concern should be low. ▪ Aggregation: it is not clear that your scheme allows for the above in the aggregate, because it does not provide that as a discrimination level in exposure. There is no good evidence that any protein (unless it is a major allergen or toxin) has a potential adverse effect when present in the ppm range in a food. That may not be true for some metabolites, allergens or toxins.
Product scope	▪ The scheme seems quite specific for transgenic plants, as suggested by the prerequisite and core information in the questionnaire. ▪ It is not clear that transgenic crops can or should be assessed by similar methods. (How can one know specific genes, proteins, etc. for induced mutations, interspecific or intergeneric crosses?). Crops modified by induced mutations (interspecific or intergeneric crosses) should have more general and thorough requirements (proteomics, genomics, metabolomics) than crops modified by rDNA. Compared to rDNA, far less is known about the changes in induced mutations which are also likely to be less predictable. ▪ In the Scope description it is said that the Risk-Based Classification Scheme should include a range of conventional and modern techniques of genetic modification but this is not apparent in the questionnaire. Including conventional ways of crop enhancement in the scheme is very challenging and will likely evoke resistance.

Theme	Detailed comments
	▪ Any thoughts on extending this to "novel foods" in general? This would extend its usefulness. ▪ Plants which produce pharmaceutical products or plants for increasing nutrient availability. Plants which potentially modify soil functionality (e.g., for bioremediation).
Usefulness of the scheme	▪ Some of the questions/requirements can only be met late in the development of a GM crop or newly crossed crop. Other aspects related to known safety should be identified early in development, as assessment may take 2 or 4 years. Because the "useful" or legally protected life of these crops is quite limited, the assessment and testing scheme should be compressed as much as possible to maximize the benefits of the crop. It is not clear when assessment using this classification scheme would be completed.
Terms used	▪ The column heading 'Potential Hazards' could be made clearer if it was changed to: "Level of Uncertainty of Potential Hazards". ▪ The definition and use of the term "GM" for the scheme is not the conventional one.
Case-by-case	▪ The method seems reasonable. However, it is always appropriate to judge each new GM food on a case-by-case basis, rather than as part of a category of foods. ▪ It is difficult to generalize about concern levels, particularly about what would constitute "low" concerns, since the assessment will be on a case-by-case basis, given the wide variation in modifications, crops, human consumption, toxic characteristics of the modified compounds etc. Products should be assessed rigorously, even in the case of "low" concerns.
Future product examples	- Similar to Starlink corn (pepsin stable protein with no other indication of hazard and expressed at low levels). - Transcription factor - Reduced allergenicity product - Addition of a major seed storage protein.
Other	▪ I think you have done a great job on this. I think it would be very interesting to study this retrospectively comparing a GM vs. mutagenised crop which we now have data on a safe history of use. This will really set things in a sound context. ▪ Nothing to add to the above comments.

Université d'Ottawa · University of Ottawa

Cabinet du vice-recteur à la recherche
Office of the Vice-Rector, Research

Annex 2-1

HEALTH SCIENCES AND SCIENCE RESEARCH ETHICS BOARD

CERTIFICATE OF ETHICAL APPROVAL

This is to certify that the University of Ottawa Health Sciences and Science Research Ethics Board has examined the application for ethical approval for PHASE 1b of the research project entitled **A Risk-Based Classification Scheme for Testing Genetically Modified Foods (file H 08-04-06)** submitted by Eunice Chao, who is supervised by Daniel Krewski, both of the Institute of Population Health. The Board found that this research project met appropriate ethical standards as outlined in the Tri-Council Policy Statement and in the Procedures of the University of Ottawa Research Ethics Boards, and accordingly gave it a Category 1a (approval). This certification is valid for one year from the date indicated below.

Rita D'Alessandro
Protocol Officer for Ethics in Research
For the Chair of the Health Sciences and Science REB

August 27, 2004
Date

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Annex 2-2. Information/recruitment letter

Date

Dear [name]:

I am writing to inform you of a research project on a *risk-based classification scheme for testing genetically modified foods* that is currently underway at the University of Ottawa. I am seeking experts to review the logical soundness of a *draft* scheme developed in part in response to an expert consultation conducted early this year.

Please feel free to respond if this project is of interest to you personally, or if you would like to suggest the names of colleagues whom you think would be interested in this topic.

Project Objective

The primary objective of the proposed research is to develop and evaluate a risk-based classification scheme for testing foods that are derived from crops genetically modified by a range of methods, including conventional (e.g., induced mutation, tissue culture) and more modern (e.g., recombinant DNA) techniques. The proposed scheme aims to (1) differentiate and classify genetically modified (GM) foods based on levels of concern about potential health risks and attendant uncertainty, and (2) guide the application of testing methods for GM foods in proportion to their potential to produce human health risks.

Expertise

I am seeking the guidance of a multidisciplinary group of experts who have expertise in health risk assessment of GM foods. Participants will be selected to reflect a balance of expertise in relevant disciplines, including plant sciences, toxicology, immunology, biotechnology, food/nutritional sciences, and health risk assessment.

Consultation 2

In this second expert consultation, participants will be asked to complete a self-administered questionnaire, by commenting on specific aspects of the draft scheme:

- the relevance of prerequisite and core information required to use the scheme
- the acceptability of criteria for assigning concern levels for the 3 component categories of the scheme (exposure, potential hazards from intended effects, and potential hazards from unintended effects)
- the reasonableness of the method of aggregation of the 3 component categories
- the appropriateness of the component and aggregate concern levels assigned to a sample product

I estimate that the questionnaire will take approximately one hour to complete. Individuals who provide detailed comments may spend more time. Normally, a respondent will have 3-4 weeks to return a completed questionnaire.

All data collection and dissemination will be conducted in accordance with confidentiality and ethics guidelines for research involving human subjects.

The research is being conducted as part of a doctoral thesis at the University of Ottawa Population Health PhD Program. The principal researcher is Eunice Chao, doctoral candidate, who is working with Dr. Daniel Krewski, Professor and Director, McLaughlin Centre for Population Health Risk Assessment, Institute of Population Health, University of Ottawa. The research project is supported by the McLaughlin Centre through a grant from the R. Samuel McLaughlin Foundation. No industry funding is involved in this project.

Contact Information

I will contact you by phone within the next week to discuss your interest and availability for participation and to answer any questions that you may have. Suggestions for other potential participants are welcome.

If you prefer to provide your response by e-mail to Eunice Chao (echao097@uottawa.ca), that would also be much appreciated. Any information on your scientific background that you are willing to provide (a short bio, full CV, or a simple statement of interests) would be helpful.

Thank you for your consideration.

Sincerely,

Eunice Chao, MSc
Doctoral candidate and project researcher
Population Health PhD Program
University of Ottawa

echao097@uottawa.ca

Tel: (613) 562-5800 ext 2367
Fax: (613) 562-5380

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Université d'Ottawa · University of Ottawa

Institut de recherche sur la santé des populations | Institute of Population Health

Annex 2-3. Project Information for Research Participants

Name of researcher(s): Eunice Chao

Institution, Faculty, Department: University of Ottawa, Population Health PhD Program

Name of supervisor: Dr. Daniel Krewski

Institution, Faculty, Department: University of Ottawa, Faculty of Medicine, Department of Epidemiology and Community Medicine, Institute of Population Health

Project Description

The research project "A risk-based classification scheme for testing genetically modified foods" is conducted by Eunice Chao of the Department of Population Health PhD Program at the University of Ottawa under the supervision of Dr. Daniel Krewski. The purpose of the research is to develop and evaluate a risk-based classification scheme for the testing of foods that are derived from crops genetically modified by a range of methods. The proposed scheme aims to (1) differentiate and classify GM foods based on levels of concern about potential health risks and attendant uncertainty, and (2) guide the application of testing methods for GM foods in proportion to their potential to produce human health risks. The research will be conducted in several phases. You are asked to participate in expert consultation 2 for the project.

Participation in Expert Consultation 2

Participants of expert consultation 2 will be asked to review the logical soundness of a *draft* scheme developed in part in response to an expert consultation conducted early this year, by completing a self-administered questionnaire and commenting on specific aspects of the draft scheme:

- the relevance of prerequisite and core information required to use the scheme
- the acceptability of criteria for assigning concern levels for the 3 component categories of the scheme (exposure, potential hazards from intended effects, and potential hazards from unintended effects)
- the reasonableness of the method of aggregation of the 3 component categories
- the appropriateness of the component and aggregate concern levels assigned to a sample product

The questionnaire will take appropriately one hour to complete. Individuals who provide detailed comments may spend more time. Normally, a respondent will have 3-4 weeks to return a completed questionnaire.

Confidentiality and Anonymity

The contents of the completed questionnaires will be used only for the purposes of developing a risk-based classification scheme for testing genetically modified foods and that a research participant's confidentiality will be respected through the following actions taken by the researcher.

All of the data collected will be tabulated and maintained by the researcher in accordance with confidentiality standards for research involving human subjects. The data will be accessible only to the researcher in a locked filing cabinet and a password protected personal computer on the premises of the Institute of Population Health, University of Ottawa.

Anonymity of a participant's individual views and responses will be protected. Questionnaires will be coded for follow-up purposes and the participant's name will not appear on the completed questionnaires. While group results of the surveys at the aggregate level will be published, and discussed within the research team, and at conferences, no names will be linked to individual views or responses in data analysis, reports or in presentations.

In order to acknowledge participants' contribution to the research project and to make the expert consultation process transparent and credible from a research perspective, their names and affiliations (the organization where one works or which one represents) will be included in a list as an appendix to the doctoral thesis. Only one combined list of participants for all phases of the research will be published without identifying the specific phase to which they made a contribution. Please see the request at the end of the page.

Research participants agree not to disclose or publish any aspects of the risk-based classification scheme developed by the researcher except with the express consent of the researcher.

Voluntary Participation
There is no direct individual benefit from participating in the study and from answering the questionnaire. All costs associated with participation in the project (Canadian postage and long-distance phone calls) will be born by the McLaughlin Centre for Population Health Risk Assessment. Return postage for participants from countries outside Canada will be paid in the form of international postal reply coupons, if requested.

Participation is completely voluntary. A participant is free to withdraw from the project at any time, before or during a survey, refuse to participate and refuse to answer certain questions.

A SUBMITTED QUESTIONNAIRE IMPLIES CONSENT.

Any information about the rights of a research participant may be addressed to **Protocol Officer for Ethics in Research, University of Ottawa, 550 Cumberland Street, Tabaret Hall, Room 159, Ottawa, Ontario, Canada, K1N 6N5, tel.: (613) 562-5387, e-mail: ethics@uottawa.ca**.

Any questions about the conduct of the research project may be directed to the researcher or his or her supervisor at the coordinates listed below.

Eunice Chao: **Telephone number:** 1-613-562-5800 ext 2367 **Fax number:** 1-613-562-5380

E-mail address: echao097@uottawa.ca

Daniel Krewski: **Telephone number:** 1-613-562-8261 **Fax number:** 1-613-562-5380

E-mail address: dkrewski@uottawa.ca

Address: McLaughlin Centre for Population Health Risk Assessment
Institute of Population Health, University of Ottawa
1 Stewart Street, Room 318B, Ottawa, Ontario, CANADA K1N 6N5

Research project: A risk-based classification scheme for testing genetically modified foods

If you have not already done so, please indicate your preference (yes / no) for acknowledging your contribution to the project. Please provide the following information to Eunice Chao with your completed questionnaire.

Research participant's name:

- □ Yes, I agree to have my name and affiliation included in the researcher's thesis
- □ No, I do not wish my name and affiliation to be included in the researcher's thesis

Annex 2-4. Questionnaire

Annex 2-4. Questionnaire

Risk-Based Classification Scheme for Testing Genetically Modified Foods - Expert Consultation 2

Risk-based classification scheme for testing GM foods: A risk-based classification scheme (RBCS) as envisioned in this research is intended to be a tool to efficiently differentiate genetically modified (GM) foods that require more extensive testing and regulatory oversight from those that do not.

- **Focus**: potential human health risks from dietary exposure to foods derived from GM crops
- **Scope**: include a range of conventional (e.g., induced mutation, tissue culture, interspecific and intergeneric crosses) and modern (e.g., cell fusion, in *vitro* and recombinant deoxyribonucleic acid - DNA) techniques of genetic modification to intentionally change the heritable traits of food crops
- **Intended uses**:
  - classify GM foods based on levels of concern about potential health risks and attendant uncertainty
  - guide the application of testing methods in proportion to their potential to produce human health risks
- **NOT intended** to be used as a health risk assessment tool *per se*, nor for decision-making regarding the approval or disapproval of a GM food

When completed, the RBCS will consist of 4 elements:

1) Prerequisite and core information required to use the scheme
2) Concern levels assessed separately for 3 component categories: a) dietary exposure, b) potential hazards from intended effects, and c) potential hazards from unintended effects
3) Aggregate concern levels that combine the 3 component concern levels using a matrix approach
4) Recommended types of testing for each aggregate concern level in proportion to the degree of concern and uncertainty and with the necessary flexibility to address specific issues identified in the assignment of concern levels for the individual component categories

The **objective of this expert consultation** is to conduct a preliminary assessment of the logical soundness of the first 3 elements of a draft scheme.

There are 4 parts to this questionnaire:

- **Part I** - **prerequisite and core information** required to use the scheme
- **Part II** - **component categories** comprising the scheme
- **Part III** - **aggregate concern levels** of the scheme
- **Part IV** - **product examples** with assigned concern levels using the scheme

Space is provided in each part for comments. Legible writing would be appreciated. Please use additional paper if necessary.

Development of this draft scheme: This consultation is the second step of Phase 1 in the development and evaluation of a RBCS for testing GM foods. This draft scheme is developed in part based on response to a previous expert consultation using a self-administered questionnaire on a list of factors and criteria considered relevant in health risk assessment of GM foods. The factors and criteria were identified from a review of the scientific literature on major approaches to genetic modification of plants and their effects on plant genome structure and gene expression and function, as well as national and international guidelines on safety assessment of GM foods, and well-established concepts of health risk assessment.

Future phases: Detailed evaluation of the utility of the scheme will be conducted using a panel of reference products with a range of characteristics, as well as the development of graded testing options.

I.D. number:

Part I – Prerequisite and Core Information Required to Use the Risk-Based Classification Scheme (RBCS)

For a RBCS to be useful in assessing the nature and level of testing needed for GM foods, the establishment of prerequisite and core information will be essential. Prerequisite information will be met by all GM crops to be assessed using the RBCS and will not provide a basis for differentiation. In contrast, core information is expected to have discriminating value in classifying GM crops and derived foods on the basis of their health risk potential. It is proposed that core information be:

- based on information required in developing the modified crop
- amenable to be easily assessed

Listed below is a proposed list of prerequisite and core information. Please

- **rate the relevance of the individual components of prerequisite and core information (yes/no); if not relevant, why not**
- **identify other information that should be included as prerequisite or core information**

Prerequisite and Core Information	Relevant		Comments (if not relevant, why not; what is missing)
	Yes	No	
Prerequisite information			
1. Stable expression of new trait for several generations, confirmed by biochemical and molecular analysis, where applicable			
2. Application of good laboratory practices and best practices in all aspects of testing, transformation and selection procedures[1]			
3. For crops modified by rDNA techniques: well-characterized sequences for transfer before and after transformation[2]			
4. Substantiation for the efficacy of the new trait			
5. Indication of the intended benefit and for whom			
Core information			
6. Unmodified host plant			
a) History of safe use as food, or intended food use if different from historical use			
b) Well-established knowledge of the biology and composition of the plant, including toxicants, allergens, nutrients, and antinutrients			

Prerequisite and Core Information	Relevant		Comments (if not relevant, why not; what is missing)
	Yes	No	
7. Donor organism, where applicable			
a) Use or presence in food supply			
b) Knowledge of the biology and composition of the donor organism, including toxicants, allergens, nutrients, and antinutrients			
c) Known or suspected adverse human health effects			
8. Gene function and related reactions or pathways that are the target of the modification, or that result in the selected modified trait			
a) Prior knowledge or experimental data			
9. Primary gene product (protein/enzyme)[3]			
a) Tissues and levels of expression in modified and unmodified crop			
b) Known or suspected adverse human effects			
c) Structure and physico-chemical properties			
10. Where applicable, secondary gene products (direct product and metabolite of modified enzyme)[3]			
a) Tissues and levels of expression in modified and unmodified crop			
b) Where the primary or secondary gene product is a novel component in the host plant: metabolic fate in the modified crop			
c) Known or suspected adverse human effects			
d) Structure and physico-chemical properties			

[1] Examples of relevant publications: U.K. ACRE. Guidance on Best Practice for the Presentation and Use of Molecular Data in Submission to the Advisory Committee on Releases to the Environment; Doyle, JJ and Persley, GJ. Enabling the Safe Use of Biotechnology - Principles and Practice. Environmentally Sustainable Development Studies and Monographs Series No. 10, The World Bank, Washington, D.C. Good practices also apply to the use of viral sequences and of antibiotic resistance genes as marker genes in food crops - see http://binas.unido.org/dtree and van den Eede et al. Food and Chemical Toxicology 2004; 42:1127-1156, respectively. [2] See for example: USDA-APHIS requirements for biotechnology crops (http://www.aphis.usda.gov/brs/notgen.html); Canada-U.S. Bilateral on Agricultural Biotechnology - Appendix I: Molecular Genetic Characterization Data, 1998. [3] Gene product and direct product of enzyme include protein/enzyme and its metabolite resulting from targeted modification by rDNA techniques as well as undirected modification by conventional methods. In the case of rDNA technique, product of novel marker gene should also be considered.

General comments (Part I):

1. In addition to new techniques in genomics, proteomics, and metabolomics that are being developed to complement current assessment of potential health risks from unintended effects in GM crops, what other emerging analytical technologies may be considered in guiding judicious toxicological testing, particularly for assessing modifications involving complex or new metabolic pathways, or modifications using conventional (non rDNA) techniques?

2. Other types of information that should be included:

a) Prerequisite information

b) Core information

3. Other comments

Part II - Component Categories

Three component categories will make up the risk-based classification scheme:

A. Exposure
B. Potential hazards from intended effects
C. Potential hazards from unintended effects

Three levels of concern are proposed for each component category: low, medium, high. The criteria for each level of concern are included for each component category in the sections to follow. Examples are provided to illustrate the application of the criteria. They are based on publicly available information on approved or experimental products and do not necessarily reflect their potential for commercialization.

You are asked to assess the acceptability of the criteria for classifying the concern levels for each component category. Please provide your ratings collectively for the group of criteria given within the same row, using the scale provided, as illustrated in the following sample response.
0 = Not acceptable 1 = Acceptable with modification 2 = Acceptable as is

SAMPLE RESPONSE ONLY

Level	Criteria	Criteria Acceptability	Comments and Suggestions (for ratings 0 and 1)
Low	□ **No new primary or secondary gene product**	0 1 **2**	
Low	□ **Existing component of edible plants and plant-derived foods** 1- Not known to be a toxicant, allergen or antinutrient at the level expressed in crop plants with a history of safe use as food a/ Expressed at levels comparable to those in crop plants, AND b/ Limited and well-characterized modification in structure/function (e.g., point mutation or site-directed mutagenesis)	0 **1** 2	b- not necessarily relevant for non-protein substances
Low	□ **Novel component of edible plants and plant-derived foods** 1- Well-characterized modification of target gene (e.g., site-directed mutagenesis), AND 2- Prior knowledge of toxicity: one or more of the following a/ Known mechanism for toxicity not applicable to humans, or extensive evidence for no adverse human health effects, OR b/ Established tolerance limit, such as maximum residue level (MRL) or equivalent, and level expressed is below MRL, OR c/Level expressed is below threshold of toxicological concern (TTC) based on aggregate exposure estimates, OR d/ History of safe use in relation to food at the level expressed in the modified	0 **1** 2	2c- aggregate exposure estimate would not be part of core information obtained for R&D; for this criterion to be useful, we need a practical tool for estimating aggregate exposure

Level	Criteria	Criteria Acceptability	Comments and Suggestions (for ratings 0 and 1)
	crop		

II-A. Exposure - Dietary

Dietary exposure means consumption by humans of food or ingredient derived from the edible part of a modified crop. Edible part refers to that anatomical part of the modified crop that is intended for consumption as food or ingredient (e.g., the flesh portion of fruits; seed portion of cereals), subject to normal level of mixing of edible and non-edible parts during harvest.

Criteria: Three levels of dietary exposure at the food level are based on a combination of qualitative criteria: (1) the potential for a substance of concern in the modified crop entering the food supply, (2) the intended use of the food or ingredient derived from a modified crop, and (3) the quantity and frequency of consumption of the food and derived ingredient.

In your judgment, are the criteria acceptable for each level of concern regarding *exposure*? Please use the following scale in rating the acceptability of the criteria. For ratings 0 and 1, please comment and provide suggestions.

0 = Not acceptable 1 = Acceptable with modification 2 = Acceptable as is

Level	Criteria	Criteria Acceptability	Comments and Suggestions (for ratings 0 and 1)
Low -1	□ Site/tissue-specific modification - primary and secondary gene products expressed, metabolized, and stored only in non-edible part of modified crop, and - food consumed in limited form as highly refined ingredient and well-defined chemical entity **Examples**: Refined oils from oilseed rape or cottonseed that expresses a novel protein only in the non-edible parts of the plant	0 1 2	
Low - 2	□ Site/tissue-specific modification - primary and secondary gene products expressed, metabolized, and stored only in non-edible part of modified crop, and - food consumed fresh, processed, or as ingredient in wide spectrum of applications **Examples**: Fruit or kernels of a modified crop that expresses a novel protein only in the non-edible part of the plant	0 1 2	
Medium	□ Food consumed in limited form as highly refined ingredient and well-defined chemical entity, or □ Common food or food ingredient consumed in very small amounts, or □ Rare food, infrequently consumed	0 1 2	

Level	Criteria	Criteria Acceptability	Comments and Suggestions (for ratings 0 and 1)
	Examples: Refined oils from oilseed rape or cottonseed; spices, seasonings; exotic fruits		
High	□ Common food, regularly or variably consumed in appreciable amounts **Examples**: Seeds, nuts; many fruits and vegetables; many cereal grains, and legumes, including corn, rice, soybean, and wheat	0 1 2	
Special case	□ GM food or GM ingredient in a food that is the sole or primary source of nutrition for extended period for vulnerable subpopulations (e.g., infants, patients unable to consume a varied diet) - excludes food and ingredient classified as low-1 for exposure **Examples**: Infant formulas, ingredients used in formulated tube-feeding products	0 1 2	

II-B. Potential hazards from intended effects

Intended effects in a modified crop include the effects of the **primary gene product** (protein, enzyme) and **secondary gene product** (direct product or metabolite of target/modified enzyme). Primary and secondary products of novel marker genes are also considered here. Levels expressed refer to *levels in the edible part* of the modified crop and of other crop plants.

Criteria: Four criteria underlie three levels of concerns (low, medium, high) for potential hazards from intended effects of the primary and secondary gene products: (1) their novelty or genetic relatedness and (2) the levels expressed relative to the host plant, other edible crops, and other sources of comparable substances in the food supply; as well as (3) prior knowledge of any adverse health effects of these substances, and (4) the extent and specificity of modification of the structure/function of the primary gene product, where applicable. The likely combinations of these four criteria are grouped into the three concern levels. Current scientific knowledge does not permit the classification of concern levels of allergenic substances based on threshold for inducing sensitization. However, the allergenic potential of the host plant and donor organism are considered as part of potential hazards from unintended effects (Part II-C).

In your judgment, are the criteria acceptable for each level of concern regarding *potential hazards from intended effects*? Please use the following scale in rating the acceptability of the criteria. For ratings 0 and 1, please comment and provide suggestions.

0 = Not acceptable 1 = Acceptable with modification 2 = Acceptable as is

Note: Concern levels are used to direct the type and intensity of further testing required in assessing the safety of the GM food. They are not a determination of the safety of the GM food in question. Similarly, the criteria for assigning concern levels are best viewed as indicators for the degree of uncertainty of potential hazards, rather than as evidence of the nature of potential hazards.

Level	Criteria	Criteria Acceptability	Comments and Suggestions (for ratings 0 and 1)
Low	□ **No new primary or secondary gene product*** **Examples**: Silenced expression of an endogenous gene *The intended silencing of a gene encoding a substance beneficial to health is possible, but unlikely in practice. It is more likely to occur as an unintended effect of gene silencing, which will be assessed separately under considerations for metabolic pathways and multiple gene modifications (Part II-C)	0 1 2	
Low	□ **Existing component of edible plants and plant-derived foods** 1- Not known to be a toxicant, allergen or antinutrient at the level expressed in crop plants with a history of safe use as food a/ Expressed at levels comparable to those in other edible crop plants, AND b/ Limited and well-characterized modification in structure/function (e.g., point mutation or site-directed mutagenesis)	0 1 2	

Level	Criteria	Criteria Acceptability	Comments and Suggestions (for ratings 0 and 1)
	Examples: Alteration of the levels of some plant-derived fatty acids that are common dietary components		
Low	□ **Novel component of edible plants and plant-derived foods** 1- Well-characterized modification of target gene (e.g., site-directed mutagenesis), AND 2- No toxicological concern based on prior knowledge: **<u>one or more</u>** of the following applies a/ Known mechanism for toxicity not applicable to humans, or extensive evidence for no adverse human health effects, OR b/ Established tolerance limit, such as maximum residue level (MRL) or equivalent, and level expressed is below MRL, OR c/Level expressed is below threshold of toxicological concern (TTC)[1] based on aggregate exposure estimates, OR d/ History of safe use in relation to food at the level expressed in the modified crop[2] **Examples**: Cry protein from Bt (a common soil bacterium) as a pesticide; active compound of a herbicide and its metabolite in a herbicide-tolerant plant that is modified to catabolize the herbicide; coat protein of common plant viruses for virus resistance	0 1 2	
Medium	□ **Existing component of edible plants and plant-derived foods** 1- Not known to be a toxicant, allergen or antinutrient at the level expressed in crop plants with a history of safe use as food a/ Expressed at levels higher than those in crop plants, AND b/ Limited and well-characterized modification in structure/function <u>OR</u> 2- Known adverse health effect in some animals or humans, *with* an established reference dose (RfD) or acceptable daily intake (ADI) or equivalent a/ Expressed at higher level in the modified crop than in the native crop, or at comparable or higher levels than in other edible crops,	0 1 2	

Level	Criteria	Criteria Acceptability	Comments and Suggestions (for ratings 0 and 1)
	AND b/ Limited and well-characterized modification in structure/function **Examples**: Increased level of lycopene in tomatoes for health benefit (lycopene is a naturally occurring constituent in tomato); increased levels of some naturally occurring alkaloids from crop plants as pesticides		
Medium	□ **Novel component of edible plants and plant-derived foods** 1- Not known to be a toxicant, allergen or antinutrient at the level present in the food supply a/ Expressed at higher level in the modified crop than other sources in the food supply, AND b/ Limited and well-characterized modification in structure/function <u>OR</u> 2- Known adverse effect in some animals or humans, *with* an established reference dose (RfD) or ADI or equivalent a/ Expressed in the modified crop at comparable or higher level than in other sources in the food supply, AND b/ Limited and well-characterized modification in structure/function **Examples**: Expression of omega-3 fatty acids in edible crops (dietary omega-3 fatty acids are primarily derived from marine food sources)	0 1 2	
High	□ **Existing component of edible plants and plant-derived foods** 1- Evidence of an adverse effect in some animals or humans, *without* an established ADI or equivalent, OR 2- Limited or lack of evidence for no adverse effect in animals or limited knowledge of mechanism of toxicity (limited data/unable to evaluate), OR 3- Extensive or inadequately characterized modification in structure/function **Examples**: Use of some phytoalexins as pesticides (phytoalexins are chemically diverse low-molecular plant secondary metabolites believed to have a plant protectant role but their mechanisms of action are not well-understood)	0 1 2	
High	□ **Novel component of edible plants and plant-derived foods**	0 1 2	

Level	Criteria	Criteria Acceptability	Comments and Suggestions (for ratings 0 and 1)
	1- Evidence of an adverse effect in some animals or humans, *without* an established ADI or equivalent, OR 2- Limited or lack of evidence for no adverse effect in animals, or limited knowledge of mechanism of toxicity (limited data/unable to evaluate), OR 3- Extensive or inadequately characterized modification in structure/function, OR 4- Primary product of a completely new gene or a completely novel protein in the host plant or in the diet[3] **Examples**: Expression in edible crop of conjugated linoleic acid (naturally occurring in mammalian or algal sources; structurally could be classified as trans fatty acids for which there is limited information on the potential for adverse health effects of increased intakes); chimeric gene to produce human milk protein in cereal; protein kinase from a mammalian source for pathogen resistance in the modified crop		

[1] Kroes et al., Food and Chemical Toxicology 2004; 42: 65-83. [2] The determination of history of safe use takes into consideration the function and exposure of the substance or a closely related substance (Codex Guidelines for the Conduct of Food Safety Assessment Derived from rDNA plants, 2002). [3] Examples include recombination of gene fragments or functional blocks of gene sequences by gene shuffling or chromosome design.

II-C. Potential hazards from unintended effects

Unintended effects are those effects arising from alterations in metabolic pathways, modification or disruption in the expression of native genes (silencing, activation, or interactions). These alterations may lead to the generation of new toxic/allergenic compounds, or modified expression of endogenous toxic substances in different tissues or at different levels. The assessment of potential hazards from unintended effects also apply to gene modification from gene deletion or gene silencing.

Criteria: Three levels of concern (low, medium, high) are based on the following criteria: (1) the availability and use of molecular characterization methods; (2) the nature of the host and donor organisms; (3) the complexity or novelty of the metabolic reactions or pathways in the modified crop involved in achieving the modified trait; and (4) ease in assessing the health consequence of an unstable modified trait. Molecular characterization methods are routinely used in rDNA transformation to assess genomic changes involving gene insertion or excision sites and junction regions – transformants with multiple insertion sites, gene copies, or interrupted open reading frame may be eliminated from further development. Molecular characterization methods are not commonly used in crops modified by conventional methods for risk assessment purposes. It is expected that the host plant being modified and any parental lines used in backcrossing will have an established breeding history and have been widely consumed for the duration of their commercial lifespan.

In your judgment, are the criteria acceptable for each level of concern regarding *potential hazards from unintended effects*? Please use the following scale in rating the acceptability of the criteria. For ratings 0 and 1, please comment and provide suggestions.

0 = Not acceptable 1 = Acceptable with modification 2 = Acceptable as is

Note: Concern levels are used to direct the type and intensity of further testing required in assessing the safety of the GM food. They are not a determination of the safety of the GM food in question. Similarly, the criteria for assigning concern levels are best viewed as indicators for the degree of uncertainty of potential hazards, rather than as evidence of the nature of potential hazards.

Level	Criteria	Criteria Acceptability	Comments and Suggestions (for ratings 0 and 1)
Low	□ **Molecular methods are available and used to localize and characterize changes in genetic elements** 1- Host plant: not known to be a source of allergen[1] or toxicant[2], AND 2- Donor organism: not pathogenic (microbial donor organism), and unrelated to toxicity and allergenicity (microbial and other donor organisms not known to be a source of allergen or toxicant), AND 3- Modified trait[3] well-defined mechanisms with **all** of the following characteristics a/ Single or limited number of enzymes b/ High specificity of target enzyme in terms of substrate and function c/ Limited number of metabolites that are not precursors or	0 1 2	

Level	Criteria	Criteria Acceptability	Comments and Suggestions (for ratings 0 and 1)
	intermediates in other pathways in the modified crop **Examples**: Introduction of Cry protein in corn by rDNA techniques		
Low	□ **Molecular methods are *not* available or used to localize or characterize changes in genetic elements** 1- Host plant: not known to be a source of allergen or toxicant, AND 2- Donor plant (same as host plant in mutation): has **all** of the following characteristics a/ Not known to be a source of allergen or toxicant b/ Edible plants with an established breeding history commercialized and widely consumed for several generations c/ Extensive published scientific literature on the biology and composition, AND 3- Modified trait: well-defined mechanisms with all of the following characteristics a/ Single or limited number of enzymes b/ High specificity of target enzyme in terms of substrate and function c/ Limited number of metabolites that are not precursors or intermediates in other pathways in the modified crop **Examples**: Point mutation of an endogenous gene encoding an enzyme such as ALS (acetolactate synthase) for herbicide tolerance in rice	0 1 2	
Medium	□ Molecular methods are available and used to localize and characterize changes in genetic elements 1- Host plant: a less common source of allergen or toxicant *not* associated with severe adverse health consequences (requiring immediate medical attention or life threatening in some individuals), OR 2- Donor organism: not pathogenic (microbial donor organism), and a less common source of allergen or toxicant *not* associated with severe adverse health consequences (microbial and other donor organisms), OR 3- Modified trait: well-defined mechanisms with **one or more** of the following characteristics	0 1 2	

Level	Criteria	Criteria Acceptability	Comments and Suggestions (for ratings 0 and 1)
	a/ Multiple enzymes b/ Low specificity of target enzyme in terms of substrate and function c/ Secondary gene product is precursor or intermediate metabolite in other pathways in the modified crop **Examples**: Alteration of plant-derived fatty acids involving complex pathways of central metabolism using rDNA technique		
Medium	□ **Molecular methods are *not* available or used to localize or characterize changes in genetic elements** 1- Host or donor plant (host plant in mutation) - a less common source of allergen or toxicant *not* associated with severe adverse health consequences, OR 2- Modified trait: mechanisms not well-established, or with **<u>one or more</u>** of the following characteristics a/ Multiple enzymes b/ Low specificity of target enzyme in terms of substrate and function c/ Secondary gene product is precursor or intermediate metabolite in other pathways in the modified crop **Examples**: Alteration of plant-derived fatty acids involving complex pathways of central metabolism using chemical mutagenesis	0 1 2	
High	□ Host plant or donor organism 1- Host or donor plant: a source of allergen with severe health consequences, OR 2- Donor organism: known pathogenicity/infectivity (microbial donor organism), or known toxicity/allergenicity (microbial and other donor organisms) and characterization insufficient to eliminate transfer of potentially harmful genetic elements, OR 3- Donor organism: unknown or insufficient knowledge regarding pathogenicity, infectivity, allergenicity, or toxicity **Examples**: Modification of peanuts (known to elicit severe allergic reactions in susceptible individuals); breeding with a wild variety of a crop species that contains some potentially toxic constituents and that has no history of safe use as food	0 1 2	

Level	Criteria	Criteria Acceptability	Comments and Suggestions (for ratings 0 and 1)
High	□ Metabolic pathway - complex or new in host plant, with **one or more** of the following characteristics 1- Multiple enzymes and branch points, regulated by multiple genes, OR 2- Secondary gene product is a modulator of enzyme activity via feedback mechanism, or is a signal for one or more regulatory genes, OR 3- Linked to secondary pathways with the potential for modified biosynthesis of toxic or bioactive secondary metabolite, OR 4- Involves redirection of one or more enzymatic reactions to alter the production of existing compounds, produce new compounds, or mediate the degradation of compounds **Examples**: Limiting production of ethylene (a plant hormone affecting many metabolic processes) for delayed fruit ripening	0 1 2	
High	□ Multiple gene modifications, with the potential for **one or more** of the following 1- Interactions among the modified genes or gene products, OR 2- Gene products sharing common or synergistic mechanisms of toxicity or pathways related to toxicant production **Examples**: Stacking of genes encoding ribosome-inactivating protein and lectin (a protein that normally cannot penetrate the brush-border membranes of the gut lumen may be transported across the gut membrane through increased membrane permeability or co-endocytosis mediated by lectin). Gene stacking may occur in the same or separate transformation events, including crossing two GM crops using conventional techniques	0 1 2	
High	□ Modified trait that has a negative health consequence if stability is not maintained during the commercial lifespan - modified trait not readily assessed **Examples**: Crops modified to reduce allergenic protein, or to express antigen protein for immunoprotection - changes in levels of antigen proteins are not readily assessed at the field level based on current practices	0 1 2	

[1]Allergenic sources are defined as those organisms for which reasonable evidence of IgE mediated oral, respiratory, or contact allergy is available (Proposed Draft Annex on the Assessment of Possible Allergenicity - Draft Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants, Appendix IV, Codex Alimentarius Commission, 25th Session, 30 June - 5 July, 2003). [2] For the purposes of the RBCS, a source of toxicant would be defined as an organism which, when consumed at a

level comparable to its counterpart in the diet, will produce an adverse health effect in humans. [3] Modified trait includes traits modified by gene deletion. [4] A less common source of allergen is a food that is not among the eight foods or food groups (peanuts, soybeans, tree nuts, milk, eggs, fish, crustacean, wheat) that account for over 90% of the documented food allergies worldwide (Taylor and Lehrer, Critical Reviews in Food Science and Nutrition 1996; 36(S): S91-S118).

General comments (Part II):

Part III – Aggregate Concern Levels

Concern levels for the 3 component categories reviewed in Part II are aggregated using a matrix approach below. You are asked to assess whether the proposed method of aggregation of the component categories is reasonable. Please provide comments and suggestions if you feel that modifications are necessary. Your response is requested at the end of the table.

Rationale: The primary aggregate concern levels correspond to four levels of potential hazards from intended effects (levels I – IV) and a special case for a food or a major ingredient in a food that is the sole or primary source of nutrition for extended period for vulnerable subpopulations (level V). In addition, provision is made for rejecting a product for which no further testing would be recommended. In order to have a manageable number of aggregate concern levels in the RBCS, we have combined two levels of dietary exposure (medium or high) and two levels of potential hazards from unintended effects (low or medium). The distinctions between these respective levels of dietary exposure and potential hazards from unintended effects are judged not to be of practical importance for the purposes of establishing aggregate concern levels. However, distinctions in these levels within the two component categories may be helpful in determining further testing and assessment of individual products.

The aggregate concern level listed in the far right hand column in the table below does not necessarily follow a ranked order of increasing level of testing. While the development of graded testing options will be the focus of a future phase of the research, it is expected that the level of testing focused on assessing intended effects will increase from level I to level IV, and that focused on assessing unintended effects will increase from level A to level B. Additional criteria may be required to guide the type and level of testing within each concern level. Level V will be treated as a special category of high dietary exposure for which the level of testing will take into consideration component concern levels for intended and unintended effects. Provisional comments on further testing based on concern levels are provided with the product example in Part IV.

Exposure (Dietary exposure to food or ingredient derived from the edible part of modified crop)	**Potential Hazards**		**Aggregate Level**
	Intended Effects	**Unintended Effects**	
Low - 1	Not Applicable	Not Applicable	**I-A**
Low - 2	Not Applicable	Low, Medium, or High	**I-B**
Medium or High	Low	Low or Medium	**II-A**
		High	**II-B**
	Medium	Low or Medium	**III-A**
		High	**III-B**
	High	Low or Medium	**IV-A**
		High	**IV-B**

Exposure (Dietary exposure to food or ingredient derived from the edible part of modified crop)	Potential Hazards		Aggregate Level
	Intended Effects	**Unintended Effects**	
Special case: sole or primary source of nutrition	Low, Medium, or High	Low, Medium, or High	**V**
Medium or High	Known or probable toxicant of high potency[1] in edible part of modified crop	High, or identified known or probable toxicant of high potency in edible part of modified crop	**Reject (do not test further)**
	Risk management options limited or unlikely to be effective		

[1] High potency toxicants could include substances with structures similar to azoxy or N-nitroso compounds, aflatoxin or other microbial or animal toxin, for which threshold of toxicological concern is not established.

In your judgement, is the method of aggregation of component categories reasonable?

☐ Yes ☐ No

If your answer is 'no', please comment and provide suggestions for modification.

General comments (You may comment on the type of future product examples that should be included in assessing the usefulness of the scheme and the type of product information that should be included in assigning component and aggregate concern levels, and other points you may wish to include)

Note: Assuming that you have made no major modifications to the criteria for concern levels II to IV of the three component categories (Part II) and that you agree with how the component categories are combined into aggregate concern levels (Part III), please proceed to Part IV. Otherwise, you may end your response to this questionnaire here without completing Part IV. Information on the return of the completed questionnaire is provided on the last page.

Part IV - Product Examples

To keep the completion of this questionnaire within a reasonable timeframe, you are asked to review one product example randomly selected among several examples prepared. **Please indicate whether you agree with the component and aggregate concern levels assigned to the product example using the product information provided and the draft RBCS. Your response is requested at the end of Product Information.**

Several product examples have been prepared to illustrate how the criteria for the concern levels of the draft RBCS can be applied. The examples include products that have been approved as food in at least one country and experimental products based on publicly available product information and the scientific literature. We have kept the product information brief to provide only essential core information. In a future phase of the project, we will expand the assessment of the application of the RBCS to include a broader range of reference products.

Example - Product A

Product Information:

Modified crop: Oryza sativa (rice)

Intended food use of modified crop: Rice, polished or brown, is used for food in various forms. In limited amounts, rice is also used to manufacture starch.

Target/modified trait: Imidazolinone herbicide tolerance

Method of gene transfer/modification: Chemically induced seed mutagenesis using ethyl methanesulfonate (EMS) and traditional cross-breeding with commercial varieties

Promoter: Not applicable

Molecular characterization: No

Donor organism: Not applicable

Prior knowledge regarding the safety of host plant and donor organism: Rice is a well-established food crop with no known toxicants in the form customarily consumed. The bran fraction contains antinutrients (phytic acid, trypsin inhibitor, lectins). Allergenic reaction (atopic dermatitis) to a 16 kD globulin fraction has been reported in Japan.

Target/modified gene and gene product (primary gene product): gene encoding acetolactate synthase (ALS), also known as acetohydroxy acid synthase (AHAS)

Direct product of target/modified enzyme (secondary gene product): acetolactate, acetohydroxybutyrate - intermediary metabolites in the biosynthesis of branched-chain amino acids (valine, leucine, and isoleucine)

Metabolic reactions/pathways: ALS enzyme modified by EMS differs by one amino acid from that of the wild-type enzyme due to single point mutation within the ALS encoding gene. The mutated enzyme is insensitive to the herbicide but maintains its catalytic function in the first reaction in the biosynthesis of branched-chain amino acids. There are 2 reactions catalyzed by ALS: conversion of pyruvate to acetolactate, and the condensation of α-keto-butyrate and pyruvate to acetohydroxybutyrate.

Levels of primary/secondary gene products and metabolites in edible parts of modified crop:

a) ALS - not altered compared to typical level in rice seed (~0.001% of total protein)

b) Acetolactate, acetohydroxybutyrate - levels not reported - see comment below

c) Branched-chain amino acids - levels not altered compared to those typical in rice seed

Prior knowledge regarding the safety of primary/secondary gene products and metabolites:

ALS and its metabolites: existing components of rice and other edible crops; not known to be toxic or allergenic at levels present in plants

Assignment of concern levels

	Concern Level	Criteria Applied
Exposure	High	▪ Common food, regularly consumed whole, or as ingredient in wide spectrum of applications
Potential hazards from intended effects	Low	▪ ALS enzyme and metabolites: existing components of edible plant and plant-derived food: not known to be toxicants and limited and well-characterized modification
Potential hazards from unintended effects	Medium	▪ Molecular methods not available/used to localize and characterize changes in genetic elements - Mutagenesis has a high likelihood of producing unintended effects[1]. However, rice, the modified plant, is not a common source of allergen or toxicant - Host plant not a common source of allergen or toxicant - Modified trait: modified enzyme restores biosynthesis of branched-chain amino acids - unlikely to induce new unintended concerns
Aggregate concern level	II-A	▪ Comments - further testing to include: - verification of structural and functional equivalence of expressed, modified enzyme to that of native enzyme (except for the known difference in one amino acid) - given that rice is not a known source of significant toxicant or allergen, potential unintended effects can be assessed by measuring changes in the levels of key nutrients and antinutrients, including the composition of other amino acids, and by assessing the presence of new proteins using an acceptable analytical method

[1] U.S. National Research Council. Safety of Genetically Engineered Foods. Approaches to Assessing Unintended Health Effects, 2004

Please respond to the following questions

Q1: Do you agree with the assignment of concern levels?

	Agreement		Please comment if your response is 'no'
Exposure	Yes	No	
Potential hazards from intended effects	Yes	No	
Potential hazards from unintended effects	Yes	No	
Aggregate concern level	Yes	No	

Q2: Is the product information provided sufficient to assess the appropriateness of the concern levels assigned using the criteria proposed for the draft RBCS? ☐ Yes ☐ No

Comments:

THANK YOU FOR YOUR PARTICIPATION IN THE STUDY. Please return the completed questionnaire to:

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Part IV - Product Examples

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Several product examples have been prepared to illustrate how the criteria for the concern levels of the draft RBCS can be applied. The examples include products that have been approved as food in at least one country and experimental products based on publicly available product information and the scientific literature. We have kept the product information brief to include only essential core information. In a future phase of the project, we will expand the assessment of the application of the RBCS to include a broader range of reference products.

Example - Product B

Product Information

Modified crop: Glycine max L. (Soybean)

Intended food use of modified crop: Mostly oil (about 94% of soy food ingredients), protein fractions, and dietary fibre. Soy-based foods are estimated to make up between 0.8% to 1.5% of a child's (ages 1-6) and an infant's diet, respectively. For infants, about 80% of the soy-based food is expected to come from oil, and 20% from soy flour (flake) products.

Target/modified trait: Modified seed fatty acid content - high oleic acid

Method of gene transfer/modification: Biolistic transformation of plant cells or tissue

Promoter: Seed-specific promoter from soybean

Molecular characterization: Yes

Donor organism: Soybean

Prior knowledge regarding the safety of host plant and donor organism:
Soybean - considered as a commonly allergenic food
Antinutrients: include trypsin inhibitors, lectins - both subject to degradation upon heating; phytoestrogens (isoflavones), stachyose and raffinose, and phytic acid.
Allergens: severe adverse gastrointestinal reactions have been attributed to the globulin fraction of soybean proteins that comprises about 85% of total protein

Target/modified gene and gene product (primary gene product): GmFad2-1 encoding fatty acid δ-12 desaturase (FAD)

Direct product of target/modified enzyme (secondary gene product): Linoleic acid

Metabolic reactions/pathways: Gene silencing - introduction of a copy of the gene encoding FAD suppresses expression of the gene. Suppressed level of FAD reduces the conversion of oleic acid to linoleic acid (18:1 to 18:2), resulting in the accumulation of oleic acid. The conversion from oleic to linoleic acid is the first step in the biosynthesis of polyunsaturated fatty acids in plants.

Levels of primary/secondary gene products and metabolites in edible parts of modified crop:
a) FAD enzyme - no detectable level of GmFad2-1 mRNA

b) Crude total fat and fatty acid composition: total fat at level comparable to unmodified soybean and literature values; significant increase in oleic acid with concomitant decreases in the levels of other fatty acids (palmitic, linoleic and linolenic) - at levels comparable to other edible oils

Prior knowledge regarding the safety of primary/secondary gene products and metabolites:
FAD enzyme, oleic acid and linoleic acid: existing components in edible plants - not known to be allergenic or toxic at levels present

Assignment of concern levels

	Concern Level	Criteria Applied
Exposure	High	▪ Common food, regularly consumed as ingredient in wide spectrum of applications
Potential hazards from intended effects	Low	▪ No new primary or secondary gene products. - Modified fatty acid: existing component of edible plant and plant-derived foods: not known to be a toxicant or allergen at the level expressed
Potential hazards from unintended effects	Medium	▪ Molecular methods available and used to localize and characterize changes in genetic elements - Host plant: a common allergenic source - Modification of target trait: alters a reaction that is part of complex metabolic pathway
Aggregate concern level	II-A	▪ Comments on further testing: - assess potential impact on fatty acid intakes at the population level, with attention also given to infants and young children - assess the presence of new proteins using an acceptable analytical method, and other unintended effects on changes in the levels of key nutrients, antinutrients and allergenic substances

Please respond to the following questions

Q1: Do you agree with the assignment of concern levels?

	Agreement		Please comment if your response is 'no'
Exposure	Yes	No	
Potential hazards from intended effects	Yes	No	
Potential hazards from unintended effects	Yes	No	
Aggregate concern level	Yes	No	

Q2: Is the product information provided sufficient to assess the appropriateness of the concern levels assigned using the criteria proposed for the draft RBCS?

☐ Yes ☐ No

Comments:

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Part IV - Product Examples

To keep the completion of this questionnaire within a reasonable timeframe, you are asked to review one product example randomly selected among several examples prepared. **Please indicate whether you agree with the component and aggregate concern levels assigned to the product example using the product information provided and the draft RBCS. Your response is requested at the end of Product Information.**

Several product examples have been prepared to illustrate how the criteria for the concern levels of the draft RBCS can be applied. The examples include products that have been approved as food in at least one country and experimental products based on publicly available product information and the scientific literature. We have kept the product information brief to include only essential core information. In a future phase of the project, we will expand the assessment of the application of the RBCS to include a broader range of reference products.

Example - Product C

Product Information

Modified crop: Zea mays L. (Maize)

Intended food use of modified crop: Field corn for the production of ingredients such as starch, sweeteners, flaking grits, flour, and oils from wet or dry milling processing. Ingredients have wide applications in foods.

Target/modified trait: Resistance to European corn borer and tolerance to glufosinate herbicide (active compound phosphinothricin, PPT)

Method of gene transfer/modification: Microparticle bombardment of plant cells or tissue

Promoter: Constitutively expressed promoter CaMV 35S

Molecular characterization: Yes

Donor organism: Bacillus thuringiensis subsp kurstaki (donor of Cry1Ac protein) and Streptomyces hygroscopicus (donor of PAT enzyme)

Prior knowledge regarding the safety of host plant and donor organism:
Maize - no significant toxicants, allergens, or antinutritional factors

Anti-nutrients: phytic acid raffinose, low levels of trypsin and chymotrypsin inhibitors not considered nutritionally significant.

Allergens: maize is not a common allergenic food, but case studies of skin, gastrointestinal and respiratory symptoms have been reported. Protein(s) responsible have not been identified.

Secondary metabolites include: a) Highly variable levels of DIMBOA (2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one), with its glycoside form being the most prominent. The deglycosylated form, DIMBOA is toxic to insects; data on effects on humans and animals are limited. b) Furfural: moderate acute toxicity and oral genotoxic carcinogen of low potency; partly removed by heating. c) Ferulic acid and p-coumaric acid: natural pesticide against insects and fungi. As phenolic acids and weak anti-oxidants, their effects (positive and negative) on mutagenic substances have been reported.

Bacterial donor organisms - a) Bacillus thuringiensis subsp kurstaki: common soil bacterium, no known adverse effects on humans or animals;. b) Streptomyces hygroscopicus: ubiquitous soil bacterium and no reports of adverse effects on humans or animals.

Target/modified gene and gene product (primary gene product): a) Cry1Ac gene encoding Cry1Ac protein, a delta-endotoxin - expressed in truncated form in modified plant; b) Bar gene encoding phosphinothricin N-acetyltransferase (PAT)

Direct product of target/modified enzyme (secondary gene product): a) not applicable; b) several - see below

Metabolic reactions/pathways[1]:

a) Cry1Ac protein: acts by selectively binding to specific sites localized on the brush border midgut epithelium of susceptible insect species, forming cation-specific pores that disrupt midgut ion flow and causing paralysis and death. No report was identified of metabolism of expressed Cry1 Ac protein in the modified crop.

b) PAT: inactivates the active compound in the herbicide, making it non toxic to the plant – by catalyzing the acetylation of PPT and preventing the inhibition of plant enzyme glutamine synthetase which converts ammonia to non toxic glutamine.

Abbreviations: PPO=4-methylphosphinico-2-oxo-butanoic acid MPP=3-methylphosphinico-proprionic acid MPHB=4-methyl-phosphinico-2-hydroxy-butanoic acid

Two metabolic pathways compete: (1) the deamination of glufosinate and subsequent conversion of PPO to MPP or to MPHB, (2) the N-acetylation of L-glufosinate by PAT. In L-PPT tolerant plants which express relatively high levels of PAT, the second pathway predominates and the main residue metabolite of L-PPT catabolism is N-acetyl-phosphinothricin. When PAT expression is low, the deamination pathway predominates, resulting in the residue metabolites MPHB and the main metabolite MPP, which is non-phytotoxic. An unstable intermediate PPO is formed via deamination, which is rapidly decarboxylated to the stable MPP. PPO can also be reduced to form MPHB, another final and stable product. Acetylated and non-acetylated forms of L-glufosinate are also formed.

Levels of primary/secondary gene products and metabolites in edible parts of modified crop:

	Per gram tissue (harvested kernels)	Oil
a) Cry1Ac	36.0 - 42.8 ng	-
b) PAT	3.1 - 6.0 µg	-
PPT + N-acetyl-L-glufosinate+ MPP	<0.05 - 0.07 µg	<0.05 ppm

Prior knowledge regarding the safety of primary/secondary gene products and metabolites:

a) Cry1Ac delta-endotoxin: an insecticidal protein of high specificity attributable to the presence of specific binding sites in target insects. Binds only to receptors on intestinal cells of a narrow range of lepidopteran and coleopteran insects. No receptors for delta-endotoxins of B. thuringiensis in mammalian intestinal cells.

b) PAT and metabolites[1,2]

PAT: highly substrate specific for L-PPT; not a known allergen or toxicant

Glufosinate-ammonium and its metabolites: certain toxic effects reported in animals.

Tolerances for combined residues of glufosinate-ammonium and its metabolites (MPP and N-acetyl-L-glufosinate) expressed as 2-amino-4-(hydroxymethylphosphinyl) butanoic acid (glufosinate-ammonium free acid) equivalents have been established in the USA for transgenic corn, at 0.2 ppm[1,2]. Maximum residual limit of 0.2 ppm for corn is also established in Canadian Food and Drug Regulations. There is no concern for mutagenic and carcinogenic activity[2].

An acceptable daily intake (ADI) value of 0.02 mg/kg body weight/day was accepted for glufosinate, and confirmed as group ADI for glufosinate-ammonium, MPP and N-acetyl-L-glufosinate[3]. Acceptable total exposure was established at 0.0063 mg/kg (acute), and 0.006 mg/kg/day (chronic) for U.S. population with 48% and 10% from food sources, respectively[2].

Assignment of concern levels

	Concern Level	Criteria Applied
Exposure	High	▪ Common food, regularly consumed as ingredient in wide spectrum of applications
Potential hazards from intended effects	Medium	▪ Novel component of edible plants and plant-derived foods a) Cry1Ac: known mechanism for toxicity not applicable to humans; no information on the metabolic fate of Cry1Ac in the modified crop (medium concern) b) PAT and associated products: evidence of adverse effect in some animals - level of glufosinate ammonium and metabolites in edible part is at the tolerance limit (medium concern)
Potential hazards from unintended effects	Low	▪ Host plant has no significant toxicants or antinutrients; possibly a minor and less common allergen ▪ Acceptable molecular methods available and used to localize and characterize changes in genetic elements - Transferred coding sequence: well-characterized genes of known function; microbial donor organism unrelated to pathogenicity, infectivity, toxicity and allergenicity - Modification of target trait: well-defined mechanisms with limited number of enzymes and reactions; high specificity of protein/enzyme that is the subject of modification
Aggregate concern level	III-A	▪ Comment - further testing to include: - biochemical/functional equivalence of novel proteins expressed in modified crop to proteins used in toxicity testing to establish ADI - implications of nondetected/nonquantified residue levels on dietary exposure of glufosinate ammonium and metabolites from multiple sources[4] - assessment of unintended effects on changes in the levels of key nutrients, antinutrients, and allergens; expression of new proteins

[1] OECD Series on Harmonization of Regulatory Oversight in Biotechnology No. 11 (1999) and No. 25(2002).
[2] U.S. EPA Glufosinate Ammonium; Pesticide Tolerance. Federal Register Vol. 68, No. 188, 55833-55849, 2003.
[3] WHO Joint FAO/WHO Meeting on Pesticide Residues, Rome, 20-29 Sept, 1999, cited in reference 1.
[4] U.S. EPA. Assigning Values to Nondetected/Nonquantified Pesticide Residues in Human Health Food Exposure, 2000.

Please respond to the following questions

Q1: Do you agree with the assignment of concern levels?

	Agreement		Please comment if your response is 'no'
Exposure	Yes	No	
Potential hazards from intended effects	Yes	No	
Potential hazards from unintended effects	Yes	No	
Aggregate concern level	Yes	No	

Q2: Is the product information provided sufficient to assess the appropriateness of the concern levels assigned using the criteria proposed for the draft RBCS?

☐ Yes ☐ No

Comments:

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Part IV - Product Examples

To keep the completion of this questionnaire within a reasonable timeframe, you are asked to review one product example randomly selected among several examples prepared. **Please indicate whether you agree with the component and aggregate concern levels assigned to the product example using the product information provided and the draft RBCS. Your response is requested at the end of Product Information.**

Several product examples have been prepared to illustrate how the criteria for the concern levels of the draft RBCS can be applied. The examples include products that have been approved as food in at least one country and experimental products based on publicly available product information and the scientific literature. We have kept the product information brief to include only essential core information. In a future phase of the project, we will expand the assessment of the application of the RBCS to include a broader range of reference products.

Example - Product D

Product Information

Modified crop: Lycopersicon esculentum (Tomato)

Intended food use of modified crop: Primarily for human consumption, either fresh or processed

Target/modified trait: Delayed ripening

Method of gene transfer/modification: Agrobacterium tumefaciens-mediated transformation

Promoter: Constitutively expressed 35S promoter derived from figwort mosaic virus

Molecular characterization: Yes

Donor organism: Pseudomonas chlororaphis

Prior knowledge regarding the safety of host plant and donor organism:

Tomato -

Toxicants: glycoalkaloids, primarily alpha-tomatine at low concentration in green tomatoes; small amounts in the ripe fruit of certain tomato varieties close to the wild form (e.g., "tomadoses" in the Antilles). The alkaloid can be easily bred out. Other glycoalkaloids include solanine and chaconine.
Antinutrients: lectins and oxalate
Allergens: not a major source of allergens, but several glycoproteins are known to be allergenic

Pseudomonas chlororaphis - a non-pathogenic soil bacterium

Target/modified gene and gene product (primary gene product): Gene encoding 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (ACCd) enzyme

Direct product of target/modified enzyme (secondary gene product): Ammonia and alpha-ketobutyrate

Metabolic reactions/pathways: ACCd enzyme catalyzes the conversion of ACC, an essential precursor for the biosynthesis of the plant hormone ethylene, to ammonia and alpha-ketobutyrate, thus reducing the level of ACC, limiting the production of ethylene, and delaying the ripening process.

Levels of primary/secondary gene products and metabolites in edible parts of modified crop:
ACCd: 20.6 - 39.4 μg/g fresh weight (0.21 - 0.39% total fruit protein). No ACCd detected in the control line. No data provided on levels of comparable microbial enzymes in the food supply.

Ammonia and alpha-ketobutyrate: levels not reported - see comment below

Prior knowledge regarding the safety of primary/secondary gene products and metabolites:
ACCd: a novel enzyme in the ethylene biosynthesis pathway in tomato. Low probability of enzymes being a toxicant[1].

Ammonia and alpha-ketobutyrate: endogenous metabolites in plants which are able to assimilate and redistribute ammonia and nitrogen compounds through amino acid pathways, of which alpha-ketobutyrate is an intermediate metabolite.

Assignment of concern levels

	Concern Level	Criteria Applied
Exposure	High	▪ Common food, regularly consumed fresh or processed, whole or as ingredients in many foods
Potential hazards from intended effects	Medium	▪ ACCd: novel component of edible plants and plant-derived foods - enzymes not likely to be toxicants at the level present in the food supply ▪ Metabolites of ACCd: existing components of edible plants and plant-derived foods: not known to be a toxicant, allergen or antinutrient at the level typically present in crop plants with history of safe use as food
Potential hazards from unintended effects	High	▪ Complex metabolic pathway in host plant: ethylene signaling pathway affects many metabolic processes in plants. The genes encoding the enzymes of the pathway are part of a multigene family. The introduction of ACCd enzyme in manipulating ethylene levels has several unintended effects with potential health-related concerns, including: increased accumulation of metals[2,3] and mycotoxin due to enhanced susceptibility of the modified plant to pathogens[4]
Aggregate concern level	III-B	▪ Comments - further testing to include: - limited toxicological testing on novel enzyme comparable to that required for enzyme as a food additive[1] - assessment of unintended effects on changes the levels of key toxicants, nutrients, antinutrients, heavy metals and mycotoxins; expression of new protein - new methods may be required to better predict unintended metabolic effects using microarray and profiling techniques and an integrated approach to the analysis of data

[1] Pariza MW and Johnson EA. Regulatory Toxicology and Pharmacology 2001; 33:173-186. [2] Grichko VP et al. J Biotechnol 2000; 81:45-53. [3] Nie L et al. J Plant Physiol Biochem 2002;40:355-61. [4] Klee H in Criteria for Field Testing of Plants with Engineered Regulatory, Metabolic, and Signaling Pathways - Workshop Proceedings, June3-4, 2002. Information Systems for Biotechnology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Please respond to the following questions

Q1: Do you agree with the assignment of concern levels?

	Agreement		Please comment if your response is 'no'
Exposure	Yes	No	
Potential hazards from intended effects	Yes	No	
Potential hazards from unintended effects	Yes	No	
Aggregate concern level	Yes	No	

Q2: Is the product information provided sufficient to assess the appropriateness of the concern levels assigned using the criteria proposed for the draft RBCS?

☐ Yes ☐ No

Comments:

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Example - Product E

Product Information

Modified crop: Solanum tuberosum L. (Potato)

Intended food use of modified crop: Potatoes are increasingly consumed in processed form (e.g., French fries and potato chips) while the direct consumption of potatoes (e.g., boiled potatoes) has declined dramatically. Potatoes should be cooked before eating because of the indigestibility of non-gelatinised starch and the presence of anti-nutritional proteins.

Target/modified trait: Enhanced resistance to potato pest (tomato moth)

Method of gene transfer/modification: Agrobacterium tumefaciens-mediated transformation

Promoter: Constitutively expressed cauliflower mosaic virus 35S promoter

Molecular characterization: Yes

Donor organism: Galanthus nivalis (snowdrop)

Prior knowledge regarding the safety of host plant and donor organism:

Potato

Toxicants: potatoes contain several types of alkaloids, with glycoalkaloids being the most important, 95% of which consist of α-chaconine and α-solanine, present in higher concentrations at the periphery, in green and sprouted tubers; not destroyed during cooking and frying. The widely accepted tolerance limit of total glycoalkaloids in tuber is 200 mg/kg, fresh weight, based on acute and/or subacute effects. New standards based on chronic effects may be warranted[1]. The susceptibility of humans to glycoalkaloids appears to be high and variable: 1-5 mg/kg body weight oral doses are marginally to severely toxic; 3-6 mg/kg body weight can be lethal.

Allergens: not generally considered as source of allergens. However, potato contains multiple heat-labile proteins which can induce immediate hypersensitivity reactions when raw potatoes are consumed. Patatin, the main storage protein in potatoes, has been associated with allergic reactions in sensitive children - allergenicity of patatin to be confirmed. In addition, IgE binding to several proteins belonging to the family of soybean trypsin inhibitors was observed.

Anti-nutrients: several protease inhibitors and lectins are present, inhibiting the activity of trypsin, chymotrypsin and other proteases, or binding to specific carbohydrate structures on cell surfaces (e.g., intestinal or blood cells) - activity largely inactivated by boiling and other thermal processes.

Snowdrop

Scientific family: Amaryllidaceae
Toxic parts: bulbs
Toxic chemicals: lycorine

Target/modified gene and gene product (primary gene product):
Snowdrop lectin or Galanthus nivalis agglutinin (GNA)

Direct product of target/modified enzyme (secondary gene product):
No report on metabolic fate of GNA in potato was identified

Metabolic reactions/pathways:

No report on the metabolism of GNA in potato was identified

Levels of primary/secondary gene products and metabolites in edible parts of modified crop:
GNA: up to 2.0% total soluble protein

Prior knowledge regarding the safety of primary/secondary gene products and metabolites:
GNA - highly resistant to digestion; a mannose-specific agglutinin for which there is insufficient evidence to determine the health significance of its binding activity on different body tissues. Information identified includes the following:

a) Gut brush border enzymes: activity of sucrase-isomaltase nearly halved and those of alkaline phosphatase and aminopeptidase significantly increased in 10 day dietary exposure at a level (~0.7% GNA isolated from snowdrop bulbs, or 42 mg/day) that provides insecticidal protection for plants, without affecting growth of young rats[2]
b) Gastrointestinal (GI) mucosal thickness and crypt length (suggestive of proliferative and antiproliferative effects): effects were inconsistent across the different GI regions examined in a subsequent 10-day rat study feeding transgenic potatoes expressing GNA at 25.4 μg/g (GM-GNA) compared to unmodified potatoes and potatoes supplemented with the same level of GNA as the GM-GNA group, raw and cooked[3]. Several limitations of the study make the results difficult to interpret[4].
c) In vitro binding of GNA to human white blood cells: strong binding of GNA (as commercially available lectin) was reported in a research letter[5]

[1] Korpan YI et al. Trends in Biotechnology 2004; 22(3):147-151.
[2] Pusztai A et al. Nutritional Biochemistry 1996; 7:677-682.
[3] Ewen SWB and Pusztai A. Lancet 1999; 354:1353-4.
[4] Kuiper HA and Noteborn HPJM. Lancet 1999; 354:1315-6.
[5] Fenton B et al. Lancet 1999; 354:1354-5.

Assignment of concern levels

	Concern Level	Criteria Applied
Exposure	High	▪ Common food, regularly consumed cooked or processed
Potential hazards from intended effects	High	▪ Novel component of edible plants and plant-derived foods - limited or lack of evidence for no adverse effect in animals
Potential hazards from unintended effects	Medium	▪ Molecular characterization methods available and used - Host plant: a source of toxicant - Metabolic fate of gene product (GNA) in host plant unknown; if metabolized, most plant pesticidal protein break down products are expected to be non-toxic peptides and amino acids
Aggregate concern level	IV-A	▪ Comments - further testing to include: - establishment of relevant endpoints for toxicological testing and dose-response to determine threshold levels - assessment of unintended effects on changes the levels of key toxicants, nutrients, antinutrients; expression of new protein

Please respond to the following questions

Q1: Do you agree with the assignment of concern levels?

	Agreement		Please comment if your response is 'no'
Exposure	Yes	No	
Potential hazards from intended effects	Yes	No	
Potential hazards from unintended effects	Yes	No	
Aggregate concern level	Yes	No	

Q2: Is the product information provided sufficient to assess the appropriateness of the concern levels assigned using the criteria proposed for the draft RBCS?

☐ Yes ☐ No

Comments:

THANK YOU FOR YOUR PARTICIPATION IN THE STUDY. Please return the completed questionnaire to:

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APPENDIX C

APPENDIX C

EXPERT CONSULTATION 3

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PHASE 2 (Testing Options Development)

Phase 2 of the research consisted of expert consultations 3 and 4 with a focus on the development of graded testing options. In expert consultation 3, one multidisciplinary group of experts (generalists) and one group of toxicologists and immunologists (specialists) were provided with a revised RBCS and a draft version of graded testing that corresponded to the concern levels in the revised RBCS. Changes were made to both the RBCS and graded testing following consultation 3. Participants of expert consultation 4 were specialists in toxicology. They were asked to review the modified version of graded testing. The corresponding version of RBCS was provided as background information.

3. Expert Consultation 3 - Assessment of revised RBCS and draft testing options

3.1. Purpose

The purpose of expert consultation 3 was to conduct a general assessment of the revised RBCS and the draft testing options. The assessment focused on the following aspects:

- the scientific and logical soundness of the conceptual basis of the RBCS,
- the appropriateness of the factors and criteria in determining concern levels for testing,
- the adequacy of the range of testing options proposed,
- the appropriateness of the proposed testing options in relation to the concern levels in the RBCS, and
- areas for improvement in both the RBCS and the testing options.

3.2. Recruitment and selection of experts

The recruitment process was reviewed by the Health Sciences and Sciences Research Ethics Board (HSSREB). The certificate of ethical approval is appended as **Annex 3-1**. Similar to previous consultations, potential participants were identified from publicly available sources. Letters informing potential participants of this phase of the research project were sent to participants in Phase 1 who did not request to discontinue contact regarding future phases of the project (when specifically advised) and others who had not been contacted previously about this project. Two groups of participants were distinguished:

1) Generalists judged to have broad knowledge of safety assessment of GM foods or health risk assessment and expertise in one or more specific discipline(s) relevant to the subject based on their publications and/or their professional positions, and

2) Specialists who publish primarily in one discipline relevant to the safety assessment of GM foods (toxicology and immunology) with or without direct involvement in agricultural biotechnology.

Eighty-five individuals (40 generalists and 45 specialists) were contacted by a letter sent electronically. The information letters sent to new contacts in groups 1 and 2, respectively is shown in **Annexes 3-2A and 3-2B**. Modified versions of the letters were prepared for individuals who participated in previous consultations. Sixty individuals were reached with one follow-up phone call or email, the maximum number of reminder permitted by the HSSREB. Thirty-nine individuals agreed to participate in the study and received information on their role as research participants (**Annexes 3-3A and 3-3B**). Three individuals withdrew after receiving the document for review due to unexpected time commitments, or judging oneself not to have the required expertise. Nineteen participants returned completed assessments after one emailed reminder.

3.3. Data collection

To provide flexibility for individual participants to complete the questions on the classification scheme and testing options, two versions of the review document were prepared, one for generalists and one for specialists. The two versions of the document had the same content but the content was organized differently so that participants could elect to answer the questions in one of three ways. For generalists, participants could answer questions on the classification scheme, or the testing options, or both. For specialists, participants could answer questions on toxic/antinutritional effects in the classification scheme and related testing options, or allergenic effects in the classification scheme and related testing options, or questions on both categories of health effects. **Annex 3-4** is the document sent to generalists for their review.

Responses from a total of 19 participants (11 generalists, 5 toxicologists, and 3 immunologists) were received, including one complete interview by phone. Several respondents provided general comments only, or comments to specific sections of the questionnaire only, without rating all components of the proposed RBCS, or the proposed testing options. A breakdown of the responses to the different sections of the questionnaire is shown in **Tables 3-1 and 3-2**. Participants included individuals affiliated with academic institutes (9), government (5), publicly funded research institutes (4), and industry (1) from 8 countries in Europe and North America. The types of self-reported expertise of the respondents are tabulated in **Table 3-3**.

3.4. Data analysis

In two separate sections, this analysis summarizes the comments on: (I) the proposed RBCS and (II) the proposed graded testing options. An overall assessment is followed by general comments, an assessment of the different components of the proposed scheme or testing options, as well as detailed comments. Given the small number of respondents in each expertise category, comments were combined with no attempt to compare the responses by expertise category. The number of respondents providing

comments in a specified grouping of response is included in brackets. Individual comments in the tables were distinguished by respondent identification number (A=generalists, B=specialists).

I. PROPOSED RBCS

1. Overall assessment.

Except for the screening step (exposure to the food derived from the modified crop), the majority of respondents rated the RBCS to be scientifically and logically sound (**Table 3-4**). For those respondents who disagreed, the following reasons were provided.

Complex scheme - The scheme should be simplified, even though the conceptual basis is good. "Rules for determining concern levels" should be taken out and described separately (1).

Allergenic effects.

- There is some duplication in the criteria for intended and unintended effects (e.g., allergenicity of the donor). Revisions are necessary although parts of it are very sound. (1).
- Products with sequence similarity with known allergens should be assigned level III. Cut-off values for the level of introduced/modified protein in a food (major/minor component) should be discussed and developed (1).

Unintended effects. The proposed scheme for unintended effects was considered *not* scientifically and logically sound by two respondents for opposing reasons.

- Unpredictable changes associated with extreme mutation rate of transfection procedure in GE plants require fixed screening procedure (such as metabolic profiling, Ames test, and multigeneration animal feeding to detect developmental problems). Except for allergens, protein is not the problem. It is the secondary metabolites that include all toxins and carcinogens, plus many we don't know (1).
- Concerns about unintended effects are exaggerated. Theoretical risks must be balanced by the likelihood of their occurrence based on available data. The few examples of unintended effects associated with increased toxic substances suggest low probability of concern. Even in wide cross hybridization of food crops in conventional breeding, we have completely different composition, cryptic pathways, and novel proteins resulting from recombination, without noticeable incidences of untoward consequences (1).

Screening step. The comments addressed 3 common concerns.

- There is a lack of clarity, particularly related to the outcome of the screening step and how it is linked to hazard identification (4).
- "Trivial part of the diet" is poorly defined; different aspects of triviality should be considered (e.g., ingredients not containing introduced DNA/protein and compositionally equivalent to conventional ingredients) (6).

- Exposure (at the food level) is given a very important role in the initial screening. In risk assessment, hazard identification and hazard characterization should come first. Otherwise, potent constituents active at low doses might be overlooked (1).
- It is somewhat dangerous if hazard characterization is triggered based on intake. Hazard assessment should be universal and should not be dependent on intake, which can change with time and with population groups (1).
- Other comments (3). One comment questioned the assumption that if a product is highly refined, a possible toxin will not co-purify with the product. Another comment questioned the basis for low level of concern about unintended effects when the edible part is not affected by genetic modification in tissue-specific modification. Specific changes were also suggested by one respondent.

2. General comments. General comments are grouped into the following themes.

Unclear distinction between levels/ability to classify all products? (6).

- Inconsistency in the labelling of concern levels for intended (I, II, III) and unintended effects (A-1, A-2, B) is confusing.
- If a distinction cannot be made between 2 levels (e.g., between levels A-2 and B for unintended effects), or if a concern level cannot be assigned based on the criteria in the scheme, then a default decision or the highest category of concern should be assigned as a precautionary measure.
- A distinction cannot always be made between levels - too detailed, creating a risk that some products will not fit it exactly. A case-by-case approach should be emphasized.

Abstract/subjective criteria (2).

- The basis of allocation to concern levels depends on some rather subjective criteria although the scheme is a good one at a rather abstract level.
- It may also be difficult to require so much data in a standardized form and to make similar demands on applicants with widely different products. The complicated assessments in the scheme may result in the need for animal studies to confirm the absence of effects in most cases!

Complex scheme (4).

- Tables are difficult to understand. Not user friendly in its present format.
- Would prefer a decision tree or similar logic flow with the relevant text for rules etc. annexed for referral as necessary.

Lack of clarity and precision of the terms used (8). Examples of ambiguous terms given by respondents included: "likely" not relevant to unpredictable events; "unusual" metabolites; "predictable" vs. "unpredictable" unintended effects; "toxin" vs. "toxicant"; history of "safe" use.

Scope of genetic modification (1). While the scheme proposes to cover a range of GM methods, the focus seems to be on GE. Risk should not be based on process or method.

Strengths (5).

- Transparency in identifying uncertainties; comprehensive and systematic assessment of a wide range of factors that impact on the potential risk of a GM food; allows one to document the process of assessing potential risk and the rationale behind why a specific concern level is assigned, or why there is an increased level of uncertainty about safety or risk.
- Potentially useful at an early stage of crop development.
- Flexible, iterative process.
- The levels of concern are logical in that they change based on the degree of information one has and thus the degree of concern identified, and the extent and type of testing to be undertaken.
- Especially liked the differentiation between the primary effects (intended) and effects coming from the genetic transformation event to the recipient organism (unintended).

Encouraging comments (3). Nice and interesting piece of work; excellent and comprehensive; very solid and scientifically sound classification and testing scheme; no substantive suggestions for improvement.

Areas for improvement (7).

- Simplify the structure and the number of levels.
- Consider a separate placement of the rules for determining concern levels.
- Suggest reprioritizing the criteria.
- Clarify instructions to users of the scheme if it is necessary to assess all factors, if meeting one critical factor would put the GM food into the highest concern level.
- Allow for additional factors to make the scheme flexible for future developments where new factors may be identified and new tests developed or required.

3. Detailed assessment by category of effects and concern level.

The factors in the RBCS were generally considered to be "appropriate" while a larger number of respondents considered the proposed criteria and rules for determining concern levels to be "appropriate with changes", particularly with respect to unintended toxic/antinutritional effects and allergenic effects from intended modification (**Tables 3-4 A-D**).

4. Detailed comments. Several common themes recurred. These comments reiterated the need for clarity and objective criteria. Inconsistencies in how concern levels are differentiated were noted, as they relate to intended and unintended changes with a potential for toxic/antinutritional effects. It was also noted that there were few criteria for unintended effects. Specific and detailed comments and suggestions

were provided for criteria for differentiating allergenic effects by two respondents. **Tables 3-5 A-D** present minimally edited comments for the four categories of effects. They are organized by factors, criteria, and rules for determining concern levels.

II. GRADED TESTING OPTIONS

1. Overall assessment.

In general, the majority of respondents considered that the range of options proposed for testing GM foods to be appropriate and adequate. Relative to those who commented on testing options for toxic/antinutritional effects, a larger proportion of respondents who reviewed testing options for allergenic effects considered that the proposed testing options to be inappropriate/inadequate. The reasons given for a negative rating for the different categories of effects are summarized below.

Unintended effects.

- The proposed options are more than necessary. Compositional analysis should be the basis of testing. Whole food toxicity testing is insensitive. Growth study using young poultry has some global diagnostic values and can add another level of assurance (1).

Toxic/antinutritional effects.

- More weight should be put on reproduction as a sensitive parameter for both toxic and antinutritional effects of intended and unintended changes in whole foods. This type of study may also pick up the potential for chronic toxicity (1).
- There is comparatively much weight being attached to animal testing (1).

Allergenic effects.

- Except for digestibility test, most of the proposed tests (such as serum screening and animal testing) are hypothetical at best. Double blind food challenge would probably not be ethical. It's also hard to say what information a negative result would provide. It's way too premature to come up with a testing scheme of this sort (1).
- Need to re-consider the use of occupational subjects, the use of animal models, or T-cell assays. Targeted serum screens are not going to help in allergenicity assessment. Specific serum screens are of value, when appropriate and when performed properly (1).
- Detailed comments on the uses and limitations of skin-prick test, animal models, T-cell epitope identification, and bioinformatics were provided by another respondent (1).

2. General comments. Several comments related to the testing of unintended effects were provided by respondents, in addition to comments on the types of testing and their relevance to risk management.

Types of testing (3).

- Consider broader immune responses – In general we should address immune response, not only allergenicity, especially with modified bacteria colonizing the gut.

- Toxicity testing methods have served society well, and if applied with due thought and consideration to the testing of GM foods, as outlined here, should provide a high level of confidence in the food supply. There was some discussion in the introduction about the interpretation of and lack of confidence in a negative test due to the absence of sufficiently sensitive biomarkers. Evidence to support this view should be cited.
- Emphasis on animal and clinical testing – The usefulness of, for example, acute oral animal toxicity studies with purified proteins, as well as animal tests with whole products and their sensitivities, are bound with limitations. Appropriate *in vitro* pre-clinical tests deserve much more emphasis here (e.g., mutagenicity and genotoxicity tests) before embarking on any chronic *in vivo* carcinogenicity test.

Testing of unintended effects (2).

- Unnecessarily complicated, particularly with respect to testing unintended effects. A fixed screening procedure should be used to detect unpredictable changes.
- Testing should be based on tangible science-based rationale. There is limited value in broad requirement for whole food toxicity tests and untargeted profiling analysis, which is useful as a research tool, but not for food safety at this time. Should focus on targeted analysis of components that are important to nutrition and safety. Analysis of a range of metabolites that are in the global metabolic network in the plant is equivalent to conducing profiling analysis. Changes in the composition of individual foods should be assessed in the context of wide variations in the composition of our diets. Even dramatic changes in food composition would have no major adverse health effects.
- The point is: science is costly to do. Scientific risk assessment should be commensurate with risk based on observations, not hypothetical hazards. From a public health perspective, we need to focus our resources on reducing those hazards that can give us additional safety.

Testing of intended vs. unintended effects (1).

- It may not be necessary to separate the testing of intended and unintended effects. Presumably foods tested for unintended effects will also be tested for intended effects, as appropriate. There is some inter-dependence between the two and perhaps this could be clarified.

Postmarket monitoring (2).

- Some form of monitoring may be necessary. It seems that one cannot be sure if some unintended effects would not be uncovered if one looks for them. Concern about allergenicity is particularly difficult to deal with.
- Postmarket monitoring should not be a substitute for pre-market assessment. It may serve to confirm essential assumptions on consumption etc. made during pre-market assessment. If there is any doubt on safety, the product should not be marketed at all.

Relevance to risk management (2).

- The scheme(s) is useful in offering a systematic approach to risk assessment of GM foods. However, risk assessment steps are not really the cause of concerns. Concerns have more to do with risk management and risk communication.
- There remains the need for risk assessors to communicate clear advice to risk managers following completion of recommended studies. There is clearly a need for interaction between these players and acceptance of approaches.

Suggestions for improvement (2).

- Separate validated and new methods – Since we need to assess GM plants with existing tools today in ways that are reassuring to consumers, it is important to separate between those methods that have already been tested and validated, from those that are in development.
- Add problem formulation step – A problem formulation step will provide the thought stimulus on what needs to be done and how, before jumping into a prescribed scheme.

3. Limitations of proposed testing options (3).

One respondent commented on the current status of metabolite profiling analysis, which is a rapidly developing field. On the question of methods for testing allergenic effects, one respondent provided detailed information on the pros and cons of relevant testing methods. Regarding methods for testing allergenic effects, one respondent noted that "nothing has been shown to work at all, much less validated". Another additional respondent suggested four areas of current methods that require attention.

- Development of "standardized" antigen-specific IgE protocols and study designs to evaluate affinity or avidity of binding.
- Development of "standardized" method for evaluating effective IgE binding that is capable of cross-linking mast cells.
- Assessment of the predictive value of different computer search approaches for predicting cross-reactivity.
- Development of guidelines on selecting patients as ideal IgE serum donors and test subjects.

4. New methods (5).

When asked, "what new methods will likely be available in the near future that could be added to the proposed range of testing options in order to mitigate or reduce current limitations?", several respondents suggested the following potential developments.

- Methods for identifying more metabolites.
- Effective biomarkers of exposure and of effects in bridging studies in animals and observations in humans.
- Increasing feasibility in conducting pre-release studies in humans.

- Methods for evaluating allergenicity: animal models and/or *in vitro* and *in silico* test systems, including

  - repositories of T-cell epitopes with a preponderance of triggering sensitization, rather than suppression,

  - a substantial increase in the number of experimentally verified protein allergen 3-D structures, opening the opportunity for characterizing 3-D motifs commonly involved in either sensitization or cross-reactivity, and

  - extensively validated and technically simplified cell-based functional assays, derived from reported basophil/mast cell IgE allergen interaction assays, likely enhancing the overall assessment of cross reactivity.
- Premarket animal studies for addressing issues of both nutritional/health benefits and health risks.
- Whole food testing using a "spiked" approach for testing both intended and unintended effects.
- Systems approach to data generation and interpretation.

5. Detailed assessment by category of effects and concern level.

For the testing options suggested for detecting toxic and antinutritional effects, slightly less than half of the respondents suggested changes, particularly concerning the testing of intended effects. For the testing options for detecting allergenic effects, approximately equal or higher number of respondents suggested changes to the suggested options or rated them to be inappropriate/inadequate. The number varied depending on the category of effects and concern level (**Tables 3-6 A-B**).

6. Detailed comments.

Several respondents questioned or provided comments on the different components of the proposed testing options. The comments are summarized under two groupings.

Testing for toxic/antinutritional effects. Comments included the testing options for protein substances, in particular, differences in the duration of testing for protein vs. non-protein substances, the range of testing and the rationale supporting the differences. As noted previously, divergent comments were expressed regarding appropriate testing approaches for and the challenges in detecting unintended effects. The lack of a common view is evident in the relative value of targeted compositional analysis vs. untargeted metabolite profiling, whole food toxicity testing, and flexible vs. fixed testing procedure. One respondent reiterated that it would be more efficient to combine the testing of intended and unintended effects where whole food testing is indicated. Individual comments are included in **Tables 3-7 A-B.**

Testing for allergenic effects. Detailed comments were provided by two respondents on specific tests in detecting allergenic effects. One respondent questioned the validity of testing methods

currently being used for identifying the potential for allergenic effects, particularly in assessing proteins derived from sources without prior dietary history (**Tables 3-7 C-D**).

3.5. Discussion

Results of consultation 3 led to the following simplification of the RBCS to make it more user-friendly.

- The screening step was eliminated. Dietary exposure was incorporated as part of the RBCS based on consumption at the level of the food, as well as at the level of the substance of interest in the food.
- There are now 3 separate categories of effects in the revised scheme (instead of 4 categories in the previous version).
- For allergenic effects, intended and unintended changes were merged into one category and the number of concern levels was also reduced (from 3 levels to 2 levels).
- For toxic/antinutritional effects, the number of concern levels was also reduced for unintended changes.
- A consistent approach was adopted in developing mutually exclusive criteria for the different concern levels across the 3 categories of effects. This should eliminate redundancies and gaps in the determination of concern levels.
- Aggregation/decision rules for determining concern levels for a category of effects are now separated from the application of criteria to individual factors within the category.
- Supplementary information for applying the RBCS has been developed as a separate document. While it is unlikely that any amount or type of information can totally eliminate the subjective nature of some criteria or replace expert judgement, the supplementary material should facilitate a common understanding of the terms used and the rationale for the factors and criteria in the scheme.

The above revisions were made to address several major comments on the scheme.

- The screening step or trigger for hazard identification based on exposure at the level of the food alone did not take into account potent constituents active at low doses and changes in food consumption with time and among different population groups.
- The scheme was complex and not user friendly.
- Several criteria were abstract and subjective.
- There were inconsistencies in how concern levels are differentiated. The distinctions between levels were unclear in some cases. Several criteria were overlapping or too detailed. As a result, some products may not fit the criteria exactly.

Comments from consultation 3 on the proposed graded testing options provided direction on how to proceed. Suggestions for improvement included the separation of validated methods from methods under development, or distinguishing required and optional testing methods. There is also the need to more clearly articulate the rationale for a proposed type of testing or an information gap that needs to be filled by testing. Comments from consultation 3 also identified several limitations of currently available methods for detecting unintended effects, as well as allergenic effects. These comments were reviewed in conjunction with those from consultation 4 (described in Appendix D) in evaluating the implications of concern levels in the RBCS for the testing of GM foods and in developing a graded approach to testing. Assignment of concern levels for a panel of reference products in Phase 3 of the project also provided concrete examples on which the appropriateness of the types of testing that are being proposed was assessed.

Table 3-1. Number of participants providing responses to RBCS.

	Comments only	Rating provided	Total
Screening step	3	16	19
Toxic / antinutritional effects	2	14	16
Allergenic effects	2	12	14

Table 3-2. Number of participants providing responses to Testing Options.

	Comments only	Rating provided	Total
Toxic / antinutritional effects	4	11	15
Allergenic effects	4	10	14

Table 3-3. Number of participants by expertise.

Expertise	Number*	Expertise	Number
Toxicology	11	Plant sciences	1
Immunology	5	Agricultural sciences	1
Pharmacology	4	Food/nutritional sciences	8
Biological sciences/molecular biology	11	Agricultural biotechnology	5
Analytical sciences	2	Health risk assessment	6
Other (microbiology, cancer research)	2		

* Participants were asked to indicate all expertise that applies.

Table 3-4. RBCS - Overall assessment.

	Number of respondents*	Yes§	No§	Not rated
Screening step	16	8	8	3
Toxic / antinutritional effects	15†	14¶	1	4
Allergenic effects	12‡	10	2	7

* Number of respondents who provided a rating for the respective sections of the questionnaire.
§ Respondents were asked the question: Is the conceptual basis of the proposed classification scheme scientifically and logically sound?
† Included 1 respondent who provided a rating for overall assessment only.
¶ Included 1 respondent who provided a "no" response for unintended effects.
‡ Included 1 respondent who did not provide a rating for unintended effects.

Table 3-4A. RBCS - Potential for toxic and antinutritional effects - Intended changes.

Number of individuals who provided a rating to the section = 14

Level	Factor			Criteria			Rules for determining concern levels		
	A	A+C	I	A	A+C	I	A	A+C	I
I	13	1	0	8	6	0	13	1	0
II	13	1	0	10	4	0	11	3	0
III	13	1	0	10	4	0	12	2	0

Table 3-4B. RBCS - Potential for toxic and antinutritional effects - Unintended changes.

Number of individuals who provided a rating to the section = 14†

Level	Factor			Criteria			Rules for determining concern levels		
	A	A+C	I	A	A+C	I	A	A+C	I
A-1	12	1	1	7	6	1	7	4	1
A-2	11	2	1	7	6	1	7	5	1
B	11	2	1	6	6	1	8	4	1

Table 3-4C. RBCS - Potential for allergenic effects - Intended changes.

Number of individuals who provided a rating to the section = 12

Level	Factor			Criteria			Rules for determining concern levels		
	A	A+C	I	A	A+C	I	A	A+C	I
I	10	2	0	7	5	0	10	2	0
II	10	2	0	7	5	0	7	5	0
III	10	2	0	7	5	0	7	5	0

Table 3-4D. RBCS - Allergenic effects - Unintended changes.

Number of individuals who provided a rating to the section = 11†

Level	Factor			Criteria			Rules for determining concern levels		
	A	A+C	I	A	A+C	I	A	A+C	I
A-1	10	0	1	7	2	1	7	2	1
A-2	9	1	1	6	2	1	7	1	1
B	9	0	2	6	2	3	7	1	2

A = Appropriate A+C = Appropriate with changes I = Inappropriate

† For some levels or components of the scheme, the number of respondents providing a rating does not equal to the number shown because not all participants provided a rating for each item.

Table 3-5A. Potential for toxic/antinutritional effects - Intended effects.

Theme	*Comments*
Factors	
Prior dietary exposure: "use" vs. "safe use"	The first factor should more correctly be worded "use" instead of "safe use" because safety is never scientifically assessed [B-05].
Factors missing	I think you miss one factor: the influence of food preparation (cooking etc.) [B-05]. Level I: GM products with same modifications previously assessed and approved for food use [A-02].
Criteria	
Prior dietary exposure	Prior dietary exposure: qualify "widely consumed" with "reported adverse effects" [A-02].
Expected change in dietary exposure	Need actual intakes [A-02].
Degradation products	Unusual metabolites should be classified as level II [A-02].
Differentiation between levels	Some overlap in criteria (e.g., history of safe use and known safety under expected conditions of consumption, when there is no increase in consumption [B-01]. "Incomplete analysis" should be moved to level III [A-02].
Need objective or quantifiable criteria	Criteria need to be made more objective or quantitative, as identified below [B-10]: - expected change in total dietary exposure: not significantly vs. significantly elevated - prior knowledge of function/safety: what does "limited data" mean? (one study, five studies?)
Rules for determining concern levels	
Level II	For level II: it should be "and/or" [B-05].
Other comments	
Secondary metabolite as end product	I think you are approaching it but I was lost again where the end product is a secondary metabolite. You need one or more new enzyme proteins to produce it. Are you going to assess both the toxicity/allergenicity of the end product as well as of the enzymes, or just the end product? I think this problem has not really been addressed in other reports [B-05].

A = Generalist
B = Specialist

Table 3-5B. Potential for toxic/antinutritional effects - Unintended effects.

Theme	*Comments*
Factors	
Novelty of gene function	Unintended effect is likely INDEPENDENT of introduced gene (function), novelty of "gene" not relevant in many cases [A-01].
Instability of modified trait	Unintended effects (2) … I am not sure what you mean by "instability of a modified health-related trait". Are you talking about expression is unstable, or gene instability? [B-03].
Complexity of pathway	Complexity of pathways – where are the data that support the contention that pathway complexity means increased concern? Changing metabolic pathways significantly does not necessarily have an impact on risk, although some people have asserted that. Human diet is highly variable, as long as it is free from toxicants etc, that can be characterized. Changes in composition of individual foods should be viewed in the context of the diet. As long as you get the essential elements and avoid the known harmful elements, there is no reason for concern, since people self-select foods in their diet all the time [A-08].
Extent of genomic disruption	No distinction between A-1 and A-2 is necessary (p.9) - where is the scientific support for this? Just as much chance of unintended effects in limited genetic change as in extensive genetic change [A-08].
Criteria	
Gradation of criteria appropriate /understandable	The gradation of criteria is appropriate. Plant breeders can understand what A-2 and B mean. However, unlikely to have products in higher level [A-08].
Safety of donor organism	I think Factor 4 should be directed towards the exact knowledge regarding the exactness of gene insert. If that is well-defined, I cannot see how other genes may be transferred. Maybe there should be a wider description of the whole gene insert for Factor 4 to exclude unintended transfer. Maybe the plant genetic relationship between the host and donor should be touched upon in a factor (factor 2)? [B-05].
Few criteria	Level A-1 and A-2: may be difficult to classify on the basis of one or two of the criteria proposed. Too few criteria for unintended effects [B-01]. Factor 2/level B "manipulation of regulatory genes" - if this has been done, does it automatically come under level B even if it comes under A1 for factor 1 (no significant change from appropriate comparator)? [A-15].
Differentiation between levels	Why should not criteria for all 7 factors be met at each level [B-05]? Distinction between level A-2 and B not clear. There need to be some means by which to distinguish an A-2 level of concern from a B level of concern for all factors identified [A-14].
Lacks clarity and objectivity	Unclear (e.g., "insufficiently characterized") [A-08]. "Validated chemical, biochemical or biological assays" (criteria for safety of donor or genetically related organisms) – what is "validated" in this context? [A-06]. "Complexity and interconnectivity …" and "extent of genomic disruption" – how do you define simple pathway (number of steps?); limited number of enzymes/metabolites (how many?); what is limited vs. extensive genomic disruption? [B-10]. The basis for allocation to a concern level depends on some rather subjective judgments according to the wording of the criteria. For example Factor 1, levels A-2 and B: "appropriate comparator" - choice has to be made, is it an actual "control" crop (several options here) grown at the same time/place as the GM crop or a reference data base of key nutrient compositions etc. (e.g., those published by OECD). "Significant" - statistical significance ($p<0.05$) or look at "fold-difference" between mean levels?

Theme	*Comments*
	How many times, places should crop be grown, what level of analysis (pooled material, individual plant, fruit etc.?) "limited" vs. "extensive" changes, "well-characterized" vs. "inadequately characterized" - how is it decided which applies? [A-15]. Need to explain how compositional analysis should be interpreted with respect to benchmark natural variability, which constituents to analyze etc.; when "substantial equivalence" is achieved; need to define when a change is significant or not [A-06; A-13, B-10].

Table 3-5C. Potential for allergenic effects - Intended effects.

Theme	*Comments*
Criteria	
Exposure history - post translational modification	Intended factor 2. You should probably clarify post translational modification. It is extremely difficult to demonstrate there is absolutely no PTM. There could be myristillation, removal of the leading Met, removal of a signal peptide, formation and rearrangement of disulfide bonds. The PTM that are most likely to be important in terms of potentially impacting allergy would be glycosylation, and primarily N-linked, with specific xylose or fucose substitutions (alpha[1,3]-fucosyl or beta[1,2]-xylose) [B-03].
Protein characterization	Intended factor 3. (Possibly the most important factor in the allergenicity evaluation.) I would add a block under concern level III protein characterization, with sequence identity over 50% for more than 80 amino acids. I would also suggest changing criteria II a/ to indicate low level sequence homology, between 35% and 50% identical over 80 or more amino acids. I would modify criteria I a/ to indicate less than 35% identity over all possible 80 amino acid segments [B-03]. Homology is a theoretical statement of probable evolutionary relatedness of two proteins. Many evolutionary biologists feel "homology" may be indicated by sequence identities as low as 25%, clearly well below the identity match of any cross-reactive proteins that I have seen published. Therefore I would change and focus on sequence identity. It would also be incorrect to indicate sequence similarity. Identity is what you want. Level II: Delete a (suspected sequence homology with known allergen). Add Level III: Amino acid sequence similarity (indicating relevance to allergenic response) with known allergens [B-22].
Non-dietary routes of exposure	Intended, factor 4...all levels. This is supposed to be a food safety assessment. It is therefore not at all clear to me why you have non-dietary routes of exposure to workers. That seems quite inappropriate as all agricultural crops have allergenic components for workers, though not everyone is affected. I would strongly argue against including airway exposure and allergenicity in a safety assessment of GM. Codex and EFSA also modified their original drafts to remove or reduce the statements about assessing airway allergy. I would suggest that you do the same. In parallel, there may also be some uses of a given crop that would be the application of relatively raw commodity fractions on the skin...dermal application. There could possibly be a slight risk that someone would transfer a latex allergen into a product only used for dermal applications, and there may be some risk then of contact allergy. However, if that crop was also used for food, the food allergy issue would be there and require addressing, so there is no need to cover non-dietary exposure risk assessment [B-03].
Level of introduced/ modified protein in food	A differentiation between concern levels I and II/III, depending on whether the xenoprotein expression level is below or above 1% is suggested. Firstly, xenoprotein expression in parts of the crop not used for consumption may not be of great relevance to food risk assessment. Secondly, small amounts of a certain protein allergen may be sufficient to mount an immune response, whereas a larger dose may be needed for another protein. Principally, the risk for sensitization and cross-reaction, as accomplished by ingestion of a food protein increases with dose within certain ranges. This circumstance can possibly justify an expression-based factor in the ranking of concern levels, but it should be used with great caution and allowed to have relatively limited influence on the overall assessment. Additionally, a differentiation level (between the concern levels) of 1% seems to me as being a bit high. In Table C (potential allergenic effects; page 9), I have made a somewhat relaxed suggestion of 0.1% as a threshold level. As I recall, this is roughly in compliance with the expression levels of xenoproteins in some already established GM crops and also in line with ranges discussed in papers in this context (II-4.1. Suggested changes for concern levels ...) [B-22].
Knowledge of functional category	Intended factor 6. As a minimum, I would suggest changing rules a/ for all 3 criteria to indicate not a member of, or belongs to, a protein family commonly associated with allergenicity (specifically prolamin, Bet v 1, cupin, profiling: see Jenkins J et al., J Allergy Clin Immunol, 2005

Theme	*Comments*
	115:163-170). Actually I would strongly argue that this factor is unnecessary because the bioinformatics search will take care of identifying any protein that is biologically meaningful close relative of the major allergen families. Membership in a family is not very "quantifiable", or measurable. Therefore the added category only provides one more way to fail a test, when the meaningful measurement of cross-reactivity, and probably sensitizing potential is the close identity (which represents overall structural shape, and likely function) [B-03].
Rules for determining concern levels	
Suggested revision	Level II: Change to "meet criteria for factor (1 or 3)". Level III: Change to "meet criteria for factor (1, 2 or 3)" [B-22].
Other comments	
Differentiation between levels	No differentiation between levels II and III in some cases (e.g., wind-borne pollen); differentiation should be made even if it is with respect to the quantity or degree of difference [A-14].
Questionable usefulness of levels II & III	Unlikely to have levels II and III. Genes from unknown allergenic sources are more difficult to assess. Level II can include known allergens. More testing for level III does not necessarily give you more certainty [A-08]. Level II - unlikely that there is some new ingredient that we can't tolerate; if they can be isolated from existing food sources but will be included in foods at higher levels but no homology with known toxin etc., would this not be level I (not sure how different are levels of testing between I and II; we already know too much about foods in level I - what we do today in terms of testing is sufficient) [A-08].
Define or reference criteria	Criteria should be defined or referenced (e.g., no sequence homology, rapid degradation) [B-01].
Introductory section	Intended (5) Magnitude of airborn release - what you do mean? How is this relevant to food safety? Abundance in food may be an important factor for some food allergens, but that is not a 100% risk factor. We know very little about concentrations and airborne allergens, and that may not have meaning for food allergy. (6) I am not sure why you indicate that the level and relatedness of to protein families associated with allergenicity is only important for "structural" proteins from an allergic source. Not all allergens are structural [B-03].
Suggested revisions	Delete: Post-translational modification (non-glycosylated proteins can also be allergenic; extremely difficult to demonstrate absolute absence of post-translational modification; most likely to be important with respect to allergenic potential would be glycosylation and primarily N-linked with specific xylose or fucose substitutions; not important if the protein was from a known source and was glycosylated similarly in the new host or if the protein is enzymatically cleaved) [A-02, B-03]. Delete: structural protein and level (level not justifiable; not all allergens are structural; structural proteins are important for allergenicity if they are from an allergenic source) [A-02, A-05, B-03].

Table 3-5D. Potential for allergenic effects - Unintended effects.

Theme	*Comments*
Factors	
Allergenicity of donor organism	I am not sure where you are going with this, but assume it is to determine whether or not the modified plant should be tested for dramatically increased allergenicity due to an insertional affect. Why list factor 1 here? It really belongs under intended effects. To determine whether or not the introduced protein is likely to be an allergen. That question does not seem to fit under unintended effects [B-03].
Genomic disruption	I believe that Factor 7 (genomic disruption of host plant and characterization of gene insertion/modification site; page 8) also applies to allergy and, accordingly, should be included in the table on page 10. This is in line with recommendations proposed by Konig A. et al., 2004 [B-22].
Criteria	
Combine Levels A-2 and B and have just 2 levels (A & B)	I simply do not see the point in separating criteria specified in A-2 from those appearing in B. The criteria of class A-2 are already alarming enough to trigger the highest level of alert. Additionally, it may be sensible to suggest a special focus on the targeted expression of well-characterized major allergen(s), in the event of occurrence of such proteins in the recipient crop, as an extension of the proposed immunochemical comparison of IgE-binding proteins [B-22].

Table 3-6. Graded testing options - Overall assessment.

	Number of respondents*	Yes§	No§	Not rated
Toxic / antinutritional effects	12†	10¶	2	6
Allergenic effects	11‡	7	4	7

* Number of respondents who provided a rating for the respective sections of the questionnaire.
§ Respondents were asked the question: Is the range of testing options proposed for testing GM foods appropriate and adequate in identifying potential toxic/antinutritional effects, or potential allergenic effects.
† Included 1 participant who provided a rating for overall assessment only.
¶ Included 1 respondent who provided a "no" response to unintended effects.
‡ Included 1 participant who did not provide a rating for unintended effects.

Table 3-6A. Graded testing options - Toxic and antinutritional effects.

Number of individuals who provided a rating to the section = 11† (intended); 10 (unintended)

Level	Intended effects			Unintended effects		
	A	A+C	I	A	A+C	I
I / A-1	6	4	1	7	2	1
II / A-2	6	5	0	6	3	1
III / B	6	4	0	7	2	1

Table 3-6B. - Graded testing options - Allergenic effects.

Number of individuals who provided a rating to the section = 10

Level	Intended effects			Unintended effects		
	A	A+C	I	A	A+C	I
I / A-1	5	2	3	8	0	2
II / A-2	6	2	2	7	1	2
III / B	5	3	2	4	2	4

A = Appropriate/adequate A+C = Appropriate/adequate with changes I = Inappropriate/inadequate

† For some levels or components of the scheme, the number of respondents providing a rating does not equal to the number shown because not all participants provided a rating for each item.

Table 3-7A. Potential for toxic/antinutritional effects - Intended effects.

Theme	*Comments*
Level I	
Protein substances: Questionable need for acute toxicity test	Delete "acute oral toxicity …" and replace with "in the case of proteins expressed in the GM plant where both the plant and the new protein have history of safe consumption, specific toxicity testing might not be required (EFSA, 2004) [A-05]. Why an acute oral toxicity on a protein that is not a pesticidal protein or has some other characteristics that one might consider as potentially having a toxic effect on a mammal? I would especially question that for low level expressed enzymes [B-03]. What is the argument for testing the protein just for acute toxicity since we are going to eat it every day? Regarding "confirm" see my other remarks! [B-05].
Level II	
28 vs. 90 day toxicity tests	It is implicit here and elsewhere that ADIs can be set for some foods on the basis of less than lifetime studies (here 28 days). Whilst this is probably justifiable, there needs to be some explicit text on this [B-01]. Difference in testing duration between protein (28 days) and non-protein (90days) needs to be justified, as well as the use of single species in these studies [B-01]. It is not clear why there is the possibility of waiving a 90-day study. Are you assuming that a 28-day study would provide an adequate MOE, but there is no mention of a 28-day study or a dose-finding pilot? [B-01]. IF there is some data that would suggest a low level protein may have toxic effects, especially through non-acute mechanism, it might be worth testing in a repeated dose 28 day. Otherwise, it is most likely a huge waste of animals, protein and money, with no gain in safety assurance. If there is a real reason to test, feed the commodity at relatively high levels for 40 (chicken) or 90 (rat) days [B-03].
Genotoxicity	Genotoxicity – why only *in vitro* tests. An agreed strategy including *in vivo* testing, if *in vitro* tests were positive, should be followed. If not, why not? [B-01]
Human studies	Human studies are not undertaken to confirm the absence of "major health effects". This would not be ethical [B-01].
Reproduction study	Since billions of people are going to eat the stuff, why not introduce a 2-generation reproduction study with 90-day feeding study in the first filial generation in order to assure the safety in relation to the very sensitive reproduction parameter? Either replace the 90-day study or conduct specific toxicity tests [B-05].
Level III	
Some tests not necessary	Some level III assays not necessary. They do not directly affect outcome in a way that could be understood. Eg. Metabolism, protein quality, absorption/bioavailability - very difficult with protein - OK with drugs [A-01].
Combine testing of intended and unintended effects using whole food	Since you are suggesting to test for un-intended effects in level B by testing whole foods in animal studies, I will suggest that you replace the suggested, very extended level III testing with the testing of whole food. By placing the test burden on the whole food combining with a spiking procedure (for the intended trait) you may end up with a much more rational testing approach giving the same answers for much less efforts and testing. Of course you will still need some shorter toxicity studies (28-days) to determine the toxicity of the gene product in order to decide on the spiking dosage [B-05].

Theme	*Comments*
Nutritional adequacy of food replacement	Whilst mention is made of the possibility of altering the intake of nutrients, consideration also needs to be given to the nutritional adequacy of a food replacement product. For example, does a novel protein provide the same nutritional adequacy of other proteins, when the balance of the diet is considered with respect to its intended use [B-01].
Types of substances included	
Inconsistency/clarification needed	Level I – toxic/antinutritional effects (p.13): The first heading under Testing includes "novel enzymes and proteins", yet to qualify for level I, foods should be from existing food sources that are widely consumed. Either this is an inconsistency or some clarification is required. If there is a history of safe use and no expected increase in exposure, why is there concern about digestibility etc. (item e/). This could apply equally well to all food. There is a danger of being over-precautionary here [B-01].

A = Generalist
B = Specialist

Table 3-7B. Potential for toxic/antinutritional effects - Unintended effects.

Theme	*Comments*
Need fixed testing procedure	I do not think that you can make any prior assumptions that relate gene to unintended effects - need fixed testing procedure: 1. Oral toxicity (short term, high dose) 2. Oral toxicity - feeding multi (2) generations in mice 3. Ames (mutagenicity) 4. Small molecule ID - metabolite profiling including flavonoids (nutrients) [A-01]. Major concerns with genetically engineered food (and food modified by other mutagenic modification methods) is with unpredicted, unintended effects of secondary metabolites; untargeted profiling and long term testing such as reproductive/teratological studies would increase the sensitivity of detecting unpredictable effects; difficulties of conducting animal toxicity testing have been exaggerated [A-01].
Focus on targeted analysis	Expanded compositional analysis should be done in a more targeted fashion and focus on classes of compounds likely to be affected (e.g., carotenoids in case of modifying provitamin A biosynthesis), as profiling methods have not yet been used on a routine basis for identifying unintended effects. Comparison and interpretation of differences observed using profiling methods will require information on the background variation of tested parameters, such as in different environments or in different crop varieties [A-08]. I will question the usefulness of metabolic fingerprinting and proteomic analysis at this stage of method development [B-05].
Whole food testing vs. profiling analysis	Include as possible alternative the option to perform whole food testing required for concern level B. It might be easier and less expensive to perform whole food testing than metabolic profiling or proteomic analysis [B-06].
Combine testing of intended and unintended effects	I think the safety of intended and un-intended effects can be assessed in one go as described previously [B-05].
Unintended effects difficult to assess	Extend your review of possibilities of screening for unintended effects (gene expression; enzyme screening) and long-term animal testing? It's really difficult to categorize and predict (e.g., unclear effects in the flanking regions) [A-16].

A = Generalist
B = Specialist

Table 3-7C. Potential for allergenic effects - Intended effects.

Theme	*Comment*
Level I	Level 1 testing: If the protein is not from an allergenic source and is not sufficiently similar to any known allergen in sequence, then there is no value in performing any serum screening, and especially not "targeted" screening. This should be dropped [B-03].
Level II	Level 2 testing: a/ Specific serum screening is performed when the gene was taken from an allergenic source, or the sequence is above a certain level of identity compared to a known allergen. Then the specific subjects are allergic to the source, or to the matched allergen, as appropriate. b/ "targeted" testing is not of value as there is almost no probability of finding anything useful [B-03].
Level III	Level 3. Testing. This sounds like an issue of definition, but it is important. Specific serum screens mean the serum sources are allergic to the source of the gene, or allergic to the source of the sequence matched allergen (and preferably have demonstrated IgE against the matched allergen). Targeted serum screening is not correctly described. Animal testing is of no value at this time, neither is T cell testing as there are no validated models for either for predicting allergenicity, and there is a high likelihood of some reaction that can not be gauged [B-03].
Types of substances	
Level I – allergenic effects (p.19):	There appears to be some contradiction between items c) and d). c) States that there must be no concerns from sequence homology or digestibility, yet d) states that it must not be more than 1% of total protein. Non-allergenic proteins >1% are not covered. Level II covers those proteins where there are suspicions of allergenicity +/- >1% of total protein. The second part of e) "not a member of a protein family with allergenicity" appears to overlap with c) [B-01].
Level 2. Types.	See level 1, no call for concern with PTM (post-translational modification) or respiratory release [B-03].
Other comments	
Targeted screening	Targeted screening: correct indication to "indicated where the donor organism is not a known source of allergenicity but related to food sources that are known to contain allergens" [A-05].
Specific screening	Specific serum screens however are of value, when appropriate, and when performed properly [B-03].
Over-reliance on sequence homology	Sequence homology might be over-rated. If there is homology with known allergen, that's a risk, but could equally be a risk if there is no homology [B-19].
Post market monitoring	Post marketing monitoring is not of value. If occupationally exposed individuals had indications of allergy to the source or to the protein, and especially if there was evidence of real reactions, then they would be used in a specific serum screen. EPA refused to use Garst Seed breeders to evaluate Cry9C because they knew there was no reasonable chance of obtaining meaningful data. It is too easy to get false positives values, and the most likely thing due to the low incidence of allergy to any one protein is a false negative, if the protein was an allergen [B-03]. I will question the ethics behind doing postmarket monitoring on the basis described for level B, but I accept postmarket monitoring in relation to a potential allergenicity [B-05].
Occupational exposure	a/ Inhalation: If there is a chance of inhalation sensitization through occupational exposure (as is the case for almost every protein that is purified, lyophilized and in some aerosol or dust in an occupational setting, that is an issue for occupational health and safety, for personal protective gear, for controlled interventions. These individuals should be avoiding contact not for food risk reasons, rather to maintain healthy lungs (and not develop obstructive pulmonary disease) [B-03].
In vivo test	*In vivo* tests such as food challenges, or even skin prick tests are not necessary to confirm a negative

Theme	*Comment*
	response (and are likely to be impossible to perform for ethical reasons, or lack of available and willing subjects). However they are also more likely to be needed to prove whether or not apparent IgE binding represents a false positive [B-03].
IgE tests	Not sure what you mean by food extract testing, but see below [B-03]. IgE tests. Whenever there is reason to suspect that a protein is potentially allergenic, the protein may be tested by *in vitro* IgE binding using pure protein, especially if it is in low concentration in the food, but should also be tested in the food in order to evaluate potential differences in folding, or processing between pure protein and "native" protein. Serum donors: Individuals with clear histories of clinically confirmed diagnosis with reactions to the specific allergen and individuals without allergies to the allergen, but with other allergic disease should be used as test subjects. The assay methods and reagents must be tested for specificity and lack of non-specific binding. Serum donors should have their serum tested by commercial assay to confirm IgE if possible. Assays should be validated and if possible calibrated against a standard of IgE. There is a high likelihood of false positive IgE binding, or such low level, low affinity binding that inhibition assays are necessary to demonstrate the relevance of apparent binding. Because of the low incidence of allergy to many allergens, it may often be impossible to find appropriate subjects to use as serum donors (or *in vivo* tests). In such cases, the likelihood of potential risk should be carefully evaluated. In some cases only children can be identified as potential donors, but the approval process or amount of serum available reduces the number or even the possibility of performing valid testing [B-03].
Skin-prick test	A negative skin-prick test (SPT) result is generally appreciated as being very useful to excluding type I food allergy to a tested protein, whereas a positive readout suggests the presence of such allergy. Consequently, SPT should be highly indicative of (especially) absence of offensive properties connected with transgene-encoded proteins present in GM crops [B-22].
Basophil activation test	Basophil activation tests (either *in vitro*-activated patient basophils or assays founded on cell lines expressing human FCεRI receptors) are, however, still associated with technical concerns and seemingly not fully comparable to SPT in diagnostic efficacy. Nonetheless, this assay format - not least that reported by Dibbern et al., 2003, J Immunol Methods 274:37-45 - shows promise for development, refinement and validation and should already in its present form provide a valuable complement to SPT as to specific/targeted serum screens, based on quantitative methodology (Concern levels II and III) [B-22].
Food challenges	Although being the gold standard in the assessment of potential allergenicity of food proteins, the DBPCFC assay is generally not always a realistic method of choice in the development of GM crops, due to ethical reasons or because of the considerable expenses associated with it [B-22].
Testing priority	Hence, it may be appropriate to suggest SPT as a mandatory format and DBPCFC as well as basophil activation as optional procedures in most cases. In those rare events of the transgene being obtained from an allergenic source and bioinformatics tests indicate allergenicity , however, (worst case scenario as appearing in revised concern level III), a very extensive test battery - including DBPCFC - would appear as sensible to request from the applicant (Concern levels II and III) [B-22].
Animal models	At the present time, several animal models for allergenicity testing purposes have been reported. No such model has, however, been satisfactorily validated or adopted as a standard procedure for regulatory risk assessment. Moreover, some critics claim that inherent immunological dissimilarities between humans and animals restrict their usefulness in risk assessment of food allergenicity, Finally, rising scepticism among the general public against animal-based testing schemes is not readily reconciled with promotion of such tests (Concern level III) [B-22].

Theme	*Comment*
T-cell epitope identification	While certain described T-cell epitope identification procedures, including that reported by Stickler et al. 2000; 2003, are scientifically very intriguing, such methods are still at research level. Additionally, to my knowledge, assay formats for this purpose, and focused on peptides occurring in existing proteins, cannot specifically identify epitopes with a preferential stimulation of naïve T lymphocytes to CD4+ T_H2 cells. Recent findings suggest the presence of highly active and antigen-specific CD4+, IL-10-secreting T_{Reg} to Bet v 1 and Der p 1 in non-allergens (Akdis, M., 2004, J Exp Med, 199, 1567-1575). Seemingly, there is considerable room for development in this area: for example, the Stickler group have assigned one segment of the Cry1Ab as being clearly stimulatory, whereas a relatively broad consensus has emerged on the absence of (food) allergenic potential of this proteins. Furthermore, exhaustive T-lymphocyte epitope mapping exercises seem to be quite expensive undertakings and are still technically advanced to conduct. On the other hand, there is no reason to altogether exclude this methodology from allergenicity assessment as a strictly optional step, which may provide some valuable information in the context of results from established assays. Absence of CD4+ T cell response, as measured in a dendritic cell/CD4 T lymphocyte/peptide co-stimulation assay, in conjunction with negative specific and targeted serum screening readouts and an inability to provoke significant SPT reaction, would collectively provide strong indication of non-allergenicity also in the event of the test protein being derived from an allergenic source and/or ambiguous scores in bioinformatics testing (Concern level III) [B-22].
Bioinformatics	Bioinformatics (or computational) testing (amino acid sequence comparison, including further data processing and interpretation) is simplistically referred to as "sequence homology to known allergens". This phrasing (homology) should be avoided in this context, since it refers to phylogenetic relationship, rather than to immunological/biochemical relatedness. Moreover, there appears to be no clarification in the document of the terms "indication" and "suspicion" of similarity to known allergens. Since the pioneering reports, appearing in the mid-late 1990s, on amino acid sequence comparison analysis in order to assess the risk of potential allergenicity of xenoproteins expressed in GM crops, a large expansion of available amino acid sequences for known allergen proteins has occurred over the recent years. There are now several publicly accessible repositories on such data. Moreover, several remarkably more advanced - and thereby more specific - systems, founded on refined algorithmic approaches, have been described. While bioinformatics methods, similar to relevant *in vitro* and *in vivo* assays, are insufficient as stand-alone techniques for the purpose of determining whether a protein possesses offensive properties, they have evolved to be considerably more accurate testing schemes, relative to early systems. Consequently, an indication of allergenicity, as obtained from dedicated computation testing systems, would have greater weight than the pioneering formats. In conclusion, I believe it to be sensible to refer to reasonably validated bioinformatics testing methods, rather than just sequence homology. Assuming adherence to the suggested three-level concern level structure, and founded on the aforementioned outline, an indication of significant amino acid sequence similarity should rather be placed in level III than in that of II. A positive scoring would simply be too serious an alert for an assignment to concern II level (Concern levels II and III). [B-22]

Table 3-7D - Potential for allergenic effects - Unintended effects

Theme	*Comments*
Level B	a/ testing, again you should use "specific" serum testing, as targeted serum testing is inappropriate. b/ specific serum testing of high risk groups is not appropriate, or meaningful see above. c/ animal testing or T cell testing...again, no models or tests have been demonstrated to be predictive over a broad spectrum of allergens, and non-allergens. As stated in Codex, these may be considered when validated [B-03].

A = Generalist, B = Specialist

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Office of the Vice-Rector, Research

Annex 3-1

HEALTH SCIENCES AND SCIENCE RESEARCH ETHICS BOARD

CERTIFICATE OF ETHICAL APPROVAL

This is to certify that the University of Ottawa Health Sciences and Science Research Ethics Board has examined the application for ethical approval of PHASE 2 the research project entitled **A risk-based classification scheme for testing genetically modified foods (file H 03-05-06)** submitted by Ms. Eunice Chao who is supervised by Dr. Daniel Krewski, both of the Institute of Population Health. The Board found that this research project met appropriate ethical standards as outlined in the Tri-Council Policy Statement and in the Procedures of the University of Ottawa Research Ethics Boards, and accordingly gave it a Category 1a (approval). This certification is valid one year from the date indicated below.

Rita D'Alessandro
Protocol Officer for Ethics in Research
For Dr. Daniel Lagarec, Chair of the
Health Sciences and Science REB

April 11, 2005
Date

550, rue Cumberland
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Annex 3-2A. Information/recruitment letter to generalists

Date

Dear Dr. [name]:

I am writing to inform you of a research project on a *risk-based classification scheme for testing genetically modified foods* that is currently underway at the University of Ottawa. I am seeking experts to participate in phase 2 of the project by reviewing and commenting on a proposed classification scheme and corresponding graded testing options.

Project Objectives

The primary objective of the research is to develop and evaluate a risk-based classification scheme as guidance for testing foods that are derived from crops genetically modified by a range of methods, including conventional (e.g., induced mutation, tissue culture) and more modern (e.g., recombinant DNA) techniques. The proposed scheme aims to (1) differentiate and classify genetically modified (GM) foods based on levels of concern about potential health risks and attendant uncertainty, and (2) guide the application of testing methods for GM foods in proportion to their potential to produce human health risks.

Expertise

I am seeking the guidance of a multidisciplinary group of experts who have expertise in one or more disciplines relevant to health risk assessment or management. Participants will be selected to reflect a range of specific expertise, including toxicology, immunology, pharmacology, biological sciences/molecular biology, plant sciences, agricultural sciences/biotechnology, analytical sciences, food/nutritional sciences, and health risk assessment.

Participation

Participants of this phase of the project will be provided with the proposed risk-based classification scheme, the graded testing options, and a list of questions. They will be asked to respond to the following types of questions on the classification scheme and the testing options.

Types of questions on the classification scheme will include:

- In your overall assessment, is the conceptual basis of the proposed classification scheme scientifically and logically sound?
- Are the factors and criteria appropriate in determining concern levels for testing?
- If you have concerns about the scientific or logical soundness of the overall scheme or the appropriateness of how concern levels are determined, please provide suggestions for changes, or reasons for your assessment.

Types of questions on the testing options will include:

- In your overall assessment, is the range of testing options proposed appropriate and adequate in identifying potential toxic/antinutritional and allergenic hazards of GM foods?
- Taking into account the classification scheme for concern levels for testing, are the testing options proposed for each concern level appropriate and adequate for each category of potential hazards?
- If you have concerns about the adequacy or appropriateness of the proposed testing options, please provide suggestions for changes, or reasons for your assessment.

Depending on an individual's expertise, one may elect to focus on either one of these two types of questions. Normally, a participant will have 2-3 weeks to complete a written response. If clarification on a written response is needed, a phone (or face-to-face) interview will be requested. A respondent may choose to provide his/her response entirely through an interview. A participant will also have an opportunity to review a summary report of the consolidated responses at the completion of data analysis to verify that his/her comments have been included.

Interviews will be audiotaped so they can be reviewed for completeness during data analysis. All data collection and dissemination will be conducted in accordance with confidentiality and ethics guidelines for research involving human participants.

It will take approximately 1-2 hours to review the classification scheme and the testing options and to respond to the questions. An interview for clarification purposes, if necessary, will take about one-half hour.

Participants will also be asked if they would be willing to participate in a future consultation by classifying 2-3 GM food products using the risk-based classification scheme and product information provided by the researcher.

The research is being conducted as part of a doctoral thesis at the University of Ottawa Population Health PhD Program. The principal researcher is Eunice Chao, doctoral candidate, who is working with Dr. Daniel Krewski, Professor and Director, McLaughlin Centre for Population Health Risk Assessment, Institute of Population Health, University of Ottawa. The research project is supported by the McLaughlin Centre through a grant from the R. Samuel McLaughlin Foundation and a fellowship awarded by the Canadian Institutes of Health Research. No industry funding is involved in this project.

Contact Information

I will contact you by phone within the next week to discuss your interest and availability for participation and to answer any questions that you may have. Any information on your scientific background that you are willing to provide (a short bio, full CV, or a simple statement of interests) would be helpful. Suggestions for other potential participants are welcome.

If you prefer to provide a response by e-mail to Eunice Chao (echao097@uottawa.ca), that would also be much appreciated. If you do not wish to be contacted in the future for a subsequent consultation of this study, please advise me that this is your preference. Thank you for your consideration.

Sincerely,

Eunice Chao, MSc
Doctoral candidate
Population Health PhD Program
University of Ottawa

echao097@uottawa.ca

Tel: (613) 562-5800 ext 2367
Fax: (613) 562-5380

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1 Stewart Street, Room 318B
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Annex 3-2B. Information/recruitment letter to specialists

Date

Dear Dr. [name]:

I am writing to inform you of a research project on a *risk-based classification scheme for testing genetically modified foods* that is currently underway at the University of Ottawa. I am seeking experts to participate in phase 2 of the project by reviewing and commenting on a proposed classification scheme and corresponding graded testing options.

Project Objectives

The primary objective of the research is to develop and evaluate a risk-based classification scheme as guidance for testing foods that are derived from crops genetically modified by a range of methods, including conventional (e.g., induced mutation, tissue culture) and more modern (e.g., recombinant DNA) techniques. The proposed scheme aims to (1) differentiate and classify genetically modified (GM) foods based on levels of concern about potential health risks and attendant uncertainty, and (2) guide the application of testing methods for GM foods in proportion to their potential to produce human health risks.

Expertise

I am seeking the guidance of a multidisciplinary group of experts who have expertise in one or more disciplines relevant to health risk assessment or management. Participants will be selected to reflect a range of specific expertise, including toxicology, immunology, pharmacology, biological sciences/molecular biology, plant sciences, agricultural sciences/biotechnology, analytical sciences, food/nutritional sciences, and health risk assessment.

Participation

Participants of this phase of the project will be provided with the proposed risk-based classification scheme, the graded testing options, and a list of questions. They will be asked to respond to the following types of questions on the classification scheme and the testing options related to 1) potential toxic/antinutritional effects, or 2) potential allergenic effects.

- In your overall assessment, is the conceptual basis of the proposed classification scheme scientifically and logically sound?
- Are the factors and criteria appropriate in determining concern levels for testing?
- If you have concerns about the scientific or logical soundness of the overall scheme or the appropriateness of how concern levels are determined, please provide suggestions for changes, or reasons for your assessment.
- In your overall assessment, is the range of testing options proposed appropriate and adequate?
- Taking into account the classification scheme for concern levels for testing, are the testing options proposed for each concern level appropriate and adequate?
- If you have concerns about the appropriateness or adequacy of the proposed testing options, please provide suggestions for changes, or reasons for your assessment.

Depending on an individual's expertise, one may elect to focus on those sections of the classification scheme and testing options that are related to one of the two categories of potential effects. Normally, a participant will have 2-3 weeks to complete a written response. If clarification on a written response is needed, a phone (or face-to-face) interview will be requested. A respondent may choose to provide his/her response entirely through an interview. A participant will also have an opportunity to review a summary report of the consolidated responses at the completion of data analysis to verify that his/her comments have been included.

Interviews will be audiotaped so they can be reviewed for completeness during data analysis. All data collection and dissemination will be conducted in accordance with confidentiality and ethics guidelines for research involving human participants.

It will take approximately 1-2 hours to review the classification scheme and the testing options and to respond to the questions. An interview for clarification purposes, if necessary, will take about one-half hour.

Participants will also be asked if they would be willing to participate in a future consultation by classifying 2-3 GM food products using the risk-based classification scheme and product information provided by the researcher.

The research is being conducted as part of a doctoral thesis at the University of Ottawa Population Health PhD Program. The principal researcher is Eunice Chao, doctoral candidate, who is working with Dr. Daniel Krewski, Professor and Director, McLaughlin Centre for Population Health Risk Assessment, Institute of Population Health, University of Ottawa. The research project is supported by the McLaughlin Centre through a grant from the R. Samuel McLaughlin Foundation and a fellowship awarded by the Canadian Institutes of Health Research. No industry funding is involved in this project.

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If you prefer to provide a response by e-mail to Eunice Chao (echao097@uottawa.ca), that would also be much appreciated. If you do not wish to be contacted in the future for a subsequent consultation of this study, please advise me that this is your preference. Thank you for your consideration.

Sincerely,

Eunice Chao, MSc
Doctoral candidate
Population Health PhD Program
University of Ottawa

echao097@uottawa.ca

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Institut de recherche sur la santé des populations
Institute of Population Health

Annex 3-3A. Project Information for Research Participants - Generalists

Name of researcher(s): Eunice Chao
Institution, Faculty, Department: University of Ottawa, Population Health PhD Program

Name of supervisor: Dr. Daniel Krewski
Institution, Faculty, Department: University of Ottawa, Faculty of Medicine, Department of Epidemiology and Community Medicine, Institute of Population Health

Project Description
The doctoral research project "A risk-based classification scheme for testing genetically modified foods" is conducted by Eunice Chao of the Population Health PhD Program at the University of Ottawa under the supervision of Dr. Daniel Krewski. The purpose of the research is to develop and evaluate a risk-based classification scheme as guidance for the testing of foods that are derived from crops genetically modified (GM) by a range of methods. The proposed scheme aims to (1) differentiate and classify GM foods based on levels of concern about potential health risks and attendant uncertainty, and (2) guide the application of testing methods for GM foods in proportion to their potential to produce human health risks. The research will be conducted in several phases. You are asked to participate in phase 2 of the project.

Participation
Participants of this phase of the project will be provided with the proposed risk-based classification scheme, the graded testing options, and a list of questions. They will be asked to respond to the following types of questions on the classification scheme and the testing options.

Types of questions on the classification scheme will include:

- In your overall assessment, is the conceptual basis of the proposed classification scheme scientifically and logically sound?
- Are the factors and criteria appropriate in determining concern levels for testing?
- If you have concerns about the scientific or logical soundness of the overall scheme or the appropriateness of how concern levels are determined, please provide suggestions for changes, or reasons for your assessment.

Types of questions on the testing options will include:

- In your overall assessment, is the range of testing options proposed appropriate and adequate in identifying potential toxic, antinutritional, and allergenic hazards of GM foods?
- Taking into account the classification scheme for concern levels for testing, are the testing options proposed for each concern level appropriate and adequate for (a) intended effects, and (b) unintended effects?
- If you have concerns about the adequacy or appropriateness of the proposed testing options, please provide suggestions for changes, or reasons for your assessment.

Depending on an individual's expertise, one may elect to focus on one of these two types of questions. Normally, a participant will have 2-3 weeks to complete a written response. If clarification on a written response is needed, a phone (or face-to-face) interview will be requested. A respondent may choose to provide his/her response entirely through an interview. A participant will also have an opportunity to review a summary report of the consolidated responses at the completion of data analysis to verify that his/her comments have been included.

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Participants will also be asked if they would be willing to participate in a future consultation by classifying 2-3 GM food products using the risk-based classification scheme and product information provided by the researcher.

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Ottawa, Ontario K1 N 6N5 Canada

(613) 562-5660 .Telec./Fax (613) 562-5659

Confidentiality and Anonymity
The contents of the completed response will be used only for the purposes of developing a risk-based classification scheme for testing genetically modified foods. A research participant's confidentiality will be respected through the following actions taken by the researcher.

All of the data collected (including written responses, tapes, transcripts, and electronic files) will be tabulated and maintained by the researcher in accordance with confidentiality standards for research involving human participants. The data will be accessible only to the researcher in a locked filing cabinet and a password protected personal computer on the premises of the Institute of Population Health, University of Ottawa. Data files will be kept for 5 years after the completion of the thesis.

Anonymity of a participant's individual views and responses will be protected. Written responses will be coded for follow-up purposes. While group results of the interviews at the aggregate level will be published, and discussed within the research team, and at conferences, no names will be linked to individual views or responses in notes and transcripts of interviews, data analysis, reports or in presentations.

In order to acknowledge participants' contribution to the research project and to make the expert consultation process transparent and credible from a research perspective, the names of individuals and affiliations (the organization where one works or which one represents) will be included in a list as an appendix to the doctoral thesis. Participants will be given the opportunity to indicate their preference as part of their response included in the list of questions. Only one combined list of participants for all phases of the research will be published without identifying the specific phase to which they made a contribution.

Research participants agree not to disclose or publish any aspects of the risk-based classification scheme developed by the researcher except with the express consent of the researcher.

Voluntary Participation
There is no direct individual benefit from participating in the study and from answering the questions. All costs associated with participation in the project (long-distance phone calls and postage, where applicable) will be born by the McLaughlin Centre for Population Health Risk Assessment. Return postage for mailed responses of participants from countries outside Canada will be paid in the form of international postal reply coupons, if requested.

Participation is completely voluntary. A participant is free to withdraw from the project at any time, refuse to participate, or decline to answer certain questions.

THE RETURN OF A WRITTEN REPONSE IMPLIES CONSENT. IF YOU PREFER TO PROVIDE YOUR RESPONSE BY AN INTERVIEW, PLEASE E-MAIL YOUR PREFERENCE TO EUNICE CHAO (echao097@uottawa.ca) TO INDICATE CONSENT. (You may copy and paste the following request in your e-mail). PLEASE ALLOW 2-3 DAYS FOR A CONFIRMATION.

I prefer to provide my comments in an interview* (□ by phone; □ face-to-face). I am available on (dates, times) _____ for an interview. My phone number is _____. Please confirm.

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Any information about the rights of a research participant may be addressed to **Protocol Officer for Ethics in Research, University of Ottawa, 550 Cumberland Street, Tabaret Hall, Room 159, Ottawa, Ontario, Canada, K1N 6N5, tel.: (613) 562-5387, e-mail: ethics@uottawa.ca**.

Any questions about the conduct of the research project may be directed to the researcher or her supervisor at the coordinates listed below.

Eunice Chao: **Telephone number:** 1-613-562-5800 ext 2367 **Fax number:** 1-613-562-5380
E-mail address: echao097@uottawa.ca

Daniel Krewski: **Telephone number:** 1-613-562-8261 **Fax number:** 1-613-562-5380
E-mail address: dkrewski@uottawa.ca

Address: McLaughlin Centre for Population Health Risk Assessment
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Université d'Ottawa · University of Ottawa

Institut de recherche sur la santé des populations | Institute of Population Health

Annex 3-3B. Project Information for Research Participants - Specialists

Name of researcher(s): Eunice Chao
Institution, Faculty, Department: University of Ottawa, Population Health PhD Program

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In order to acknowledge participants' contribution to the research project and to make the expert consultation process transparent and credible from a research perspective, the names of individuals and affiliations (the organization where one works or which one represents) will be included in a list as an appendix to the doctoral thesis. Participants will be given the opportunity to indicate their preference as part of their response included in the list of questions. Only one combined list of participants for all phases of the research will be published without identifying the specific phase to which they made a contribution.

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Address: McLaughlin Centre for Population Health Risk Assessment
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1 Stewart Street, Room 318B, Ottawa, Ontario, CANADA K1N 6N5

Annex 3-4. Questionnaire

Respondent I.D.

Annex 3-4. Questionnaire

Risk-Based Classification Scheme for Testing Genetically Modified Foods
Assessment of Classification Scheme and Testing Options

The objective of this expert consultation is to conduct an assessment of the risk-based classification scheme (RBCS) and testing options proposed for each concern level in the scheme contained in this document.

The organization of this document is as follows.

- Part I: Risk-based classification scheme
  - A. Exposure to the food derived from the modified crop (screening step)
  - B. Intended effects
  - C. Unintended effects
- Part II: Graded testing options
  - A. Intended effects
  - B. Unintended effects
- Part III: List of questions
- Part IV: Appendices
  - A: Background information on the intended uses and the development of the RBCS
  - B: References

Intended and Unintended Effects in both Parts I and II are divided into 2 subsections:

- 1- Potential for toxic and antinutritional effects
- 2- Potential for allergenic effects

You are asked to review this document and respond to the questions listed in Part III. There are two types of questions:

- Questions on the risk-based classification scheme
- Questions on the graded testing options

Depending on your expertise, you may elect to focus on one of these two types of questions.

If you would be interested in reviewing this document and providing comments, a response date of June 15th, 2005 would be appreciated. Please let me know if this would be a problem. Please return your response to the questions to: Eunice Chao (echao097@uottawa.ca).

If you provide a written response, an interview may be requested for clarification purposes. You may also choose to provide your response entirely through an interview. Please refer to p. 23 of this document and "Project Information for Research Participants" e-mailed to you along with the document.

If you have any questions, please do not hesitate to contact Eunice Chao by e-mail or by phone (613-562-5800 ext 2367). Collect calls will be accepted.

Respondent I.D.

PART I - RISK-BASED CLASSIFICATION SCHEME (RBCS)

The proposed classification scheme comprises
(1) a screening step for determining the need to use the scheme in whole or in part, and
(2) factors and criteria for differentiating GM foods into levels of concern for testing.

The factors are those aspects of the pathway between the introduction of modified genetic elements in the crop and their entry into the food supply that may affect the production of health risks. The criteria for differentiating GM foods into different concern levels are based on the concepts of novelty, prior knowledge, dietary role, health consequence, directedness of gene modification, and genetic relatedness between donor organism and host plant. The criteria also take into consideration characteristics of major classes of compounds important in crops modified for food use and the type and level of testing required in assessing these characteristics.

Three levels of concern are proposed independently for intended effects and unintended effects and separately for toxic/antinutritional effects and allergenic effects. Intended effects focus on the primary and secondary gene products introduced or modified in GM foods. Unintended effects focus on the food derived from the modified crop and the potential for adverse health effects that may arise from pleiotropic and secondary metabolic changes following genetic modification of the host plant.

In the tables that follow, the factors and criteria used in developing the RBCS are organized by concern levels. Provided below is a summary of the considerations underlying the criteria used in differentiating GM foods based on the factors identified for each category of potential effects.

Intended effects from primary and secondary gene products introduced/modified

1- Potential for toxic and antinutritional effects

(1) level of novelty of the gene product of interest as a dietary constituent
(2) characteristics of the major classes of primary and secondary gene products important in the genetic modification of crops for food use
(3) type and level of testing required in assessing these characteristics
(4) dietary role of the compound

Criteria (2) and (3) are particularly relevant in determining the concern levels for the testing of enzymes and proteins at low expression levels. Toxic proteins typically act via acute mechanisms and are non-mutagenic. The classes of food-related proteins and plant-derived amino acids that are toxic are generally known [FIFRA-SAP, 2000]. Further, variation in enzyme structure and function that improves desired enzyme function is unlikely to result in the creation of a toxic protein [Pariza and Johnson, 2001; Sjoblad et al., 1992].

2- Potential for allergenic effects (protein substances only)

(1) extent of prior knowledge of the allergenicity of the donor organism
(2) extent of prior exposure and sensitization to the protein
(3) potential for post-translational modification
(4) similarity to characteristics of known allergens
(5) magnitude of airborn release
(6) level in the modified food and relatedness to protein families associated with allergenicity if the protein substance is a structural protein from an allergenic source

Unintended effects in the modified food from pleiotropic and secondary metabolic changes resulting from genetic modification of the host plant

1- Potential for toxic and antinutritional effects

(1) adequacy of data with respect to basic targeted compositional analysis and the extent of novelty of the introduced/modified gene function or pathway in the host plant

(2) seriousness of the health consequences of instability of a modified health-related trait
(3) extent of prior knowledge of the safety or biology of the donor/genetically related organism or the host plant, depending on the type and directedness of the genetic modification and the likelihood or extent of metabolic and genomic disruptions

2- Potential for allergenic effects

(1) extent of prior knowledge of the allergenic potential of the donor organism and/or the host plant

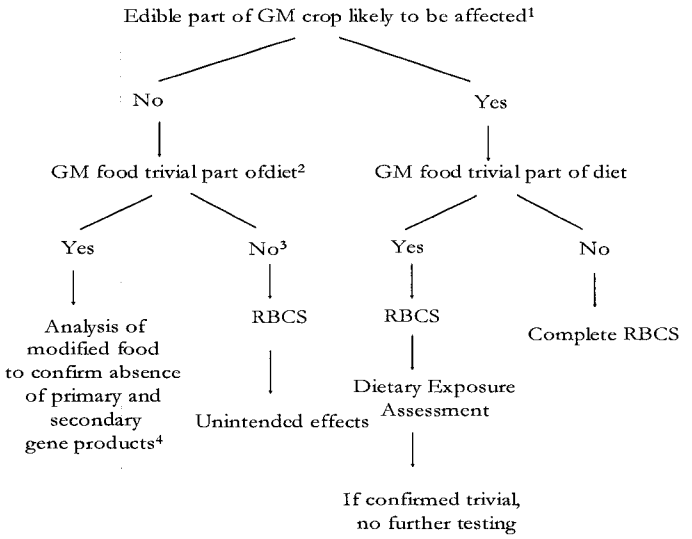
Rules for determining concern levels

For each category of potential effects, the factors and criteria are aggregated into three concern levels for testing that are progressively more extensive. The highest concern level selected for any factor for each category of effect will determine the concern level for that category. In determining the concern level for a factor, one compares the core information available on the GM food with the criteria for each factor in the RBCS sequentially from the lowest to the highest concern level and selects the criterion/criteria in a specific level that best describe the product. Core information emphasizes those differentiating elements of information that are normally compiled or generated as part of developing the desired crop that can be easily assessed (see Appendix A).

The proposed testing of both intended and unintended effects follows a progressively extensive gradation. Briefly, GM foods in the first concern level will require no or minimal further testing, primarily to confirm the absence of adverse health effects expected based on available information. For intended effects, the second concern level is intended for GM foods containing primary and/or secondary gene products that have prior dietary exposure but are modified in exposure level, or are novel enzymes or proteins at low expression levels, for which there is some prior knowledge about the compounds in question to guide the type of testing. For unintended effects, an expanded compositional analysis of the GM food in a second concern level is indicated. The third concern level includes GM foods containing substances that are novel or substantially different from known substances in comparable foods or in prior dietary exposure. The types of substances and GM foods included in each concern level are described in more detail in Part II of this document, as well as the type and extent of testing.

Respondent I.D.

A. EXPOSURE TO THE FOOD DERIVED FROM THE MODIFIED CROP - This is a screening step to determine if the use of the classification scheme in whole or in part would be necessary.



[1] The edible part of a GM crop is considered *not* to be affected in tissue-specific modification where there is
(1) no expression, metabolism, or storage of the modified substance in the edible part, and
(2) no significant contamination from other parts of the modified plant during post-harvest handling and processing, or
(3) the edible part is used as a food ingredient in a limited form as a highly refined and well-defined chemical entity (e.g., refined oil from oilseed rape and cottonseed).

[2] A food consumed in very small amounts (e.g., spices and seasonings) would be considered a trivial part of the diet and assessment of unintended effects would not be necessary. Where the edible part of a GM crop is affected, the exposure level of gene product(s) introduced or modified should be estimated using detailed dietary exposure assessment and compared to a toxicologically insignificant level (e.g., threshold for toxicological concern for the chemical class where applicable) to determine the need for further testing.

[3] In the case of a highly refined ingredient (e.g., refined oil), assessment of unintended effects would not be necessary.

[4] The analysis should include samples representative of post-harvest handling and processing.

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B. INTENDED EFFECTS FROM PRIMIARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED

1- POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - applicable to both protein and non-protein substances, unless stated otherwise.

	Criteria for Determining Concern Levels for Testing		
Factors	**I**	**II**	**III**
Rules for determining concern levels	Meet criteria for <u>all</u> factors listed.	Meet criteria for factor 2, *or* factor 1 <u>and</u> any of the other factors listed.	Meet criteria for factor 1 <u>and</u> any of the other factors (3-6).
Dietary exposure to primary and secondary gene products			
1. Prior dietary exposure (history of safe use in relation to food)	a/ From existing food sources that have been widely consumed	a/Not from existing food sources that have been widely consumed - enzymes or proteins at low expression level[1]	a/Not from existing food sources that have been widely consumed - excluding enzymes or proteins at low expression level, *or* b/ Secondary gene product resulting from a completely new gene
2. Expected change in total dietary exposure (based on theoretical maximum estimates)	a/ Not significantly elevated/altered OR b/ Elevated, <u>and</u> c/ Wide margin of exposure, or below established upper limit (e.g., ADI, tolerance level or equivalent), or below toxicologically significant levels (e.g., threshold for toxicological concern for the chemical class where applicable)	a/ Significantly elevated/altered in exposure from existing food sources that have been widely consumed, <u>and</u> b/ Insufficient prior knowledge regarding safety at elevated/altered levels of consumption	a/New dietary exposure
Nature of primary and secondary gene products			
3. Structure-activity characterization	*Enzymes or proteins expressed in a different species, genus or cell type*: a/ No concerns with all essential elements of protein characterization: ▪ no sequence homology with known toxins ▪ rapid and complete digestion in standard tests ▪ structural and functional similarity to comparator/source protein	*Enzymes or proteins at low expression level*: a/ Concerns with or insufficient data on one or more of the essential elements of protein characterization (listed for level I)	a/ Structurally or functionally dissimilar to substances that have been widely consumed from foods, or b/ Not well characterized

[1] Include proteins with enzymatic activity or are expressed at low levels (less than 0.1% of the total protein or less than 1 ppm in a food, or intake of the protein of less than 0.1 mg/kg bw/day [König et al., 2004].

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	Criteria for Determining Concern Levels for Testing		
Factors	**I**	**II**	**III**
Rules for determining concern levels	Meet criteria for all factors listed.	Meet criteria for factor 2, *or* factor 1 and any of the other factors listed.	Meet criteria for factor 1 and any of the other factors (3-6).
	Other substances: b/ Structurally and functionally similar to natural food constituents c/ Not applicable (no primary gene product expressed due to gene silencing)		
4. Prior knowledge of function/safety (including food ingredients derived from the modified crop, impurities or by-products of ingredient processing) * Relevant where modified food is prepared/processed differently from a food it replaces	a/ Not implicated in mammalian toxicity or antinutritional effect under expected conditions of consumption of the modified food, or b/ Specificity of biological function or mode of action well-established with no potential for adverse human health effects	*Enzymes or proteins at low expression level*: a/ Implicated in or potential for mammalian toxicity or antinutritional effect, or b/ Specificity of biological function or mode of action not well-established, or c/ Insufficient evidence to support mitigation of toxicity or antinutritional effects by food preparation/processing methods under expected conditions of consumption of the modified food*	a/ Limited data on mammalian toxicity or antinutritional effect under expected conditions of consumption of the modified food, or b/ Specificity of biological function or mode of action not well-established, or c/ Macro-substances of nutritional or physiological relevance
5. Degradation products			
5.1. Metabolism in the edible part of the modified plant	a/ No unusual metabolites expected, or b/ Unusual metabolites identified - levels below established upper limit or toxicologically significant levels	*Enzymes or proteins at low expression level*: a/ Unusual metabolites expected or identified (e.g., biologically active peptides)	a/ Unusual metabolites expected or identified
5.2. In mammalian metabolism	a/ No unusual metabolites expected, or b/ Unusual metabolites identified - levels below established upper limit or toxicologically significant levels	*Enzymes or proteins at low expression level*: a/ Unusual metabolites expected or identified, or b/ Potential transfer into body tissues	a/ Unusual metabolites expected or identified
6. Interactions among gene products introduced/modified (in the same or separate transformation events) and/or with food constituents	Potential for synergistic toxicity, anti-nutritional effects, or cumulative effects on biochemical pathways - All substances		
	a/ Not applicable, or b/ Can be ruled out based on known functions of the gene products	a/ Unlikely based on known functions of the gene products	a/ Likely/probable, or b/ Undetermined /unable to assess based on available information
Concern level =			

Respondent I.D.

2- POTENTIAL FOR ALLERGENIC EFFECTS - *Applicable to protein primary or secondary gene products*

	Criteria for Determining Concern Levels for Testing		
Factors	**I**	**II**	**III**
Rules for determining concern levels	Meet criteria for <u>all</u> factors listed.	Meet criteria for factor (7 or 9). Other factors can be used to guide specific testing.	Meet criteria for factor 7 or 8. Other factors can be used to guide specific testing.
Nature of donor organism (gene source)			
7. Allergenicity of donor organism (gene source) or genetically related organism (if allergenicity of donor organism is not known)	a/ Not a known source of allergenicity or gluten-sensitive enteropathy, or b/ Not applicable (donor and host plants are the same)	a/ Source of allergenicity or gluten-sensitive enteropathy	a/ Source of allergenicity or gluten-sensitive enteropathy - uncertain or undetermined
Nature of primary and secondary gene products			
8. Exposure history	a/ Prior exposure <u>and</u> b or c b/Endogenous and well characterized modification, or c/Post-translational modification not applicable or can be ruled out, or d/ Not applicable (not expressed due to gene silencing)	a/ Prior exposure <u>and</u> b and c b/ Novel to the host plant (from a non-plant source), <u>and</u> c/ Post-translational modification identified or not ruled out	a/ No or limited documented prior human dietary exposure
9. Protein characterization	a/ No sequence homology with known allergen, <u>and</u> b/ Rapidly digested in pepsin digestion	a/ Suspected sequence homology with known allergen, or b/Not rapidly digested in pepsin digestion, or c/ Not adequately characterized	
10. Non-dietary routes of exposure to workers	a/ No concerns	a/ Anemophilous host plant, and b/ High quantity of wind-born pollen OR c/ Potential for significant grain dust exposure	
Applicable if secondary gene product is a structural protein from an allergenic source			
11. Level of introduced/ modified protein in food	a/ Minor component (<1% total protein)	a/ Major component (>1% total protein)	
12. Knowledge of functional category of introduced/ modified protein in food	a/ Not a member of a protein family associated with allergenicity	a/ Belongs to a protein family associated with allergenicity b/ Uncertain or undetermined	
Concern level =			

Respondent I.D.

C. UNINTENDED EFFECTS IN THE MODIFIED FOOD FROM PLEIOTROPIC AND SECONDARY METABOLIC CHANGES RESULTING FROM GENETIC MODIFICATION OF THE HOST PLANT

1- POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS

†Basic targeted compositional analysis includes: (1) key nutrients, toxicants, antinutrients, and endogenous allergens of the *host* plant and of processed fractions, where applicable, (2) other constituents predicted from molecular characterization and biochemical pathways potentially affected, (3) microbial toxin (if increased disease susceptibility of the modified plant is a concern), and (4) toxic heavy metals (if increased uptake/accumulation in the modified plant is a concern).

	Criteria for Determining Concern Levels for Testing		
Factors	**A-1**	**A-2**	**B**
Rules for determining concern levels	Meet criteria for <u>all</u> factors (1-4).	Meet criteria for <u>any one</u> of the factors (1-4).	Meet criteria for <u>any one</u> of the factors (1-4).
1. Basic targeted compositional analysis of modified food†	a/No significant change from appropriate comparator	a/ Incomplete analysis, or b/Consistent and significant change from appropriate comparator - limited characterized changes with defined concerns	a/Consistent and significant change from appropriate comparator - extensive changes
2. Novelty of introduced/ modified gene function or pathway in the host plant (expected degree of regulatory control in the modified plant)	a/ Gene encoding product(s) that have well-characterized function in the modified plant	a/ Function of the gene modified not adequately characterized	a/ Completely new gene, or b/ Extensively modified (mutated) gene, or c/ Manipulation of regulatory gene(s) or signal transduction pathway, or d/ Inadequately characterized complex pathway(s) of secondary metabolism
3. Health consequences of instability of the modified trait‡ ‡Therapeutic health trait is outside the scope of RBCS for foods derived from GM crops	a/ Not applicable - modified trait not health-related, or b/ Nutrition-related trait, and the food is not a major source of the affected nutrient in the diet		a/ Modified trait (e.g., reduced allergenicity) has potentially serious health consequences, or b/ Nutrition-related trait and the food is a major source of the affected nutrient in the diet
4. Safety of donor (source) or genetically related organisms, or of the host plant (in the case of induced mutation or gene silencing) - as a source of known toxicants, antinutrients at normal consumption levels or patterns by normal consumers	a/ Not a source, or b/ A source - transfer to or expression of undesirable characteristics in the host plant can be ruled out (based on validated chemical, biochemical or biological assays)	a/ A source - transfer to or expression of undesirable characteristics in the host plant cannot be ruled out (based on validated chemical, biochemical or biological assays) See factors 5-7	a/ Safety uncertain or undetermined

Respondent I.D.

	Criteria for Determining Concern Levels for Testing		
Factors	**A-1**	**A-2**	**B**
If (1) the donor organism (or genetically related organism, or the host plant in the case of induced mutation) is a source of known toxicant or antinutrient in an uncharacterized gene modification (e.g., induced mutation, conventional wide-cross hybridization), OR (2) the donor organism is a source of known toxicants or antinurtrients and the transfer of undesirable characteristics of the donor organism to the host plant cannot be ruled out in modification involving gene silencing, or the introduction of a novel substance in the modified crop, the following additional factors will apply.			
	Meet criteria for <u>all</u> factors (5-7).	Meet criteria for <u>any one</u> of the factors (5-7).	
5. Substrate specificity of gene product (if it has enzymatic activity)	a/ High, or b/ Not applicable (does not have enzymatic activity)	a/ Low, or b/ Uncertain or undetermined	
6. Complexity and interconnectivity of pathway introduced/modified (as characterized by biochemical or metabolic fate studies in the modified plant)	a/ Well-defined simple pathway, e.g., - single or limited number of enzymes, and - limited number of metabolites that are not precursors or intermediates in other pathways in the modified crop	a/ Well-defined complex pathway, e.g. - multiple enzymes, and - shared enzymes and intermediates	
7. Extent of genomic disruption of host plant and characterization of gene insertion/modification site	a/ Limited and well-characterized (e.g., absence of fusion protein predicted by bioinformatics analysis or confirmed by acceptable protein detection methods)	a/ Extensive, or b/ Insufficiently characterized, or c/ Uncharacterized	
Concern level =			

Respondent I.D.

2- POTENTIAL FOR ALLERGENIC EFFECTS - ***Applicable if the modified food meets any of the criteria for level A-2 or B above regarding the potential for unintended toxic and antinutritional effects.*** For a summary of the characteristics of GM foods in level s A-2 and B, see p.20-21.

The focus is to assess the potential for (1) unintended transfer of an allergenic protein from an allergenic donor source to the modified food, (2) increase in the level or potency of an endogenous allergenic protein in the modified food if the host plant is an allergenic source.

	Criteria for Determining Concern Levels for Testing		
Factors	**A-1**	**A-2**	**B**
Rules for determining concern levels	Meet criteria for <u>both</u> factors listed.	Meet criteria for <u>any one</u> of the factors listed.	Meet criteria for <u>any one</u> of the factors listed.
8. Allergenicity of donor or genetically related organism (if that of the donor organism is unknown)	a/ Not a known allergenic source b/ An allergenic source - potential for transfer to host plant can be ruled out (e.g., by IgE binding assays on food extracts, as described for testing allergenic potential from intended effects) c/ Not applicable (donor and host plants are the same)	a/ An allergenic source - potential for transfer to host plant not ruled out	a/ Uncertain or undetermined
9. Allergenicity of host plant	a/ Not a known allergenic source	a/ An allergenic source	a/ A novel food source in target population
Concern level =			

Respondent I.D.

PART II - GRADED TESTING OPTIONS

The proposed graded testing options outline the types of testing, the testing objectives, and the indications when certain types of test may be required. They correspond to the respective concern levels for testing in the risk-based classification scheme and are built on applicable guidelines, recommendations, and testing practices for a range of food chemicals and related products.

The testing options provided here are intended to illustrate the progressively extensive nature of testing from level I to level III (intended effects) and from level A to level B (unintended effects). A system of graded testing can also provide a framework for enhancing the transparency in communicating premarket product review decisions. They are not intended to be prescriptive or rigid, as regulatory requirements may vary among jurisdictions. The proposed testing options indicate the baseline information that will likely be required in evaluating a GM food in a specific concern level for a particular category of potential hazard. In some cases, if product-relevant data are available from the literature, actual testing of a given GM food may be reduced. In other cases, further testing may be required to resolve issues that are raised in baseline testing. It is also recognized that as new testing methods are developed and validated, the range of testing options will expand and some testing options suggested here may be replaced.

When tests are conducted on the purified form of the protein that is from a source other than the modified crop, demonstration of structural, biochemical and functional equivalence to the protein modified in the plant will be needed before conducting further testing.

Respondent I.D.

A. INTENDED EFFECTS FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED

1- POTENTIAL TOXIC AND ANTINUTRITIONAL EFFECTS

Toxicity tests are to be conducted on the purified form of the test substance (typically in one rodent species for protein substances), unless whole food is specified.

Level	Types of substances included in each level and testing for each concern level
I	**Types of substances included in level I** To be classified as level I, a protein or non-protein substance must meet the criteria for all the factors. a/ History of safe use in relation to food and no toxicologically significant increase in expected exposure based on theoretical maximum estimates b/ Known safety under expected conditions of consumption of the modified food c/ No unusual degradation metabolite expected or identified at significant levels in the modified plant and in mammalian metabolism d/ No interactions with adverse health implications expected among gene products and with food constituents e/ For enzymes or proteins expressed in an alternate species, genus, or cell type: no concern with essential elements of characterization (no sequence homology with known mammalian toxins; rapid and complete digestibility under simulated conditions; biological activity comparable to an appropriate comparator or source protein with known safety profile) **Testing** ***Protein substances (novel enzymes and proteins at low expression levels)***[2] a/ **Acute oral toxicity**: to confirm the absence of toxicity (as suggested by information obtained from protein characterization) ***Non-protein substances (including metabolites of gene products)*** a/ **No toxicity testing** (only non-protein substances that are of no toxicological concern based on all aspects of their exposure and nature will be classified as level I, including their direct metabolites, if applicable)

[2] Protein at low expression level may be defined as less than 0.1% of the total protein or less than 1 ppm in a food, or intake of the protein of less than 0.1 mg/kg bw/day intake [König et al., 2004]. Relative to the types of proteins likely to be introduced in GM crops for food use, toxic proteins are known to act via acute mechanisms. Microbial toxins that produce long-term toxic effects are also acute toxins. Variation in enzyme structure and function that improves desired enzyme function is unlikely to result in the creation of a toxic protein. Chronic toxicity and mutagenicity testing are not required if appropriate quality assurance measures and a systematic approach to safety assessment are taken [Sjoblad et al. 1992; Pariza and Johnson, 2001]. Longer tem (90-day) study may be conducted to increase the level of confidence in deriving an ADI.

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Level	Types of substances included in each level and testing for each concern level

II Types of substances included in level II

Substances in level II consist of 2 main groups.

a/ Existing constituents in the diet: significant increase or alteration in expected dietary exposure for which insufficient prior knowledge exists regarding their safety at elevated/altered levels of consumption

b/ Enzymes and proteins at low expression level that do not meet the criteria for level I for one or more of the factors in the scheme, including: concerns or uncertainty with (i) toxic or antinutritional effects in mammals, or (ii) some elements of protein characterization, or (iii) metabolism in the modified plant or in mammals

c/ Unlikely interactions among gene products introduced/modified and/or with food constituents

Testing

Protein substances (novel enzymes and proteins at low expression levels)[2]

a/ **Repeated dose oral toxicity** (28-day, in one rodent species): to detect potential toxicity associated with the source organism; to identify NOEL (no observed effect level) and ADI (acceptable daily intake)

Non-protein substances (including metabolites of gene products)

a/ **Acute oral toxicity**: to explore the toxic potency of a test substance

b/ **Repeated dose oral toxicity** (90-day in a rodent species)[3]: to identify NOEL and ADI for a test substance with prior dietary exposure or prior knowledge of function and/or toxic properties (level II); to assess the need for longer term studies (level III)

c/ **Specific toxicity tests**: may replace general 90-day oral toxicity test based on prior knowledge of function and/or toxic or antinutritional properties (e.g., toxicological effects on the gastrointestinal tract)

d/ **Genotoxicity** (*in vitro*)

Macro-substances of nutritional or physiological significance (including protein substances)

a/ **Digestibility/Growth/Performance trial (animals)**: to conduct preliminary assessment of nutritional quality

b/ **Digestibility/Metabolic/Tolerance studies (humans)**[4]: to confirm the absence of major adverse health effects supported by animal testing; to assess nutritional quality and/or metabolic effects in some at-risk groups (e.g., testing polysaccharides with altered absorption properties in individuals with diabetes)

[3] Subchronic studies may not be required if a very wide margin of exposure (>2000-4000 times the estimated human exposure in 28-day short term or acute oral toxicity tests) [U.S. FDA, 2003].

[4] If animal tests reveal any toxicological concerns, these should be resolved before conducting studies in humans. Pharmacodynamic or effect studies, if indicated, are restricted to the study of acute and inherently reversible changes [Wilks, 2001].

Level	Types of substances included in each level and testing for each concern level

III Types of substances included in level III

Substances in level III are primarily novel substances (other than novel enzymes and proteins at low expression levels) that do not have a history of widespread dietary exposure in the target population. They include novel substances not from existing food sources that fit one or more of the following descriptions, including substances of nutritional or physiological relevance.

a/ Products of a completely new gene

b/ Structurally or functionally dissimilar to dietary substances that have been widely consumed

c/ Implicated in mammalian toxicity or antinutritional effect, or insufficient prior knowledge regarding their safety or specificity of biological action, including their metabolism and degradation products in the modified plant or in mammalian metabolism

d/ Likely, probable or undetermined potential for synergistic toxic or antinutritional effects among gene products or with food constituents

Testing

Non-protein substances (including metabolites of gene products)

In addition to tests in level II:

a/ **Lifetime feeding/Carcinogenicity/Reproductive/ Metabolism studies** (animal studies, including a second species or specific animal model): *normally required for novel micro-substances and isolated novel food components as part of core studies*[5]*, unless otherwise justified, or based on chemical structure and exposure level considerations*[6]*, or indicated by results of 90-day oral toxicity*

Macro-substances of nutritional or physiological significance (including protein substances)

In addition to tests in level II:

a/ **Repeated dose oral toxicity** (90-day in a rodent species), supplemented by absorption and metabolism studies in animals and humans[7]

b/ **Lifetime feeding/Carcinogenicity/Reproductive/Metabolism studies** (animal studies, including a second species or specific animal model): *may be indicated depending on prior knowledge, the level of concern and biological effects anticipated from pre-test considerations and/or the results of 90-day study*

c/ **Protein quality studies**: *may be applicable to a protein substance introduced/modified in the GM food that is a source of dietary protein if there are concerns from growth/performance studies*

d/ **Absorption/Bioavailability study**: *indicated if the potential for interactions among gene products and food constituents exists*

e/ **Clinical growth studies and other types of testing**: *applicable to GM ingredients used in foods as the sole or primary source of nutrition subject to specific regulations or guidelines, e.g.,* infant formulas - a hierarchical approach to premarket safety assessment and in-market surveillance has been proposed[8].

Toxicity tests on whole food

a/ **Repeated dose oral toxicity** (90-day in a rodent species): *indicated if there is the potential for interactions among gene products or with food constituents*

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[5] European Commission. Recommendation of 29 July 1997 (97/618/EC); The Scientific Committee on Food, 2001.

[6] U.S. FDA, 2003.

[7] For macro-substances of nutritional or physiological relevance, animal toxicity studies are not likely to be universally applicable due to difficulties in maintaining nutritional balance when fed at high doses in animal diet. The type and extent of animal studies required is considered on a case-by-case basis [U.K. Food Standards Agency, 2001]. Typically, a 90-day subchronic study in a rodent species is likely to be the minimum reasonable duration, and may often be the sufficient duration necessary, to provide data adequate for use in evaluating safety or to determine whether there is need for further studies [Howlett et al., 2003]. To compensate for the lack of dose accentuation for macro-substances in toxicity testing, studies of absorption, distribution, metabolism and elimination become more important in animals as well as in humans [Hatton, 1996; LSRO Report, 1999].

Level	Types of substances included in each level and testing for each concern level
II and III	Additional tests may be applicable ***Macro-substances of nutritional or physiological significance*** a/ **Fermentation studies**: *applicable to poorly digested and absorbed substances to assess potential changes in gut morphology or biochemistry, or composition or nature of the gut flora* ***All substances*** Dietary exposure assessment - detailed: to identify (1) distribution of exposure and population subgroups likely to have high intakes of the substance of interest, and (2) alteration in the intakes of nutrients, where the modified food replaces other foods that are important dietary sources of the nutrients; *indicated if changes are expected in the consumption of (1) the modified food, (2) a food or foods replaced by the modified food, (3) the gene product(s) of interest, or (4) if there is new dietary exposure to a novel substance of nutritional or physiological significance*

[8] The proposed hierarchical approach to premarket safety assessment includes: a two-level assessment approach to assess organ, immunological, and endocrinological systems, and a three-level approach to assess developmental outcomes [U.S. Institute of Medicine, 2004].

Respondent I.D.

2- POTENTIAL ALLERGENIC EFFECTS - *Applicable to protein primary and secondary gene products*

Allergenicity tests in applicable guidelines focus on assessing the potential for IgE-mediated allergenic effects of introduced/modified protein substance. The combined use of IgA and IgG anti-gliadin antibodies are available in *in vitro* formats for assessing gluten-sensitive enteropathy. Recent demonstration that tissue transglutaminase is the main target of the autoimmune response has led to the development of several specific IgA ELISAs based on guinea pig, or preferably, human tTG, with high sensitivities and specificities [Ahlstedt et al., 2003].

No single test can fully predict the potential allergenicity of any specific novel protein. A weight of evidence approach [Codex Alimentarius, 2003; König et al., 2004; Poulsen 2004] is increasingly favoured over a strict sequential decision tree application of testing strategies [FAO/WHO, 2001]. In practice, a positive result in an earlier test may result in the termination of further development and testing of the GM food. Alternatively, negative results from successive testing may be required to demonstrate that an earlier test produced a false positive result.

For a protein with prior dietary exposure, when the source organism is a known allergenic source or when sequence homology with a known allergen is indicated, a skin prick test and/or food challenge following negative specific serum screen is generally recommended where ethically practical. Alternatively, an *ex vivo* functional test (e.g., cell-based assay using human or humanized animal basophils, Dibbern et al., 2003; Ebo et al., 2004; Hamilton and Adkinson, 2004) would be appropriate in assessing the biological activity of IgE binding [Codex Alimentarius, 2003; European Food Safety Authority, 2004; FAO/WHO, 2001].

For a protein with no or limited prior dietary exposure, where sequence homology with a known allergen is suspected, additional tests are encouraged following negative targeted serum screen [European Food Safety Authority, 2004; FAO/WHO, 2001] in order to assess the potential for sensitization, to increase confidence in the absence of cross-reactivity with known allergen, or to assess the likelihood of a false positive serum screen result. Animal models and T cell epitope identification have been proposed as possibly useful approaches for assessing the potential for sensitization to novel proteins [Helm, 2002; Harding, 2003; Stickler et al., 2003; 2004; Tryphonas et al., 2003]. At present, *ex vivo* functional tests, animal models and T cell epitope studies are considered research methods and are not standard methods for GM food assessment.

Level	Types of substances included in each level and testing for each concern level
I	**Types of substances included in level I** To be classified into level I, a protein substance must meet the criteria for all the factors. a/ Derived from a donor organism that is not known to be a source of allergenicity or gluten-sensitive enteropathy b/ Not subject to post-translational modification of protein structure c/ No concerns arising from sequence homology with known allergens and pepsin digestibility assays d/ No concerns with respiratory routes of release e/ Not a structural protein at a level of more than 1% of total protein in the food and not a member of protein family associated with allergenicity **Testing** ***Proteins with prior dietary exposure*** a/ **Targeted serum screening**: to confirm the absence of cross-reactivity with known allergenic proteins related to the donor organism; *indicated where the donor organism is not a known source of allergenicity or gluten-sensitive enteropathy and there are no other concerns related to allergenicity*

Level	Types of substances included in each level and testing for each concern level

II Types of substances included in level II

A protein substance in level II has one or more of the following characteristics.

a/ Derived from a donor organism that is a known source of allergenicity or gluten-sensitive enteropathy, or concerns with sequence homology with known allergens and/or pepsin digestibility assays.

Additional criteria considered in specific testing include the following:

b/ Potential for post-translational modification of protein structure

c/ Concerns with respiratory routes of release

d/ A structural protein at a level of more than 1% of total protein in the food and a member of protein family associated with allergenicity

Testing

Proteins with prior dietary exposure

a/ **Specific serum screening**: to assess allergenic potential; *indicated where donor organism is an allergenic source*

b/ **Targeted serum screening**: to confirm the absence of cross-reactivity with known allergenic proteins related to the donor organism; *may be indicated where the donor organism is a known source of allergenicity or gluten-sensitive enteropathy and there is a concern with cross-reactivity with known allergenic proteins from other sources*

Additional tests

c/ **Skin prick/double-blind placebo-controlled food challenge**: to confirm the absence of allergenic potential where practical; *indicated if serum screening tests were negative and where human testing can be ethically justified in order to confirm unlikely allergenic potential*

d/ **Functional assay** (e.g., *ex vivo*/passive basophil activation): to confirm the absence of allergenic potential; to assess the clinical relevance of positive serum screening tests; *indicated where skin prick test or food challenge is not practical*

Level	Types of substances included in each level and testing for each concern level
III	**Types of substances included in level III** A protein substance in level III is derived from a novel source with no, limited, or uncertain prior dietary exposure, or there is uncertain or undetermined prior knowledge regarding its potential as a source of allergenicity or gluten sensitive enteropathy. It shares similar characteristics with protein substances in level II in one or more other respects. **Testing** ***Proteins with no or very limited prior dietary exposure*** a/ **Targeted serum screening**: to assess cross-reactive potential; *indicated in case of sequence homology with known allergen* b/ **Specific serum screening of high risk groups**: to assess allergenic potential where practical (e.g., occupational groups with non-dietary exposure); *indicated in case of sequence homology with known allergen* c/ **Animal testing and/or T cell epitope identification**: to assess sensitization potential; *indicated in case of novel protein with more than one characteristic that poses concerns* Post-market monitoring a/ **At-risk groups** (e.g., occupationally exposed groups): to further assess the potential for allergenicity informed by prior testing
II and III	Specific considerations a/ **Inhalation** sensitization: *indicated if the gene product of interest has high level of respiratory exposure* Allergenicity tests on food extracts or partially digested/denatured extracts a/ **IgE binding** (partially digested or denatured protein, or extract of the modified food as commonly consumed)[9]; *indicated if food matrix effect or the effect of food or ingredient processing on allergenic potential is considered important to confirm negative results of testing purified protein; or if there is a concern with pepsin digestibility or heat stability of the protein*

[9] IgE bearing sera raised in the brown Norway rat may be used in place of human sera for comparing the IgE binding capacity of protein fragments at different states of digestion or denaturation [Oehlschlager et al., 2001].

B. UNINTENDED EFFECTS IN THE MODIFIED FOOD FROM PLEIOTROPIC AND SECONDARY METABOLIC CHANGES RESULTING FROM GENETIC MODIFICATION OF THE HOST PLANT

Our limited knowledge about foods that contain naturally occurring toxicants and in particular, the lack of sensitive methods for testing their delayed or chronic effects [Committee on Comparative Toxicity, 1996, p.36; Speijers and van Egmond, 1997], remains a major source of contention and uncertainty in assessing unintended effects of GM foods. While interference with transcriptional regulators for genes encoding biosynthetic enzymes can "wake up" dormant biochemical pathways, the ability to synthesize particular classes of secondary metabolites is commonly restricted to selected plant groups [Osbourn et al., 2003]. For example, by nature cereal grains have relatively few intrinsic toxins, a characteristic also maintained by the domestication process, compared to some other major food crops [Cheeke, 1998, p.87]. Major classes of plant chemicals and their occurrence are reasonably well established [Beier, 1990; Harborne, 1999; Manners, 1996]. It is also well known that chemical diversity and metabolic flexibility is a common feature of plant metabolism. However, the production of secondary metabolites with potent biological activity is likely a rare property of any one molecule [Firn and Jones, 2003]. These observations would support the premise that unintended effects of genetic modification of food crops that have a history of traditional breeding, when they occur, would result in a change in the level of expression of already recognized toxicants in the plant. It is less likely that critical toxicants, inherent in the plant but hitherto unexpressed, will be unintentionally induced by genetic modification [WHO, 1995]. In contrast to traditional breeding of crops with a history of food use, wide-cross hybridization involving untargeted transfer of genetic material from plants without a breeding history for food use will require additional scrutiny. In cases where the safety of the donor plant is uncertain, genetic relatedness among plant species can be a useful guide in identifying botanical chemical constituents of concern [Franck-Oberaspach and Keller, 1997; U.S. IOM and NRC, 2004a].

An emphasis on an analytical approach (including molecular characterization) to assessing the potential for unintended adverse health effects in GM foods has been justified based on its greater sensitivity than animal testing in identifying individual compounds of relevance. For crops modified by conventional techniques, molecular characterization methods have not generally been applied to safety assessment. Applicable to all GM crops regardless of modification method, compositional analysis of basic and judiciously selected constituents in the modified plant, in concert with observable phenotype and function, are judged to be highly sensitive indicators of unintended effects because over 99% of total food composition will be covered. When compared to unmodified comparator plants grown under the same conditions, compositional analytes are part of a highly integrated and interconnected set of regulated enzymatically catalyzed pathways that should reveal metabolic disruptions if levels of selected metabolites are outside of normal ranges [FIFRA-SAP, 2004]. Targeted compositional analysis remains a major component of the assessment of unintended effects of GM foods and can provide a basis for assessing changes in the levels of known antinutrients and inherent plant toxins [Novak and Haslberger, 2000]. Broad application of general profiling techniques is considered to be of limited value in reducing uncertainty associated with unintended effects in the absence of an improved knowledge base of defined chemical food constituents, their natural variations, biological effects, and responses to environmental stress [U.S. IOM and NRC, 2004b]. Premature termination of further development and testing of a product may result due to uncertain interpretation of profiling analysis. In addition, animal testing of complex food products presents considerable technical challenges (e.g., maintaining nutritional balance of the test diet for long duration, low sensitivity without new biomarkers, exaggerated doses, or large sample size) that make interpretation of questionable results difficult [Hattan, 1996]. Toxicological testing of whole GM foods for unintended adverse health effects is therefore not a standard requirement but may be required in specific cases [European Food Safety Authority, 2004].

As part of the assessment of unintended effects, it is generally recognized that basic targeted compositional analysis should include: (1) key nutrients, toxicants, antinutrients, and endogenous allergens of the *host* plant, and of processed fractions, where applicable, (2) other constituents predicted from molecular characterization (e.g., fusion protein) and biochemical pathways potentially affected, (3) microbial toxin (if increased disease susceptibility of the modified plant is a concern), and (4) toxic heavy metals (if increased uptake/accumulation in the modified plant is a concern).

Respondent I.D.

1- POTENTIAL TOXIC AND ANTINUTRITIONAL EFFECTS

Toxicity tests for unintended effects are generally conducted in one rodent species on the whole food (edible part of the modified crop), unless there are particular concerns about certain fractions processed as food ingredients. Testing the effects of specific unintended, novel compound(s) is probably not practical. First, obtaining and reproducing sufficient quantities of the purified form of the compound(s) for testing is not likely to be feasible, even if they are defined and limited. Second, unless how these compounds are produced in the modified plant is understood, their permanent removal from the modified food is unlikely to be successful. In the context of all available information, a positive toxicity test may serve to eliminate the product from further development. A negative test also requires cautious interpretation, where the ability to detect subtle toxic effects may be limited without the use of sensitive biomarkers.

Level	Types of food included in each level and testing for each concern level

A-1 Types of food included in level A-1

Modified foods in level A-1 must meet criteria for all the factors.

a/ No significant alterations in the levels of compounds analyzed in basic compositional analysis

b/ Well-characterized function of the gene product in the modified plant

c/ The modified trait is (i) not health-related, or (ii) nutrition-related but the food is not a major source of the affected nutrient in the diet

d/ The host plant and/or donor organism are not sources of known toxicants and antinutrients, or the transfer of undesirable characteristics from donor organism to host plant can be ruled out

If (1) the donor organism (or genetically related organism, or the host plant in the case of induced mutation) is a source of known toxicant or antinutrient in an uncharacterized gene modification (e.g., induced mutation, conventional wide-cross hybridization), OR

(2) the donor organism is a source of known toxicants or antinurtrients and the transfer of undesirable characteristics of the donor organism to the host plant cannot be ruled out in modification involving gene silencing, or the introduction of a novel substance in the modified crop, all of the following conditions must also be met.

e/ High substrate specificity if the gene product has enzymatic activity

f/ The biochemical pathway modified is simple and the specificity of the introduced/modified enzyme is high

g/ The genomic disruption in the modified host plant can be reasonably characterized

Testing

No further testing

A-2 Types of food included in level A-2

Modified foods in level A-2 are those for which basic targeted compositional analysis may not be sufficient in identifying the presence of compounds of concern, including foods that fit one of the following descriptions.

a/ Incomplete basic targeted compositional analysis

b/ Limited and characterized changes with defined concerns in the altered levels of constituents identified in basic targeted compositional analysis

Level	Types of food included in each level and testing for each concern level

c/ Inadequately characterized function of the gene product in the modified plant

If (1) the donor organism (or genetically related organism, or the host plant in the case of induced mutation) is a source of known toxicant or antinutrient in an uncharacterized gene modification (e.g., induced mutation, conventional wide-cross hybridization), OR

(2) the donor organism is a source of known toxicants or antinurtrients and the transfer of undesirable characteristics of the donor organism to the host plant cannot be ruled out in modification involving gene silencing, or the introduction of a novel substance in the modified crop, one of the following conditions will apply.

d/ Low or uncertain substrate specificity if the gene product has enzymatic activity

e/ The biochemical pathway modified is complex

f/ Genomic disruption in the modified host plant is not sufficiently characterized

Testing

Expanded compositional analysis[10]: includes one or more of the following.

a/ **Metabolite profiling**: to identify and quantify critical toxicants and antinutrients of donor or related organisms based on targeted chemical classes

b/ **Metabolic fingerprinting**: untargeted screening for differences in metabolites between modified and unmodified food and subsequent identification of substances of potential relevance for health based on chemical classes

c/ **Proteomic analysis**: to identify the presence of novel proteins and to quantify their levels

B Types of food included in level B

Modified foods in level B include those that fit any one of the following descriptions

a/ Consistent and significant differences in basic or expanded compositional analysis (compared to an appropriate comparator(s) (from A-1 or A-2)

b/ Containing a completely new or extensively modified gene

c/ The modified trait is health-related with (i) potentially serious health consequences if stability is not maintained, or (ii) potentially significant changes in nutrient intakes

d/ Safety of donor (or genetically related) organism is uncertain or undetermined

e/ Modification involving manipulation of regulatory or signal transduction pathways or inadequately characterized complex pathways of secondary metabolism

Testing

Toxicity testing of whole food or food ingredient[11]

[10] Expanded compositional analysis may include analysis of processed food ingredient derived from the edible part of the modified crop if there is concern that unexpected substances at very low levels in the whole food will be concentrated in the processed fraction.

[11] In practice, toxicokinetic and genotoxicity studies will not be useful in testing whole foods with extensive changes. For complex foods, some assurance of the absence of a potential for genotoxicity may be gained by appropriate investigations of bone marrow or peripheral lymphocytes undertaken as additional elements of repeat feeding studies in rodents [Howlett et al., 2003].

Level	Types of food included in each level and testing for each concern level
	a/ **Repeated dose oral toxicity** (90-day in a rodent species) b/ **Specific toxicity test**: *indicated if changes identified from composition analysis are limited and defined* c/ **Animal growth study**: to assess nutritional effects; *indicated when unintended effects involve substances of nutritional or physiological relevance*
	Postmarket monitoring: to monitor a health-related trait with potentially serious health consequences if stability of the trait is not maintained, including: a/ Regular in-field and post-harvest analysis of marker of interest b/ Establishment and maintenance of segregation and identity preservation systems

Respondent I.D.

2- POTENTIAL FOR ALLERGENIC EFFECTS

Testing of unintended allergenic effects will focus on the whole food. Where gene transfer is not well-characterized, the introduction of allergenic protein from an allergenic donor source should be assessed. Unintended allergenic effects are also related to elevated levels or altered structure or potency of endogenous allergenic proteins in the modified food if the host plant is an allergenic source.

Level	Types of food included in each level and testing for each concern level

A-1 Types of food included in level A

a/ Neither the host plant or the source organism is a source of allergen, or the transfer of allergenic protein from the donor organism to the host plant can be ruled out

Testing

No further testing

Types of GM foods that meet the description for level A-2 or B for unintended effects related to toxic and allergenic potential will also be evaluated for their potential for allergenicity resulting from unintended effects.

A-2 Types of food included in level A-2

a/ The host plant or the source organism (or a genetically related organism) is a source of allergen

Testing

Immunochemical comparison of IgE binding proteins among the modified food, food from the host plant, and food from the donor organism (or applicable fractions): to identify any changes in the level of known allergens; *indicated when the host (modified) plant is a source of allergen and/ or allergenicity of source organism is uncertain*

B Types of food included in level B

a/ The allergenicity of the host plant or the donor organism is uncertain or unknown

Testing

Tests for proteins with no or very limited prior dietary exposure (similar to those for purified protein for intended effects); *indicated if novel protein is detected in clinically significant levels*[12] *in expanded compositional analysis*

a/ **Targeted serum screening**: to assess cross-reactive potential; *indicated in case of sequence homology with known allergen*

b/ **Specific serum screening of high risk groups**: to assess allergenic potential where practical (e.g., occupational groups with non-dietary exposure); *indicated in case of sequence homology with known allergen*

c/ **Animal testing and/or T cell epitope identification**: to assess sensitization potential; *indicated in case of novel protein with more than one characteristic that poses concerns*

[12] Experience with double-blind, placebo-controlled trials suggests that the threshold dose is individually variable but in the range of 1 mg of the allergenic protein per serving of food for the most sensitive individuals [Taylor et al., 2002]. Limited information exists on threshold doses for sensitization for dietary exposure to allergens. It is expected that the induction of allergic sensitization in a previously naïve individual invariably requires exposure to concentrations of the allergen that are greater than those necessary to elicit a response in a sensitized individual [IPCS, 1999].

PART III - LIST OF QUESTIONS

Please use additional paper if necessary for open-ended questions if you are providing comments on a hard copy.

My comments focus on

- □ questions on the risk-based classification scheme (p. 24-26)
- □ questions on the graded testing options (p. 27-28)
- □ questions on both the classification scheme and the testing options (p. 24-28)

Please also complete the following background and other information.

BACKGROUND INFORMATION

a) My expertise (check all that apply)

- □ Toxicology
- □ Immunology
- □ Pharmacology
- □ Biological sciences/Molecular biology
- □ Analytical sciences
- □ Other (please specify)
- □ Plant sciences
- □ Agricultural sciences
- □ Food/Nutritional sciences
- □ Agricultural Biotechnology
- □ Health risk assessment

b) My primary affiliation (check one)

□ Academic □ Government □ Industry □ Other (please specify)

OTHER INFORMATION

Please indicate if you would be willing to participate in a future consultation by assigning concern levels for 2-3 GM food products using the risk-based classification scheme and product information (3-4 pages for each product) provided by the researcher.

□ Yes □ No

If you have not already done so, please indicate your preference (yes / no) for acknowledging your contribution to the project.

- □ Yes, I agree to have my name and affiliation included in the researcher's thesis
- □ No, I do not wish my name and affiliation to be included in the researcher's thesis

THANK YOU FOR YOU PARTICIPATION IN THE STUDY. Please return your response to the questions to: Eunice Chao (echao097@uottawa.ca).

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If you prefer to provide your response by an interview, please e-mail your preference to Eunice Chao (echao097@uottawa.ca) to indicate consent. You may copy and paste the following request in your e-mail. Please allow 2-3 days for a confirmation.

I prefer to provide my comments in an interview* (□ by phone; □ face-to-face). **I am available on (dates, times) _____ for an interview. My phone number is _____. Please confirm.**

* Interviews will normally be conducted by phone. Face-to-face interviews with local participants will be arranged if requested. Please include your name in the request.

QUESTIONS ON THE RISK-BASED CLASSIFICATION SCHEME (PART I)

I-1. Section A. Exposure to the food derived from the modified crop is proposed as a screening step to determine if, or which aspects of, the classification scheme is applicable. Is this screening step useful and clearly laid out? □ Yes □ No

If no, please comment.

For the following questions (I-2-3 on sections B and C of the classification scheme) please indicate your ratings using the scale provided.

I-2. Section B. Intended effects from primary and secondary gene products introduced/modified

1- Toxic and antinutritional effects

I-2.1. Are the **factors** appropriate in determining concern levels for testing toxic and antinutritional effects?

Concern level	Appropriateness		
I	□ Appropriate	□ Appropriate with changes	□ Inappropriate
II	□ Appropriate	□ Appropriate with changes	□ Inappropriate
III	□ Appropriate	□ Appropriate with changes	□ Inappropriate

I-2.2. Are the **criteria** (described on p.2-3 and applied in the classification scheme) appropriate in determining concern levels for testing toxic and antinutritional effects?

Concern level	Appropriateness		
I	□ Appropriate	□ Appropriate with changes	□ Inappropriate
II	□ Appropriate	□ Appropriate with changes	□ Inappropriate
III	□ Appropriate	□ Appropriate with changes	□ Inappropriate

I-2.3. Are the **rules for determining concern levels** for testing toxic and antinutritional effects appropriate?

Concern level	Appropriateness		
I	□ Appropriate	□ Appropriate with changes	□ Inappropriate
II	□ Appropriate	□ Appropriate with changes	□ Inappropriate
III	□ Appropriate	□ Appropriate with changes	□ Inappropriate

2- Allergenic effects

I-2.4. Are the **factors** appropriate in determining concern levels for testing allergenic effects?

Concern level	Appropriateness		
I	□ Appropriate	□ Appropriate with changes	□ Inappropriate
II	□ Appropriate	□ Appropriate with changes	□ Inappropriate
III	□ Appropriate	□ Appropriate with changes	□ Inappropriate

I-2.5. Are the **criteria** (described on p.2-3 and applied in the classification scheme) appropriate in determining concern levels for testing allergenic effects?

Concern level	Appropriateness		
I	□ Appropriate	□ Appropriate with changes	□ Inappropriate
II	□ Appropriate	□ Appropriate with changes	□ Inappropriate
III	□ Appropriate	□ Appropriate with changes	□ Inappropriate

I-2.6. Are the **rules for determining concern levels** for testing allergenic effects appropriate?

Concern level	Appropriateness		
I	□ Appropriate	□ Appropriate with changes	□ Inappropriate
II	□ Appropriate	□ Appropriate with changes	□ Inappropriate
III	□ Appropriate	□ Appropriate with changes	□ Inappropriate

I-3. Section C. Unintended effects in the modified food from pleiotropic and secondary metabolic changes resulting from genetic modification of the host plant

1- Toxic and antinutritional effects

I-3.1. Are the **factors** appropriate in determining concern levels for testing toxic and antinutritional effects?

Concern level	Appropriateness		
A-1	□ Appropriate	□ Appropriate with changes	□ Inappropriate
A-2	□ Appropriate	□ Appropriate with changes	□ Inappropriate
B	□ Appropriate	□ Appropriate with changes	□ Inappropriate

I-3.2. Are the **criteria** (described on p.2-3 and applied in the classification scheme) appropriate in determining concern levels for testing toxic and antinutritional effects?

Concern level	Appropriateness		
A-1	□ Appropriate	□ Appropriate with changes	□ Inappropriate
A-2	□ Appropriate	□ Appropriate with changes	□ Inappropriate
B	□ Appropriate	□ Appropriate with changes	□ Inappropriate

I-3.3. Are the **rules for determining concern levels** for testing toxic and antinutritional effects appropriate?

Concern level	Appropriateness		
A-1	□ Appropriate	□ Appropriate with changes	□ Inappropriate
A-2	□ Appropriate	□ Appropriate with changes	□ Inappropriate
B	□ Appropriate	□ Appropriate with changes	□ Inappropriate

2- Allergenic effects

I-3.4. Are the **factors** appropriate in determining concern levels for testing allergenic effects?

Concern level	Appropriateness		
A-1	□ Appropriate	□ Appropriate with changes	□ Inappropriate
A-2	□ Appropriate	□ Appropriate with changes	□ Inappropriate
B	□ Appropriate	□ Appropriate with changes	□ Inappropriate

I-3.5. Are the **criteria** (described on p.2-3 and applied in the classification scheme) appropriate in determining concern levels for testing allergenic effects?

Concern level	Appropriateness		
A-1	□ Appropriate	□ Appropriate with changes	□ Inappropriate
A-2	□ Appropriate	□ Appropriate with changes	□ Inappropriate
B	□ Appropriate	□ Appropriate with changes	□ Inappropriate

I-3.6. Are the **rules for determining concern levels** for testing allergenic effects appropriate?

Concern level	Appropriateness		
A-1	□ Appropriate	□ Appropriate with changes	□ Inappropriate
A-2	□ Appropriate	□ Appropriate with changes	□ Inappropriate
B	□ Appropriate	□ Appropriate with changes	□ Inappropriate

I-4. If your ratings above include "appropriate with changes" or "inappropriate", please provide suggestions for changes, or reasons for your ratings.

I-4.1 Suggested changes for concern levels (please identify level, e.g., intended effects, factor 4, level III)

I-4.2. Reasons for ratings

I-5. Overall assessment. Is the conceptual basis of the proposed classification scheme scientifically and logically sound?

I-5.1) for potential toxic, antinutritional effects □ Yes □ No

I-5.2) for potential allergenic effects □ Yes □ No

If no, please comment.

I-6. General comments.

QUESTIONS ON GRADED TESTING OPTIONS (PART II)

Taking into account the classification scheme for determining concern levels for testing and the types of gene products and GM foods included in the respective concern levels, please indicate the appropriateness and adequacy of the testing options proposed for each level, using the scale provided.

II-1. Section A. Intended effects - Testing primary and secondary gene products introduced/modified

II-1.1 - Toxic and antinutritional effects

Concern level	Appropriateness/Adequacy of Proposed Testing Options		
I	□ Appropriate/adequate	□ Appropriate/adequate with changes	□ Inappropriate/inadequate
II	□ Appropriate/adequate	□ Appropriate/adequate with changes	□ Inappropriate/inadequate
III	□ Appropriate/adequate	□ Appropriate/adequate with changes	□ Inappropriate/inadequate

II-1.2 - Allergenic effects

Concern level	Appropriateness/Adequacy of Proposed Testing Options		
I	□ Appropriate/adequate	□ Appropriate/adequate with changes	□ Inappropriate/inadequate
II	□ Appropriate/adequate	□ Appropriate/adequate with changes	□ Inappropriate/inadequate
III	□ Appropriate/adequate	□ Appropriate/adequate with changes	□ Inappropriate/inadequate

II-2. Section B. Unintended effects - Testing whole food for pleiotropic and secondary metabolic changes resulting from genetic modification of the host plant

II-2.1 - Toxic and antinutritional effects

Concern level	Appropriateness/Adequacy of Proposed Testing Options		
A-1	□ Appropriate/adequate	□ Appropriate/adequate with changes	□ Inappropriate/inadequate
A-2	□ Appropriate/adequate	□ Appropriate/adequate with changes	□ Inappropriate/inadequate
B	□ Appropriate/adequate	□ Appropriate/adequate with changes	□ Inappropriate/inadequate

II-2.2 - Allergenic effects

Concern level	Appropriateness/Adequacy of Proposed Testing Options		
A-1	□ Appropriate	□ Appropriate/adequate with changes	□ Inappropriate/inadequate
A-2	□ Appropriate	□ Appropriate/adequate with changes	□ Inappropriate/inadequate
B	□ Appropriate	□ Appropriate/adequate with changes	□ Inappropriate/inadequate

II-3. If your ratings above include "appropriate/adequate with changes" or "inappropriate/inadequate", please provide suggestions for changes, or reasons for your ratings.

II-3.1. Suggested changes for testing options (please identify level, e.g., unintended allergenic effects, level B)

II-3.2. Reasons for ratings

II-4. Overall assessment. Is the range of testing options proposed for testing GM foods appropriate and adequate in identifying

II-4.1) potential toxic, antinutritional effects □ Yes □ No

II-4.2) potential allergenic effects □ Yes □ No

If no, please comment.

II-5. What are the potential limitations of the proposed testing options based on available methods?

II-6. What new methods that will likely be available in the near future would you add to the proposed range of testing options in order to mitigate or reduce current limitations?

II-7. General comments.

PART IV

Appendix A - Background on risk-based classification scheme

A risk-based classification scheme (RBCS) as envisioned in this research is intended to be a tool to efficiently differentiate genetically modified (GM) foods that require more extensive testing and regulatory oversight from those that do not.

- **Focus**: potential human health risks from dietary exposure to foods derived from GM crops. Crops modified to produce therapeutic substances are outside the scope of the scheme
- **Scope**: includes a range of conventional (e.g., induced mutation, tissue culture, interspecific and intergeneric crosses) and modern (e.g., cell fusion, in *vitro* and recombinant deoxyribonucleic acid - DNA) techniques of genetic modification to intentionally change the heritable traits of food crops
- **Intended uses**:
  - classify GM foods based on levels of concern about potential health risks and attendant uncertainty
  - guide the application of testing methods in proportion to their potential to produce human health risks
- **NOT intended** to be used as a health risk assessment tool *per se*, nor for decision-making regarding the approval or disapproval of a GM food

Development of the proposed scheme: This consultation is part of Phase 2 in the development and evaluation of a RBCS as guidance for testing GM foods. The proposed scheme and testing options are developed based on a review of the scientific literature and national/international guidelines on risk assessment of GM foods and related products, while taking into account responses received from previous expert consultations. In a future phase of the project, detailed evaluation of the applicability and utility of the classification scheme will be conducted using a panel of reference products with a range of characteristics.

In developing the risk-based classification scheme, two types of information are distinguished.

(1) Prerequisite information will be met by all GM crops and will *not* provide a basis for differentiation. This includes information on genetic stability, data quality, and the intended purpose and performance of the modified trait, currently required in the assessment of GM crops and derived foods in many jurisdictions.

(2) Core information, in contrast, should have discriminating value for classifying GM foods. For the classification scheme to be useful, the factors and criteria underlying the classification scheme emphasize those differentiating elements of the core information normally compiled or generated as part of developing the desired crop that can be easily assessed.

Core information will be required of all modified crops and derived foods before the risk-based classification scheme can be applied and provides the basis for the factors and criteria underlying the classification scheme. Core information can be based on prior knowledge or basic testing of the modified crop and derived foods as described below.

Prior knowledge

1. Biology of host plant, donor organism (or genetically related organism), including associated toxicity and allergenicity

2. Prior history of food use

3. Function and safety of primary and secondary gene products of interest, including metabolic breakdown products in plant and mammalian metabolism, and processing effects, where applicable

Testing of the modified crop and derived food

4. Structure-activity characterization - focus on protein gene product (including sequence homology with known toxins and allergens, digestibility, biological/enzymatic activity and specificity)

5. Metabolic breakdown products and related metabolic pathways in modified host, where relevant

6. Expression levels of primary and secondary gene products of interest in modified host plant, with focus on edible parts

7. Basic compositional analysis of edible part

8. Expected dietary exposure of the food derived from the modified crop (based on theoretical maximum estimates)

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APPENDIX D

APPENDIX D

EXPERT CONSULTATION 4

Assessment of revised testing options

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4. Expert Consultation 4 - Assessment of revised testing options

4.1. Purpose

The purpose of expert consultation 4 was to conduct a detailed assessment of the proposed testing options that were revised based on results of consultation 3. The assessment focused on the following aspects of the testing options:

- the adequacy of the range of testing options proposed,
- the appropriateness of the proposed testing options in relation to the concern levels in the RBCS,
- areas for improvement,
- potential limitations of the testing options based on available testing methods, and
- identifying new methods that can potentially reduce or mitigate current limitations.

4.2. Recruitment and selection of experts

The recruitment process was reviewed by the Health Sciences and Sciences Research Ethics Board. Approval was included as part of the certificate of ethical approval which is appended as Annex 3-1. A letter informing potential participants of this phase of the research project (**Annex 4-1**) and the consent form (for participants of interviews) (**Annex 4-2**) and equivalent (for participants who provided written comments) (**Annex 4-3**) are also included in the respective annexes. For logistical reasons, selection of participants focused on expert groups and special meetings when individuals with specific expertise were scheduled to convene.

Members of the U.S. National Research Council Committee on *Toxicity Testing and Assessment of Environmental Agents* were contacted for their potential interest in reviewing the proposed testing options and in attending a focus group interview. The committee was selected for the members' expertise in toxicity testing methods and health risk assessment. One structured focus group interview was conducted with four members of the committee. A separate interview was conducted with one member who provided verbal comments. Five additional members who were unable to attend a scheduled committee meeting provided written comments. Among the nine participants, four were affiliated with academic institutions, two with government, two with industry/consulting, and one was retired (formerly with industry). When asked to identify their expertise (all that apply), participants noted the following disciplines: toxicology (6), immunology (1), pharmacology (2), biological sciences/molecular biology (2), health sciences (2), health risk assessment (4), food/nutritional sciences (1), agricultural biotechnology (1), and other (1 - dietary influence on colon cancer).

4.3. Data collection

Participants were provided, in advance of the interviews, the proposed graded testing options with the RBCS as background information, along with a list of 13 questions and a form for rating the appropriateness and adequacy of the proposed testing options and concern levels in the RBCS (**Annex 4-4**). With the exception of two participants who provided their responses to the proposed testing options primarily by answering the listed questions, other participants commented on various aspects of the testing options. During both the focus group and individual interviews, participants sought clarification on the nature and objectives of the project, as well as information relevant to the assessment of foods derived from genetically modified crops.

4.4. Data analysis

Comments provided by respondents were analyzed qualitatively as only one participant responded to the rating form. In order to combine the responses provided by the different data collection formats, the results of consultation 4 were consolidated by grouping the comments provided on various aspects of the proposed testing options obtained from interviews and written responses into themes. The themes were organized to answer the questions that were posed, along with participants' responses to the respective questions. This consolidation of responses is appended as **Table 4-1**. Detailed comments provided by participants that underlie the themes are also included in the table.

The following summary will begin with an overall assessment of the testing options. This will be followed by an outline of the comments grouped into four headings that correspond to the major objectives of this consultation and comments related to specific testing options.

Overall assessment

Participants of the group interview noted that while the conceptual basis of the RBCS was good, the scheme lacked operational details. Written comments from other respondents also noted this limitation. The complexity of the scheme, the absence of clear objectives, and the obscurity of intended users (target audience) also contributed to the difficulty some respondents had in reviewing the document.

Adequacy of the range of testing options proposed

Comments suggest additional areas that require attention: processing effects and applications, future products, sensitive populations, endocrine disrupting and reproductive effects. One respondent noted the lack of context for the testing options, including: dietary context, toxicity concerns in the broader context of the views expressed by opponents of genetic modification, and reasons for genetic modification of crops for food and non-food production.

Appropriateness of the proposed testing options that correspond to the concern levels in the RBCS

One major concern was the absence of an explicit governing principle that underlies the gradation of testing. Doubts were expressed about the practicality of level III on the basis that products in this classification would not be commercially viable. Closely linked to this concern was the proposed "cookbook" approach to testing, with a suggestion that a more tailored approach would be preferable, particularly for level III. The lack of clarity of some criteria and distinctions among concern levels that form the basis for testing is another related concern.

Areas for improvement

The need for flexibility in testing appears to be the main message. It was suggested that developing general strategies for testing and the types of questions that should be answered in reducing uncertainty would be more useful than specifying the types of test to be performed. In addition, a targeted approach was suggested given that one cannot screen for everything. In choosing a test, it is necessary to have some idea about what to look for.

Potential limitations of the testing options based on available testing methods

The limitations, as well as indications and uses, of several testing options were discussed, including duration of toxicity testing, extrapolation of animal toxicity tests to human application, and post-market surveillance. The issue of testing whole food vs. purified substance generated considerable discussion during group interview as well as comments from individual respondents. Questions were also raised about the proposal for separate testing of intended and unintended effects.

Comments related to specific testing options

Novelty. Novelty in relation to history of food use is a major factor in determining the extent of testing of substances present in foods. Comments from three respondents generally supported the proposed criteria for novelty. One respondent noted that the criteria for protein should be more explicit in recognizing that proteins with known structure and function would be similar to those in established food sources even though the gene source of the protein may be from an organism without a history of food use. This takes into consideration that the structure and function of some proteins have been conserved across a variety of species.

Testing of novel proteins. There are currently few guidelines on the toxicological testing of food-related protein substances, with the exception of microbially derived enzymes as food additives. Respondents generally agreed that readily digested proteins are unlikely to pose health hazards with respect to toxic effects. One respondent provided detailed comments on the inappropriateness of the proposed testing

options for novel proteins and proteins suspected to have toxic or antinutritional effects. It was noted that a 28-day study is sufficient even for these categories of protein. In addition, biologically active peptides from incomplete digestion would also constitute a low probability of risk for the majority of proteins introduced into crops, even though it would be difficult to assess whether or not biologically active peptides would be formed in sufficient quantities during normal digestion to have a pharmacologic effect.

Testing of non-protein substances with a history of consumption from foods. Two comments were provided on this question, noting that some toxic non-protein substances are known to occur in foods although they are rare and that where the exposure is increased beyond what is seen naturally, the need for toxicity testing cannot be reduced.

Testing for unintended effects. Respondents who commented on this question generally recognized the value of compositional analysis in guiding the need for further testing. Test sensitivity and validity should be considered in advocating a specific approach. Concerns about unintended effects (e.g., fusion proteins) should also be viewed in the context of their natural occurrences in normal plant physiology.

Validated methods for screening for non-genotoxic carcinogens. Limited comments were received on this question. One comment noted the limitations of validated methods in that these methods were derived and validated with known carcinogens and some entirely new mode of action might escape detection.

Role of in vitro testing. Three respondents who commented on this question generally suggested a limited role, particularly with respect to studying reproductive effects and mutagenic effects of whole food.

Allergenicity testing. Limitations of current methods were noted in the comments, with respect to methods focusing on assessing cross-reactivity and the *in silico* approach to comparing amino acid sequence identity and the basis for the respective cut-off values. One respondent also noted the need for a clear rationale for further testing and the need to evaluate the predictive value of proposed tests.

Scope of adverse health effects considered. Two respondents commented on the need to more clearly define "adverse health effects" and the need for serious attention to be given to the question in view of concerns about GM foods among some population segments.

4.5. Discussion

One of the limitations of consultation 4 was the assumption that the proposed graded testing options could be reviewed without a clear understanding of the objectives and development of the RBCS. While

the characteristics of GM foods in the different concerns levels determined by the scheme were described, the underlying rationale, factors and criteria for concern levels in the scheme were provided only as background information in an appendix. This could make reviewing the appropriateness and adequacy of the proposed testing options more difficult for toxicologists who do not have direct experience in evaluating GM foods.

The proposal is also limited by available and validated methods. One objective of this expert consultation was to explore the potential for new methods to mitigate or reduce current limitations in testing GM foods. No suggestions were provided by respondents in the group interview or in written comments.

There were several similarities in the comments received in this consultation and those from consultation 3. Comments related to the complexity of the scheme were addressed by simplifying the RBCS to make it more user-friendly (see "Discussion" of consultation 3 in Appendix C). To address the deficiency related to the lack of operational definitions, supplementary information was prepared that can be used as a guidance document in applying the RBCS. This document was included in the evaluation of the simplified RBCS in Phase 3 of the project.

As noted in the comments of both consultation 3 and this consultation, there is a need for flexibility in the type of testing that can be used to fill the gaps in information on a product being evaluated, as well as a need for clear articulation of the rationale for proposing a gradation of testing. Reference products developed for Phase 3 of the project provided examples for how the proposal for graded testing can be applied.

Table 4-1. Consultation 4 – Combined focus group and individual comments.

Note: page numbers are those in the questionnaire as given to participants.

Overall assessment

Theme	*Comments*
Structure/construct/concept	▪ Structure is fine; construct/concept is good [G].
Lacks operational details	▪ Question remains: how do you operationalize the scheme? What would be the consequences [G]? - Uncertainty (and the consequences) are hard to deal with, but should be discussed. - Recognize that qualitative criteria require expert judgment. ▪ Novel, physiologically significant, extensively modified, dissimilarity, limited data, unusual degradation metabolites, toxic, well documented, not readily digested, likely/probable/uncertain - these terms illustrate a general problem of your scheme. They will need an operational definition before your scheme can be applied (p.14, level III) [R-3]. *See also "Unclear terms" on the last page.* ▪ One problem is the difficulty of testing food substances at high doses and the lack of dose-response data (e.g., high amount of normal protein can be toxic even if you can test purified protein) [R-1].
Complex scheme	▪ The scheme was complex [R-2].
Objectives not clear	▪ Intent of RBCS and testing scheme not clear [G]. ▪ Difficult to review in the absence of a clear statement of your goals and objectives, the target of your findings etc. [R-3]. ▪ It's helpful to set up your conceptual framework to describe what you are trying to do, so that there is a structure to fit various products [G]. ▪ Is your intention to introduce a paradigm that is different from pharmaceuticals or environmental toxicants [G]? ▪ If your intent is to increase the level of confidence by using this system, I am not sure how much more confidence your system will give you [G].
Intended users (target audience) of the scheme not clear	▪ Different audiences may well require different kinds of evidence, given that the controversies over GM, and GM foods in particular, are very much broader than purely scientific issues [R-3]. - No concern - by whom? In general, you omitted mention of the decision-making process, though that will be critical in practice (p. 11, point #4). - I am afraid no amount of testing will satisfy most of the critics because fundamental concern is not what is presented to be. It's not safety alone. I would still try to involve members of that community in developing any testing program so that some, at least, will be committed to acting on the results. ▪ Level of concern - needs elaboration - whose perspective (I take it that you do not mean level of concern to the general public?) [R-3].

Q1. In your overall assessment, is the range of testing options proposed appropriate and adequate in identifying potential health hazards of GM foods?

- One problem is the difficulty of testing food substances at high doses and the lack of dose-response data (e.g., high amount of normal protein can be toxic even if you can test purified protein) [R-1].

- Effect of cooking and processing should be considered - e.g., protein denaturing can have an effect on allergenicity [R-1].

- No [R-4].

Theme	*Comments*
What's missing	
Processing effects and applications	- Need to consider applications, processing and uses of the GM-crop derived food - i.e. stability, changes in the balance of substances in the food [G].
Future products	- Will future products be accommodated [G]?
Sensitive populations	- Are sensitive populations covered (other than allergenicity)? Consider who will be exposed - e.g., if used by children - should there be a separate category? People with metabolic diseases - issue of labelling [G]. - The likelihood that you have a modification that affects subpopulations with extreme vulnerability is remote, except for allergenicity - do you have an example? Even with allergenicity, there is an environmental component. Unless you are exposed, you probably will not develop reactions [R-1].
Endocrine disrupting effects	- For unintended effects, should assess whether phytoestrogens levels have been inadvertently increased. Substances with endocrine effects may be more common than currently recognized. As for screening for effects of endocrine disruption, the methods have not been figured out [G].
Reproduction effects	- Early screening of reproductive effects needs some attention (although this is not a unique problem of GM food) [R-2].
Dietary context	- Significant increase in the levels of a non-protein natural metabolite in a single food due to modification of a metabolic pathway via genetic engineering does not necessarily increase food safety risk since comparable or higher exposures can be achieved from the consumption of other foods (p. 11, note 2) [R-5].
Reasons for GM	- You should somewhere discuss the reasons for GM, and how they might affect testing - improve nutritional content, pest resistance, extend climate range for growth etc. Discuss also the risks of and testing for GM of products not intended for food use, e.g., corn for alcohol production [R-3].
Toxicity testing in broader context	- Concerns related to toxicity are not a driving force for people who oppose GM. There may be some value in putting toxicity in the broader context in your dissertation [R-3]. - This scheme seems to omit concerns that drive much of the opposition to GM: a) concerns about entirely new kinds of adverse health effects that might not be foreseen, and might not be detected (by tests focused on standard endpoints) b) concerns about gene transfer and ecology c) concerns about nutritional value of foods

Q2. Taking into account the classification scheme for concern levels for testing, are the testing options proposed for each concern level appropriate and adequate for (a) toxic/antinutritional effects, and (b) allergenic effects?

- Complete job [R-1].
- No [R-4]

Theme	*Comments*
What underlies the gradation of testing?	▪ Is the concept of uncertainty/familiarity an underlying trigger for the gradation of testing [G]? - The scheme appears not to assume something is safe if there is uncertainty. - Unfamiliarity and unknown/unpredictable consequences are related - your "high" category is a default of non familiarity - not an independent category, but a dependent variable. ▪ How to gain familiarity is one way of asking questions: what kind of tests do you do to gain familiarity [G]? - Why sequential testing: does determining what tier you are in helps guide what test to start? ▪ There should be an explanation concerning further testing based on the indications (e.g., neurotoxicity found in the basic repeated dose toxicity study) [R-2]. ▪ It takes many years before a GM crop is marketed during which research is conducted to characterize the crop and knowledge is gained. This does not appear to be reflected in the structuring of the scheme and testing options [G].
Level I	▪ Explain that majority of products will be Level I and can move quickly. As background for your scheme, would be useful to acknowledge that current products would fit into level I [G]. ▪ Fixed protocol more appropriate for Level I [G]. ▪ It's hard for me to imagine anything that will meet all the criteria for Level I (p.11) [R-3].
Level II	▪ Too much uncertainty - should go back to the bench; not destined for the marketplace until you are convinced that any effects you see are not relevant to humans [G].
Level III	▪ Need to more clearly identify what will push a product into level III [G]. ▪ Level III testing - not economically worthwhile; eliminates novelty/ advancement [G]. - If the potential for hazard was high, why would you do any testing, unless there is a potential benefit that is large enough to balance a totally unpredictable risk? - From commercial perspective, level III does not exist; the point of testing, including toxicity testing, is to gain familiarity/reduce uncertainty so you can move to level I. - Should level III be more tailored ?
Unclear criteria/distinction between levels or choice of testing	▪ Difference between categories I and II not clear - both have a history of consumption (p.10, box) [R-5]. ▪ Table showing 3 levels of concern - No unusual metabolites expected at pharmacologically or toxicologically significant levels - level of concern II? [R-1] ▪ You have not explained and defended the cut-offs that define the classes or intensity of screening recommended for each class [R-3]. ▪ Pepsin digestibility - no cut-off values for in vitro digestibility [R-1]. ▪ Minimal testing - depends on having answers to some questions that are pretty fuzzy or will have graded answers. E.g., "safely consumed from foods" - how safe is safe if there

Theme	*Comments*
	have been very <u>few</u> reports of problems? If no problems, how much evidence do you need to conclude "safely" consumed (p.10) [R-3]? ▪ Short term oral toxicity vs. digestibility test - are there criteria for the choice of either test (Summary table on p.3) [R-2]?

Q3. If you have concerns about the adequacy or appropriateness of the proposed testing options, please provide suggestions for changes, or reasons for your assessment.

▪ Concern with lack of dose-response; difficult to extrapolate from rat study - human range of intake greater than rat [R-1].

▪ I have two major concerns.
1) The first is that I would not eliminate the use of a second species in testing for toxic/antinutritional effects and allergenic effects. In the former case, a second rodent species or a non-rodent species might provide a sensitivity or specificity of response lacking in the first species chosen. You are in largely uncharted waters and caution is suitable. In the latter instance (allergenicity), the species of choice is one which will respond to allergens. In my experience, neither the rat nor the mouse is a good model.
2) My second concern relates to the classification system. For toxic and antinutritional effects-intended effects, Type II, 2 substances are "Non-protein substances that are naturally occurring food constituents". I would prefer to have these Type II, 2 materials described as "Non-protein substances present at levels that are higher than naturally occurring". If they are present at levels which occur naturally, they might better be classed as Type I substances [R-4].

Theme	*Comments*
Suggestions	
Develop general strategies for testing	▪ Be flexible about what tests to use [G]. - Level III - need tailored set of tests that would answer specific questions and NOT part of a fixed protocol - should not be one size fits all. - Questions rather than protocols: You need to answer a list of questions (there are certain concerns about uncertainty that the society has), but there should be scope for how you answer a question. Focus on the concerns that should be dealt with, and not so much on what type of tests. - Example, rather than standard reproduction test, ask the question: how comfortable are you that the product would not adversely affect reproduction? How to get there may be 2-generation reproduction study, or may be other approaches. - Typically, you need to use established methods, but there should be options for innovators to provide data in any way they want to try to clarify uncertainty and improve safety. ▪ May replace "high/low" with "yes/no" approach (although most things would have a potential for unintended effects) [G].
Not cookbook toxicology	▪ Having a list to do everything is too conservative [G]. ▪ Even for non-proteins, the comprehensive testing should be considered on a case-by-case basis, based on their similarity (or lack thereof) to substances normally present in food, and the levels of exposure. No one formula fits all situations, and this should not be cook book toxicology [R-5]. ▪ Basic vs. expanded targeted compositional analysis - as suggestions, one may include: approaches such as X and Y that one could use to elucidate an uncertainty, rather than a cookbook approach to what one needs to do [G].
Need targeted approach	▪ Recognize that we can't test for everything: test what you suspect [G].

Theme	*Comments*
	- You can have a few general screens - you can't screen for everything. - You need to have some idea what you are looking for before you choose your test. ▪ Why are some tests (e.g., reproduction toxicity) automatically required [G]? - Should look for markers or signals of problems: accumulation, proliferative changes. - Standardized testing does not allow for differences in polymorphisms or genetic diversity (should testing allow for individual variability or assume everyone to be the same?). ▪ General toxicity testing gives you clue how to hone in; pharmacodynamics or biological target - receptor binding also give you clues (e.g., in drug testing) [G]. ▪ Use structure-activity as signal (some hints of this approach in the scheme) [G].

Q4. What are the potential limitations of the testing options based on available methods?

- Whole food testing has many pitfalls. The efforts to establish the safety of irradiated foods involved whole food testing or diets containing large percentages of irradiated foods. The result was serious confounding because of nutritional adequacy or palatability of the diets offered to the experimental animals. How many rats would enjoy a diet consisting largely of strawberries? [R-4].

Theme	*Comments*
Types of testing	
Duration and dose	▪ Duration of study - why 90-day instead of 28-day - what drives that decision/rationale [G]? - What would likely make a substance more hazardous: uncertainty or longer exposure? If not toxic at 28-day, less likely to be toxic longer term unless it accumulates; if there are concerns with 28-day tests, then proceed to 90-day. - Limitation of 28-day - does not allow you to check for *in utero* or child exposure. ▪ High doses or longer duration - if unable to dose at high level, it may be necessary to do longer term study [G].
Susceptibility	▪ Non-protein substances - not clear why additional toxicity testing (28 day study) is needed if the substance is a natural component of food with a modest increase in exposure. Unless the target group is thought to be much more susceptible to the substance than other population groups (p.13 (2)) [R-5].
Practicality	▪ For certain non-protein substances which are intermediates in cellular metabolism, it may not be practically possible to generate sufficient quantities of the substance to support testing in rodents for 90 days [R-5].
Testing whole food vs. purified substance	▪ 2 different issues to consider: effect of the dose vs. effect of the food modifying the effect [G]. - Basic test: is it absorbed when given in purified form vs. bound in food form; absorption efficiency; do toxicokinetics, partitioning, absorption, and mechanistic studies before doing health effect studies. ▪ Whole food testing - I agree with the need for testing whole foods, but there may be some advantages in testing components as well., e.g., if there is reason to think that fat and/or carbohydrate are innocuous, remove them so as to concentrate possible toxins in the residue (p.17) [R-3].
Sequencing the testing of whole food and purified substance	▪ Which test first - testing whole food or purified substance [G]? - Purified substance first: characterize the substance - based on what you know about the purified substance, decide whether you need to do whole food testing. However, with this approach, you may not have tackled the uncertainty with unintended effects (you need to know first what the substance of interest is). - Whole food first: depending on the extent of changes, use that as signal for testing purified substance; expensive to extract and separate substance.
Indications for testing whole food	▪ Whole food testing (for unintended effects) is appropriate if other food components could affect the effect of the substance of interest; it may be possible that unintended genetic consequences would be attenuated by other food components [G]. ▪ Whole food testing may be indicated for synergistic effects or interactions, potential unintended effects, and when it is not technically feasible to produce proteins for testing such as with membrane-bound proteins (p.10, para 3) [R-5]. - Whole food testing … is to identify …[add:] and any products not identified and tested separately [R-3].
Food-specific considerations	▪ For some foods (cereal grains or processed oilseed meal), it is possible to feed rats dietary levels many times higher than humans (75X and higher) (p.13 (a) testing) [R-5].

Theme	*Comments*
Limitations of whole food testing for unintended effects	▪ An unintended substance would have to be exceedingly toxic to be detected in whole food testing; may give you some scoping capacity; more powerful to spike whole food [G].
Alternative to using purified compounds	▪ Purified form of compounds - may not need to be purified if they can be adequately concentrated (p. 20, note 1, para 2) [R-3].
ADME (absorption, distribution, metabolism and elimination)	▪ Not sure if this would be useful for food [G]. - For purified chemicals: can determine if a chemical is bioavailable or if it accumulates; if the substance is not bioavailable, there would be less risk. - Useful for comparison and extrapolation between species. - Start with: a/ chemical assays that can determine if it has properties that allows it to cross intestinal cells and likely to be absorbed; b/ in vitro tests for absorption and permeability.
Protein	▪ Measuring ADME of proteins would be nearly impossible (most proteins are extensively degraded in the gut), unless the protein is highly resistant to digestions, and if small quantities of intact protein could make it past the intestinal barriers, you could monitor the presence of intact protein in the blood using an immunologic method (p.12, testing (c)) [R-5].
Necessary to separate the testing of intended and unintended effects?	▪ It was not clear why separate concern levels were developed for intended and unintended effects. Terms such as "secondary gene products" or "gene products" were confusing. The need to distinguish the testing schemes to the extent proposed is not clear [R-2]. ▪ Given that a specific GM may introduce both intended and unintended effects, you should integrate the testing into a full test package (p.16) [R-3]. ▪ The last point about testing for intended effects (Summary table on p.3, additional tests) - why are they not relevant in general? I find this point relevant for unintended effects as well [R-2].
Post-market surveillance	▪ Questionable usefulness - people can't tell what they ate; more useful to do labelling [G]. ▪ Recognize that that will be something you can't pick up before widespread use (e.g., lettuce that promotes Salmonella growth) [G]. ▪ A product with a concern for serious health consequences should not be left to postmarket surveillance to "take care of the problem" (p. 20, last paragraph, 5th line) [R-2]. ▪ Who will do this? Are there commercial incentives? [R-1].
Pharmacological effects	▪ Pharmacological effects are not generally looked for in food [G].

Q5. What are some new methods that will likely be available in the near future with the potential to mitigate or reduce current limitations, particularly with respect to testing unintended effects?

No response.

Q6. Novelty - are the proposed criteria for novelty appropriate for (a) protein, and (b) non protein substances?

- Novelty criteria appropriate for proteins; not aware of non-proteins being changed in GM crops [R-1].

- Yes [R-4].

- Does GM produce any entirely new products, or sharply increase the amounts of some. You also need to define "food" in terms of GM. A crop might be modified in dozens of ways - is each of these a new food? [R-3].

Theme	*Comments*
Novelty - protein	▪ Known safety profile may be interpreted to mean having a related structure and function to known proteins that poses no safety concerns. A protein with this property should not be considered novel, even if the protein was derived from a non-food source. This takes into consideration that the structure and function of some proteins have been conserved across a wide variety of species (p.10, last sentence). See also comment regarding comprehensive testing of proteins [R-5].

Q7. Novel proteins

a) Is the differentiation of novel proteins into two different concern levels based on expression level justified?
b) Are there specific aspects of novel proteins that require special attention (e.g., immunotoxicity/ immunogenicity) or that are not adequately addressed by the proposed testing scheme, especially proteins that resist digestion?
c) What is the current state of knowledge with respect to prediction of protein toxicity using in silico approaches?

- Novel proteins should be completely digested. For undigested peptides - less likely that they would be a problem, but should be considered [R-1].

- a) Yes b) Not aware of any c) No Comment [R-4].

- Rather than asking about "current" state of knowledge, you should be asking about the expected state of knowledge 10-20 years down the road [R-3].

Theme	*Comments*
Protein testing	▪ Questions were raised about differences in proposed testing for protein vs. non-protein substances (i.e., emphasis only on testing for acute effects) [G]. - Less likely to be a problem if they are readily digested; bioinformatics analysis can be used to identify peptide products of concern. - When does a protein become "novel" (e.g., is a modified protein with higher catalytic efficiency a novel protein?). *See also "novelty" under "Unclear terms".* ▪ Testing of proteins - the proposed battery is not appropriate for proteins, as noted earlier, whether they are novel or not. "Novel" proteins that are readily digested and cannot be systemically absorbed in meaningful quantities in most cases would not pose a meaningful food safety risk. Even if they are structurally related to known toxic proteins, their potential toxicity can be readily manifest in short term feeding studies [R-5]. ▪ Similar comments also apply to the testing of protein macro-substances (p.15, notes) [R-5].
Confirmatory testing	▪ Confirmatory toxicity testing of proteins is generally limited to high dose, acute testing, since the majority of known toxic proteins act through acute mechanisms to exert toxicity (p. 11, last para) [R-5].
Repeat dose testing	▪ Repeat dosing for most proteins is not needed. The only exception would be proteins that may be structurally and functionally related to certain protease inhibitors (e.g., trypsin inhibitors) or lectins, Even for these proteins, their toxicity is manifest after short term

Theme	*Comments*
	dosing for 1-2 weeks. I am not aware of any toxic proteins that require any longer dosing periods to manifest their intrinsic toxicity (p. 11, last para) [R-5]. ▪ The recommendation for the highest concern level for proteins that are structurally and functionally similar to known toxic proteins is overkill and will not generate any more useful information than a short term feeding study. A 28-day study is sufficient even for a lectin, protease inhibitor, neurotoxic protein etc. (p.12, note 2) [R-5]. ▪ Comprehensive testing - A "synthetic gene" and its gene product are not necessarily toxic. If the function and structure of the protein is not known to pose a safety concern, then it does not belong in concern level III even if it is deemed to be "novel" (p.14) [R-5].
Biologically active peptides	Biologically active peptides do not necessarily pose a safety concern, if there is a history of exposure. There may be a need to consider whether the exposure would be additive, or comparable to existing exposures. For reasons stated earlier, this is a rather low probability safety concern (p.14, 4(a)) [R-5].
Metabolic fate	▪ Core information - not reasonable to suggest that you can follow the metabolic breakdown of a protein into all of its peptide fragments and amino acids etc.; also it may or may not be possible to follow the metabolic fate of the catabolic product formed by an introduced protein (enzyme) in the plant or in mammalian tissues if ingested. Such profiling of metabolic fate should be based on a case-by-case need, if there are compelling safety reasons to do so (p.7) [R-5].
Incompletely digested proteins	▪ Potential hazards of proteins that are not readily digested: immunologic and inflammatory responses that could promote neoplasia [G]. ▪ Size of protein is important [G]. ▪ Biologically active peptides from incomplete digestion - constitute a low probability of risk for the vast majority of proteins introduced into crops. Also difficult to assess. You could sometimes identify peptide fragments that might share some similarities with short chain "active peptides". However, whether or not such peptides could be formed during normal digestion in sufficient quantities to have a pharmacologic effect could be difficult to test (p.11, no.5) [R-5].

Q8. Non-protein substances naturally occurring in foods with a history of consumption from foods and modest increased exposure and no similarity in chemical structure to substances of toxic concern: can the need for toxicity testing be reduced (relative to novel substances without a history of dietary exposure)?

▪ Some toxic non-protein substances are known to occur in foods although they are rare - e.g., oils can be modified to be harmful [R-1].

▪ The need for toxicity testing can not be reduced for substances where the exposure is increased beyond what is seen naturally [R-4].

Q9. Unintended effects

a) In your assessment, is the rationale for and emphasis on compositional analysis as the basis for determining the need for toxicity testing justified? If not, what alternative approaches would you recommend?
b) Are there any advantages to "expanded compositional analysis" as proposed in this document, compared to untargeted compositional analysis (using untargeted metabolic profiling/fingerprinting)?
c) How can the sensitivity and power of toxicity testing of whole foods be enhanced?
d) To what extent would "omics"/profiling methodologies be applicable to testing "complex mixtures" such as foods?

- Emphasis on compositional analysis is justified - I don't have alternative approaches [R-1].

- a) Yes (This is fundamental to toxicity testing generally) b) No Comment c) See comments on question 4 [R-4].

- I do not see that compositional analysis will really settle much (though it should). There will be a need to test the modified food product in realistic ways [R-3].

Theme	*Comments*
Testing for unintended effects	
Inherent limitations	▪ How can you figure out unpredictable effects [G]? - Despite an apparently extensive amount of information known about food crops on major constituents and that many crops do not produce toxicants, does the knowledge that already exists allow one to do an extensive constituent analysis [G]? ▪ Sensitivity of analysis is key to detecting unintended effects [R-1].
Test validity	▪ Before advocating untargeted profiling - know the statistical problems/ limitations; otherwise, it would just lead to more testing rather than answering a question [G]. ▪ *In vitro* testing with biotech crops or protein and non-protein substances - the few tests that have been attempted have not been validated and are not considered to have predictive value at this time. Future research may help assess whether *in vitro* tests will provide useful tools in the safety assessment of biotech crops [R-5].
Analytes in testing for unintended effects	▪ Testing of mycotoxins - It is not appropriate to include mycotoxins among analytes unless there is a basis to believe that the inserted gene would contribute to mycotoxin production (p.18) [R-5].
Unintended novel protein	▪ Potential for fusion protein production - be aware that plants normally make aberrant proteins in the cells due to gene mutations, errors in processing proteins, and the cell is able to quickly identify these proteins and degrade them before they can harm the cell. Since this occurs naturally, it puts into perspective the low risk that a fusion protein might pose to the cell [R-5].
Potential for instability of modified trait	▪ Seeds saved at the farm level - some, may be all, contracts for the sale of GM seeds and plants in the US prohibit the saving and replanting of seeds (p. 20, note 2) [R-3].

Q10. Are there validated methods for screening for non-genotoxic carcinogens?

- Non-genotoxic carcinogenesis is developed as a result of cell proliferation (e.g., in inflammation, irritation, infection) as tissue tries to repair itself with increased mitotic rate and increased likelihood of error in replication; tissues susceptible to constant stimulation (e.g., large bowel) are more likely to develop hyperplasia. There are species differences in susceptibility [R-1].

- None validated by a regulatory agency of which I'm aware [R-4].

- Unfortunately, the available methods have been derived and validated with known carcinogens, and some entirely new mode of action might escape detection [R-3].

Q11. In the context of identifying potential toxic/antinutritional effects of GM foods (intended and unintended effects),

a) what should be the role for *in vitro* testing?

b) how can *in vitro* testing be better integrated with in vivo animal and human testing in food safety assessment?

- *In vitro* tests are not useful for studying reproductive effects - many endpoints (> 25) to be examined; need whole animal testing [R-1].

- *In vitro* testing of whole food would be difficult to conduct and to give negative result for mutagenicity [R-1].

- *In vitro* testing would have, at best, a limited role. Better integration will follow validation [R-4].

Q12. Given the limitations of current methods for assessing the sensitizing potential of novel proteins in inducing allergic reactions in susceptible individuals, how should the issue be addressed?

- Need to examine protein breakdown products if they are allergenic; any animal tests? [R-1].
- You have not described the limitations, so I would not know how to respond [R-3].

Theme	*Comments*
Allergenicity testing	
Basis for cut-off in amino acid sequence comparison	▪ Why no concern with amino acid sequence identity < 35% - is this established? What is the basis for this [G]? - Until better methods for secondary and tertiary structure are developed, you can have single amino aid change that alters folding with an antigenic consequence. - Alternative - qualitative criteria? ▪ How will sequence similarity be assessed? This may be a critical item. Structure-activity relationships in toxicology have not been very predictive (p.12) [R-3].
Current limitations	▪ Preventing cross-reactivity is not enough. We must prevent primary reactions to the extent feasible (p.21) [R-3]. ▪ Should there some consideration of the type/severity of allergy (p.23) [R-3]?
Test conditions	▪ More specifics needed (p.22) [R-3]. - Stability - does heat increase or decrease allergenicity, or both? - With reference to the general patterns of stability reported - what are the conditions of the *in vitro* digestibility test? - Something seems wrong between this and the top of the next page.
Need to test purified protein?	▪ Nice if possible, but I think not necessary (p.23) [R-3].
Reasons for further tests unclear	▪ You lay out conditions for adding tests, presumably because some degree of concern persists, but you do not discuss the stopping of tests part way through a sequence because what is already in hand is sufficient to make the product unapprovable (p.23) [R-3].
Consider predictive value	▪ What rates of false positives and false negatives would make such a test useful in this context (p.25) [R-3]? - What would be reliable enough to use in deciding on the next step - a more different or expensive test, approval of GM food for general use, or whatever. A test that has no further impact cannot be useful.
Add background information	▪ Before you get into testing, it might be good to say something about whatever is known regarding the nature and extent of protein changes in GM. I would not worry much about (say) doubling any protein, whatever its initial level, but I would worry about a 10-fold increase (p.22) [R-3].

Q13. Are adverse health effects defined too narrowly in current approaches to identifying potential hazards of GM foods? (e.g., should more attention be given to pharmacological effects of food constituents at high levels; sensitive subpopulations; chronic adverse health effects that are difficult to be attributed to individual components in complex foods)?

- How do you define adverse health effects - do you include drug interactions? [R-1].
- Each of these will surely be brought up by the anti-GM crowd, so each will need serious attention [R-3].

See also comments regarding "narrow focus" in response to Q1.

Unclear terms/other comments

Theme	*Comments*
Low expression vs. physiologically significant	▪ Baseline and control not clear - what constitutes "difference", "physiologically significant - not defined, especially at high level", "increase in exposure - do we know what people are eating" [G]?
	▪ Need sharper differentiation [R-3].
	▪ What is the basis of selecting 1 ppm etc as the threshold for low expression level (p.12, note 1)? [R-5].
Exposure	▪ Exposure of what, to what - if your concern is limited to human health, say so (p. 6, para 2) [R-3].
Susceptible populations	▪ Susceptible populations should include individuals with health impairments - e.g., hypersensitivity and general disability from unrelated diseases (p. 8, para 2) [R-3].
Toxic/antinutritional effects	▪ Not sure if you are trying to distinguish toxicological vs. nutritional effects observed in testing [R-2].
Safety of donor organism	▪ If a gene is obtained from a donor organism that has no history of consumption, you need to confirm that the gene is not related to genes that code for toxic proteins. One has to adequately characterize what has been obtained from non-food sources. However, if the gene codes for proteins of similar structure and function as those commonly consumed in foods, it would not pose a safety risk (p.17) [R-5].
Equivalence	▪ It is likely to be next to impossible to show equivalence in a way that will satisfy critics (p. 10, para 2) [R-3].
Organization/readability of document	▪ It would help the reader if you would put in a table the different safety tests appropriate for each level of concern. The text is hard to follow [R-5].
	▪ Difficult to read -addition of examples would help [R-3].
	▪ Overall - very good. Appendix D - excellent and complete [R-1].

Annex 4-1
Letter to New Contacts - Focus Group - Toxicity Testing

Date

Dear Dr. (name):

As announced by Dr. Daniel Krewski at the last meeting of the Committee on Toxicity Testing and Assessment of Environmental Agents, a research project on a "risk-based classification scheme for testing genetically modified foods" that is currently underway at the University of Ottawa. You are invited to participate in a group interview to provide comments and advice on the proposed classification scheme and corresponding graded testing options.

Project Objectives

The primary objective of the research is to develop and evaluate a risk-based classification scheme as guidance for testing foods that are derived from crops genetically modified by a range of methods, including conventional (e.g., induced mutation, tissue culture) and more modern (e.g., recombinant DNA) techniques. The proposed scheme aims to (1) differentiate and classify genetically modified (GM) foods based on levels of concern about potential health risks and attendant uncertainty, and (2) guide the application of testing methods for GM foods in proportion to their potential to produce human health risks.

Participation

Participants of the group interview will be provided with the graded testing options and the proposed risk-based classification scheme as background information. They will be asked to participate in a focus group interview of 4-8 experts to answer the following types of questions on the graded testing options. The focus of the planned interview will be on the testing of potential toxic and antinutritional effects of genetic modification on foods derived from modified crops. A tentative list of questions for discussion at the group interview is given below.

- In your overall assessment, is the range of testing options proposed adequate in identifying potential toxic and antinutritional hazards of GM foods?
- Taking into account the classification scheme for concern levels for testing, are the testing options proposed for each concern level appropriate for (a) intended effects, and (b) unintended effects?
- If you have concerns about the adequacy or appropriateness of the proposed testing options, please provide suggestions for changes, or reasons for your assessment.
- What are the potential limitations of the testing options based on available methods?
- What are some new methods that will likely be available in the near future with the potential to mitigate or reduce current limitations, particularly with respect to testing unintended effects?

Each participant will receive a copy of the testing options, the classification scheme, and a list of questions about 2 weeks before the group session is held. It will take approximately 1 hour to review the documents. The group session will be limited to 2 hours. A participant will also have an opportunity to review a summary report of the group session he/she attended to verify that his/her comments have been included.

The group session will be audiotaped so it can be reviewed for completeness during data analysis. All data collection and dissemination will be conducted in accordance with confidentiality and ethics guidelines for research involving human participants.

Depending on participants' availability, I plan to conduct the 2-hour group session on

- the evening before the start of the next meeting of the Toxicity Testing Committee, on Monday, July 18, 2005, between 5-9 pm, or
- immediately following the completion of the next meeting of the Toxicity Testing Committee, on Wednesday, July 20, between 5-9 pm

The exact time, location and other details will be provided to confirmed participants.

For individuals who are interested in reviewing the proposed classification scheme and testing options but are unable to attend the group interview, it would also be possible to provide comments in writing or through an individual interview by phone.

The research is being conducted as part of a doctoral thesis at the University of Ottawa Population Health PhD Program. The principal researcher is Eunice Chao, doctoral candidate, who is working with Dr. Daniel Krewski, Professor and Director, McLaughlin Centre for Population Health Risk Assessment, Institute of Population Health, University of Ottawa. The research project is supported by the McLaughlin Centre through a grant from the R. Samuel McLaughlin Foundation and a fellowship awarded by the Canadian Institutes of Health Research. No industry funding is involved in this project.

Contact Information

I will contact you by phone in the coming week to discuss your interest and availability for participation and to answer any questions that you may have. If you prefer to provide a response by e-mail to Eunice Chao (echao097@uottawa.ca), that would also be much appreciated.

If you do not wish to be contacted in the future regarding this study, please advise me that this is your preference. Thank you for your consideration.

Sincerely,

Eunice Chao, MSc
Doctoral candidate
Population Health PhD Program
University of Ottawa

echao097@uottawa.ca

Tel: (613) 562-5800 ext 2367
Fax: (613) 562-5380

McLaughlin Centre for Population Health Risk Assessment
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1 Stewart Street, Room 318B
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CANADA K1N 6N5

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Institut de recherche sur la santé des populations | Institute of Population Health

Annex 4-2. Consent Form - Group Interview

Name of researcher(s): Eunice Chao
Institution, Faculty, Department: University of Ottawa, Population Health PhD Program

Name of supervisor: Dr. Daniel Krewski
Institution, Faculty, Department: University of Ottawa, Faculty of Medicine, Department of Epidemiology and Community Medicine, Institute of Population Health

Project Description
The doctoral research project "A risk-based classification scheme for testing genetically modified foods" is conducted by Eunice Chao of the Population Health PhD Program at the University of Ottawa under the supervision of Dr. Daniel Krewski. The purpose of the research is to develop and evaluate a risk-based classification scheme as guidance for the testing of foods that are derived from crops genetically modified (GM) by a range of methods. The proposed scheme aims to (1) differentiate and classify GM foods based on levels of concern about potential health risks and attendant uncertainty, and (2) guide the application of testing methods for GM foods in proportion to their potential to produce human health risks. The research will be conducted in several phases. You are asked to participate in phase 2 of the project.

Participation
Participants will be provided with the graded testing options, a list of questions, and the proposed risk-based classification scheme as background information. They will be asked to participate in a semi-structured focus group interview of 3-8 experts to respond to the following types of questions on the graded testing options.

- In your overall assessment, is the range of testing options proposed appropriate and adequate in identifying potential health hazards of GM foods?
- Taking into account the classification scheme for concern levels for testing, are the testing options proposed for each concern level appropriate and adequate for identifying (a) toxic/antinutritional effects, and (b) allergenic effects?
- If you have concerns about the adequacy or appropriateness of the proposed testing options, please provide suggestions for changes, or reasons for your assessment.
- What are the potential limitations of the testing options based on available methods?
- What are some new methods that will likely be available in the near future with the potential to mitigate or reduce current limitations?

Normally, a participant will receive a copy of the testing options, the classification scheme, and a list of questions about 2 weeks before the focus group session is held. It will take approximately 1 hour to review the documents. The focus group session will be limited to 2 hours. Focus group interviews will be audiotaped so they can be reviewed for completeness during data analysis. A participant will also have an opportunity to review a summary report of the focus group session he/she attended to verify that his/her comments have been included.

Confidentiality and Anonymity
The contents of the completed focus group discussion will be used only for the purposes of developing a risk-based classification scheme for testing genetically modified foods. A research participant's confidentiality will be respected through the following actions taken by the researcher.

1, rue Stewart | 1 Stewart St.,
Ottawa (Ontario) K1 N 6N5 Canada | Ottawa, Ontario K1 N 6N5 Canada
(613) 562-5660 .Telec./Fax (613) 562-5659

All of the data collected (including written responses, tapes, transcripts, and electronic files) will be tabulated and maintained by the researcher in accordance with confidentiality standards for research involving human participants. The data will be accessible only to the researcher in a locked filing cabinet and a password protected personal computer on the premises of the Institute of Population Health, University of Ottawa. Data files will be kept for 5 years after the completion of the thesis.

Anonymity of a participant's individual views and responses will be protected. No full names will be used in focus group interviews. While group results of the interviews at the aggregate level will be published, and discussed within the research team, and at conferences, no names will be linked to individual views or responses in notes and transcripts of interviews, data analysis, reports or in presentations.

In order to acknowledge participants' contribution to the research project and to make the expert consultation process transparent and credible from a research perspective, the names of individuals and affiliations (the organization where one works or which one represents) will be included in a list as an appendix to the doctoral thesis. Only one combined list of participants for all phases of the research will be published without identifying the specific phase to which they made a contribution.

Research participants agree not to disclose or publish any aspects of the risk-based classification scheme developed by the researcher except with the express consent of the researcher.

Voluntary Participation
There is no direct individual benefit from participating in the study and from answering the questions. All costs associated with participation in the project (long-distance phone calls) will be born by the McLaughlin Centre for Population Health Risk Assessment. We will reimburse local transportation expenses (including taxi or parking costs) when a focus group session takes place at a site outside of a participant's place of employment. For a focus group interview involving travel by participants, the session will be arranged such that it will be adjacent to a scheduled meeting of the participants so any additional travel and related costs for participants will be kept minimal. Where applicable, additional expenses incurred by participants (e.g., accommodation and meals) will be reimbursed.

Participation is completely voluntary. A participant is free to withdraw from the project at any time, refuse to participate, or decline to answer certain questions.

Any information about the rights of a research participant may be addressed to **Protocol Officer for Ethics in Research, University of Ottawa, 550 Cumberland Street, Tabaret Hall, Room 159, Ottawa, Ontario, Canada, K1N 6N5, tel.: (613) 562-5387, e-mail: ethics@uottawa.ca.**

Any questions about the conduct of the research project may be directed to the researcher or her supervisor at the coordinates listed below.

Eunice Chao: **Telephone number:** 1-613-562-5800 ext 2367 **Fax number:** 1-613-562-5380
E-mail address: echao097@uottawa.ca

Daniel Krewski: **Telephone number:** 1-613-562-8261 **Fax number:** 1-613-562-5380
E-mail address: dkrewski@uottawa.ca

I, (please print) _____ (*name of participant*), agree to participate in this study.

Please indicate your preference (yes / no) for acknowledging your contribution to the project.
- □ Yes, I agree to have my name and affiliation included in the researcher's thesis
- □ No, I do not wish my name and affiliation to be included in the researcher's thesis

Researcher's signature: _____ Date: _____

Participant's signature: _____ Date: _____

Annex 4-3. Project information for research participants providing written comments

Name of researcher(s): Eunice Chao
Institution, Faculty, Department: University of Ottawa, Population Health PhD Program

Name of supervisor: Dr. Daniel Krewski
Institution, Faculty, Department: University of Ottawa, Faculty of Medicine, Department of Epidemiology and Community Medicine, Institute of Population Health

Project Description
The doctoral research project "A risk-based classification scheme for testing genetically modified foods" is conducted by Eunice Chao of the Population Health PhD Program at the University of Ottawa under the supervision of Dr. Daniel Krewski. The purpose of the research is to develop and evaluate a risk-based classification scheme as guidance for the testing of foods that are derived from crops genetically modified (GM) by a range of methods. The proposed scheme aims to (1) differentiate and classify GM foods based on levels of concern about potential health risks and attendant uncertainty, and (2) guide the application of testing methods for GM foods in proportion to their potential to produce human health risks. The research will be conducted in several phases. You are asked to participate in phase 2 of the project.

Participation
Participants will be provided with the graded testing options, a list of questions, and the proposed risk-based classification scheme as background information. They will be asked to respond to the following types of questions on the graded testing options.

- In your overall assessment, is the range of testing options proposed appropriate and adequate in identifying potential health hazards of GM foods?
- Taking into account the classification scheme for concern levels for testing, are the testing options proposed for each concern level appropriate and adequate for identifying (a) toxic/antinutritional effects, and (b) allergenic effects?
- If you have concerns about the adequacy or appropriateness of the proposed testing options, please provide suggestions for changes, or reasons for your assessment.
- What are the potential limitations of the testing options based on available methods?
- What are some new methods that will likely be available in the near future with the potential to mitigate or reduce current limitations?

Depending on an individual's expertise, one may elect to focus on those questions that are related to the individual's expertise. Normally, a participant will have 2-3 weeks to provide comments. A participant will also have an opportunity to review a summary report of the consolidated responses at the completion of data analysis to verify that his/her comments have been included.

Confidentiality and Anonymity
The contents of the completed focus group discussion will be used only for the purposes of developing a risk-based classification scheme for testing genetically modified foods. A research participant's confidentiality will be respected through the following actions taken by the researcher.

1, rue Stewart 1 Stewart St.,
Ottawa (Ontario) K1 N 6N5 Canada Ottawa, Ontario K1 N 6N5 Canada
(613) 562-5660 .Telec./Fax (613) 562-5659

All of the data collected (including written responses, tapes, transcripts, and electronic files) will be tabulated and maintained by the researcher in accordance with confidentiality standards for research involving human participants. The data will be accessible only to the researcher in a locked filing cabinet and a password protected personal computer on the premises of the Institute of Population Health, University of Ottawa. Data files will be kept for 5 years after the completion of the thesis.

Anonymity of a participant's individual views and responses will be protected. No full names will be used in focus group interviews. While group results of the interviews at the aggregate level will be published, and discussed within the research team, and at conferences, no names will be linked to individual views or responses in notes and transcripts of interviews, data analysis, reports or in presentations.

In order to acknowledge participants' contribution to the research project and to make the expert consultation process transparent and credible from a research perspective, the names of individuals and affiliations (the organization where one works or which one represents) will be included in a list as an appendix to the doctoral thesis. Only one combined list of participants for all phases of the research will be published without identifying the specific phase to which they made a contribution.

Research participants agree not to disclose or publish any aspects of the risk-based classification scheme developed by the researcher except with the express consent of the researcher.

Voluntary Participation
There is no direct individual benefit from participating in the study and from answering the questions. All costs associated with participation in the project (long-distance phone calls) will be born by the McLaughlin Centre for Population Health Risk Assessment. Return postage for mailed responses of participants from countries outside Canada will be paid in the form of international postal reply coupons, if requested.

Participation is completely voluntary. A participant is free to withdraw from the project at any time, refuse to participate, or decline to answer certain questions.

THE RETURN OF A WRITTEN RESPONSE IMPLIES CONSENT.

Please indicate your preference (yes/no) for acknowledging your contribution to the project*.

□ Yes, I agree to have my name and affiliation included in the researcher's thesis.
□ No, I do not wish my name and affiliation to be included in the researcher's thesis.

*You may include the above information in your response to the questions in the review document.

Any information about the rights of a research participant may be addressed to **Protocol Officer for Ethics in Research, University of Ottawa, 550 Cumberland Street, Tabaret Hall, Room 159, Ottawa, Ontario, Canada, K1N 6N5, tel.: (613) 562-5387, e-mail: ethics@uottawa.ca**.

Any questions about the conduct of the research project may be directed to the researcher or her supervisor at the coordinates listed below.

Eunice Chao: **Telephone number:** 1-613-562-5800 ext 2367 **Fax number:** 1-613-562-5380
E-mail address: echao097@uottawa.ca

Daniel Krewski: **Telephone number:** 1-613-562-8261 **Fax number:** 1-613-562-5380
E-mail address: dkrewski@uottawa.ca

Annex 4-4. Questionnaire

Annex 4-4. Questionnaire

Risk-Based Classification Scheme for Testing Genetically Modified Foods Assessment of Graded Testing Options (July, 2005)

Thank you for agreeing to participate in the evaluation of our risk-based classification scheme for testing genetically modified foods. You are asked to provide comments and advice on the graded testing options proposed in this document for testing genetically modified foods. Please refer to the list of questions on pp. 4-5 (*see note below*) to which specific guidance is being sought. Depending on your expertise, you may wish to focus on selected questions in providing comments.

To facilitate the review of this document, the proposed classification scheme and testing options are summarized on p.2. An outline of this document is provided as follows.

- Summary of the proposed classification scheme and testing options (p. 2-3)
- List of questions (p. 4-5)
- Introduction (p. 6-8)
- Proposed graded testing options (p. 9)
    - Potential for toxic and antinutritional effects

      1- Intended effects (p. 10-15)
      2- Unintended effects (p. 16-20)

    - Potential for allergenic effects (p. 21-23)
- Potential limitations of current methods and future developments (p. 21-23)
- Appendices*

  A. Summary of factors and criteria underlying the assignment of GM foods into concern levels (p. 26)
  B. Proposed risk-based classification scheme for testing GM foods (p. 27-31)
  C. Current approaches to assessing unintended effects in GM crops and derived foods (p. 32-34)
  D. Testing of allergenic potential of GM foods: current limitations (p. 35-36)

- References (p. 37-41)

* Appendices are provided as background information. It is not essential to review the appendices in order to answer the questions or to participate in the group interview.

SUMMARY OF THE PROPOSED CLASSIFICATION SCHEME AND TESTING OPTIONS

POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS

1- Intended effects

Types of substances

I. Substances (protein and non-protein) that have a history of known or presumed safe consumption from foods and have minimal concerns with respect to expected exposure.

II. 1. Novel protein substances at low expression levels.
2. Non-protein substances that are naturally occurring food constituents.

III.1. Novel protein substances expressed at physiologically significant levels in the modified food.
2. Novel non-protein substances that do not have a history of consumption from foods.

Testing

Corresponding to these broad classes of substances are three proposed levels of testing.
I. Minimal testing (beyond testing that provides the core information for classification).
II. Basic general toxicity testing and/or specific assessments.
III.Comprehensive testing.

2- Unintended effects

Types of GM foods

I. GM foods derived from sources with a history of consumption as food *or* from crops modified with well-characterized transferred/modified genetic elements *and* low potential for unpredictable adverse health effects.

II. GM foods derived from sources without a history of consumption as food, from crops modified with inadequately characterized transferred/modified genetic elements, *or* high potential for unpredictable adverse health effects.

Testing

The corresponding levels of testing for these two classes of GM foods are as follows.
I. Minimal testing (beyond testing that provides the core information for classification).
II. Expanded compositional and biochemical analyses, and toxicity testing of whole food where warranted.

POTENTIAL FOR ALLERGENIC EFFECTS

Types of allergenic proteins

I. Proteins with low likelihood* of allergenic activity.

II. Proteins with moderate or high likelihood* of allergenic activity.

**with respect to amino acid sequence identity with known allergens, protein stability, allergenicity of gene source and structural/functional characteristics.*

Testing

The corresponding levels of testing for these two classes of allergenic proteins in GM foods are as follows.
I. Minimal testing (beyond testing that provides the core information for classification).
II. Specific serum screening.

Proposed types of testing for GM foods based on concern levels

Types of Testing	I	II		III
		1	2	
POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS				
1- Intended effects				
▪ Minimal testing (beyond testing that provides the core information for classification)	x			
▪ Short term oral toxicity (28-day in one rodent species), or digestibility test, triggered by specific concerns		x		
▪ Specific toxicity (may replace general toxicity tests if triggered by specific concerns)		x		
▪ ADME			x[a,b]	x
▪ Detailed dietary assessment			x[b]	x
▪ Subchronic oral toxicity (90-day in one rodent species)			x	x
▪ Genetic toxicity			x	x
▪ Two-generation reproduction/development				x
▪ Additional tests, triggered by specific concerns or from above tests. Examples of additional tests (not a complete list):				x
All substances: toxicity testing of whole food (90-day in one rodent species)				
Non-protein substances: chronic toxicity, carcinogenicity, bioavailability (micronutrients)				
Macro-substances of nutritional or physiological significance: fermentation, human tolerance, protein quality, growth, specific metabolic study				
2- Unintended effects				
▪ Minimal testing (beyond testing that provides the core information for classification)	x			
▪ Expanded compositional/biochemical analyses		x[b]		
▪ Toxicity testing of whole food (90-day in one rodent species), triggered by characteristics of the GM food or results of expanded compositional/biochemical analysis		x[b]		
POTENTIAL FOR ALLERGENIC EFFECTS				
▪ Minimal testing (beyond testing that provides the core information for classification)	x			
▪ Specific serum screening (IgE binding tests)		x		

[a] ADME is considered desirable for non-protein substances in level II-2 with respect to intended effects. These substances have a limited empirically based toxicity profile but no identifiable cause for concern at the expected increased exposure.

[b] Applicable test depends on defined characteristics. For non-protein substances with a known toxicity profile and an established ADI (or equivalent) but limited data on expected exposure, toxicity testing would not be required.

LIST OF QUESTIONS

GENERAL QUESTIONS

*1) In your overall assessment, is the range of testing options proposed appropriate and adequate in identifying potential health hazards of GM foods?

*2) Taking into account the classification scheme for concern levels for testing, are the testing options proposed for each concern level appropriate and adequate for (a) toxic/antinutritional effects, and (b) allergenic effects?

* see Rating Form on next page.

3) If you have concerns about the adequacy or appropriateness of the proposed testing options, please provide suggestions for changes, or reasons for your assessment.

4) What are the potential limitations of the testing options based on available methods?

5) What are some new methods that will likely be available in the near future with the potential to mitigate or reduce current limitations, particularly with respect to testing unintended effects?

SPECIFIC QUESTIONS

6) Novelty - are the proposed criteria for novelty appropriate for (a) protein, and (b) non protein substances?

7) Novel proteins
   a) Is the differentiation of novel proteins into two different concern levels based on expression level justified?
   b) Are there specific aspects of novel proteins that require special attention (e.g. immunotoxicity/ immunogenicity) or that are not adequately addressed by the proposed testing scheme?
   c) What is the current state of knowledge with respect to prediction of protein toxicity using in silico approaches?

8) Non-protein substances naturally occurring in foods with a history of consumption from foods and modest increased exposure: can the need for toxicity testing be reduced (relative to novel substances without a history of dietary exposure)?

9) Unintended effects
   a) In your assessment, is the rationale for and emphasis on compositional analysis as the basis for determining the need for toxicity testing justified? If not, what alternative approaches would you recommend?
   b) Are there any advantages to "expanded compositional analysis" as proposed in this document, compared to untargeted compositional analysis (using untargeted metabolic profiling/fingerprinting)?
   c) How can the sensitivity and power of toxicity testing of whole foods be enhanced?
   d) To what extent would "omics"/profiling methodologies be applicable to testing "complex mixtures" such as foods?

10) Are there validated methods for screening for non-genotoxic carcinogens?

11) In the context of identifying potential toxic/antinutritional effects of GM foods (intended and unintended effects),
   a) what should be the role for in vitro testing?
   b) how can in vitro testing be better integrated with in vivo animal and human testing in food safety assessment?

12) Given the limitations of current methods for assessing the sensitizing potential of novel proteins in inducing allergic reactions in susceptible individuals, how should the issue be addressed?

13) Are adverse health effects defined too narrowly in current approaches to identifying potential hazards of GM foods? (e.g., should more attention be given to pharmacological effects of food constituents at high levels; sensitive subpopulations; chronic adverse health effects that are difficult to be attributed to individual components in complex foods)?

Rating Form - General Questions 1 & 2

Assessment of the appropriateness and adequacy of the proposed range of testing options in identifying potential health hazards of GM foods.

1. Overall assessment by types of potential health hazards

	Appropriate/Adequate	Appropriate/Adequate with changes	Inappropriate/Inadequate
1) Toxic/antinutritional effects			
a) Intended			
b) Unintended			
2) Allergenic effects			

2. Assessment by concern levels in the proposed risk-based classification scheme

	Appropriate/Adequate	Appropriate/Adequate with changes	Inappropriate/Inadequate
1) Toxic/antinutritional effects			
a) Intended			
I			
II			
III			
b) Unintended			
I			
II			
2) Allergenic effects			
I			
II			

If you are providing comments without participating in a group interview, please indicate your preference (yes/no) for acknowledging your contribution to the project.

□ Yes, I agree to have my name and affiliation included in the researcher's thesis.
□ No, I do not wish my name and affiliation to be included in the researcher's thesis.

THANK YOU FOR YOUR PARTICIPATION IN THE STUDY. Please return your response to the questions to: Eunice Chao (echao097@uottawa.ca).

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INTRODUCTION

Definition of genetic modification

A broad definition of genetic modification is adopted for the development of a risk-based classification scheme (RBCS) for testing genetically modified (GM) foods. It encompasses all forms of *intentional manipulation* in changing the heritable traits of an organism, including conventional methods (such as induced mutation and wide-cross hybridization of sexually incompatible plants) and more modern methods (such as recombinant nucleic acid technologies) used in plant breeding.

Transgenic methods using recombinant nucleic acid or rDNA techniques allow a greater diversity of genes that can be transferred and an enhanced range of novelty and potent effects not achievable by conventional plant breeding. The ability to insert the same gene into many cultivated species has also led to greater concerns about exposure. From a health risk perspective, these concerns are primarily related to the intended or expected effects of rDNA techniques. Yet, as will be discussed in more detail later, the types of unintended effects are, in many respects, qualitatively similar regardless of the method of genetic modification [NRC, 2000].

On the other hand, the boundaries between conventional and modern methods are being blurred as new approaches are emerging such as the stacking of transgenes using conventional breeding, site-specific deletion of inserted gene, and directed molecular evolution using DNA shuffling. Including a broad definition of genetic modification will provide a more complete context for assessing factors relevant to health risks of modifying food crops by genetic means.

Public discourse tends to focus on those aspects of rDNA technology that heighten concerns about its potential risks. Yet a range of GM foods exist. It is proposed that the potential for health risks may be differentiated among GM foods using defined criteria. The type and extent of testing can then be applied in proportion to the health risk potential. The factors and criteria used in differentiating GM foods into concern levels for testing in the RBCS are summarized in Appendix A.

Scope of the RBCS

The application of the RBCS will focus on crops modified for food use. The modification of food crops for the production of pharmaceutical and industrial chemicals is outside the scope of the scheme.

GM food testing and assessment will also focus on the edible part of a crop plant consumed by humans. It is recognized that some food crops have very limited food use for human consumption. If their human food use is restricted only to the production of highly refined ingredients of well-defined chemical composition (e.g., the only food use of oilseed rape is the production of oil by extraction using a highly refined process), currently available standards for the testing and assessment of food ingredients will apply. Similarly, in tissue-specific expression of introduced/modified genes that do not affect the edible parts of a food crop, verification of the absence of intended gene products in the extracted fractions should be sufficient testing for a crop with a limited food use. In these specific cases, the application of a RBCS in its entirety would not be necessary.

Information used in classification

In developing the RBCS, two types of information are distinguished.

(1) Prerequisite information will be met by all GM crops and will *not* provide a basis for differentiation. This includes information on genetic stability, data quality, and the intended purpose and performance of the modified trait, currently required in the assessment of GM crops and derived foods in many jurisdictions.

(2) Core information, in contrast, should have discriminating value for classifying GM foods. Core information is a subset of all the information that is typically required in premarket assessment. It provides the basis for the factors and criteria underlying the classification scheme and is based on prior knowledge or basic testing of the modified crop and derived foods.

Core information includes prior knowledge related to:
(a) biology of host plant, donor organism (and/or genetically related organism), including associated toxicity and allergenicity,
(b) prior history of food use,
(c) function and safety of the introduced/modified gene products and their principal metabolites and breakdown products in plant and mammalian metabolism, and processing effects, where applicable.

Testing of the modified crop and derived food includes:
(a) structure-activity characterization with a focus on protein gene products (comparison of sequence identity with known toxins and allergens, digestibility, heat stability, biological/enzymatic activity and specificity),
(b) levels of gene products and principal metabolites of interest in the edible parts of the modified crops,
(c) basic compositional analysis of the edible part,
(d) metabolic breakdown products from affected metabolic pathways, where relevant,
(e) expected dietary exposure to the food derived from the modified crop and to gene products and principal metabolite(s) of interest.

For the classification scheme to be useful as guidance for (toxicity) testing, the factors and criteria underlying the classification scheme emphasize information normally compiled or generated as part of developing the desired crop that can be easily assessed and used to differentiate among GM foods of varying levels of concern.

Categories of health risks
Two specific categories of health risk potential are the focus of GM food testing and assessment: toxic/antinutritional effects and allergenic effects. For each of these two categories of specific health effects, two general types of effects of genetic modification can be considered: *intended* effects and *unintended* effects.

Intended effects refer to the effects of the primary gene product (protein in nature) and secondary gene product (principal metabolite of interest of the target/modified enzyme). Primary and secondary products of marker genes are also part of the considerations. *Unintended* effects are those effects arising from alterations in metabolic pathways and modifications or disruptions in the expression of native genes (silencing, activation, or interactions).

General principles
In classical toxicology involving chemically defined substances, the type and extent of testing will depend on the nature and the level of exposure of the substance of interest. This principle is also applicable to gene products that are intentionally introduced or modified in foods derived from GM crops. In addition, prior knowledge of the safety of the genetic source of the introduced or modified substance, and that of the host or recipient plant, as well as the nature of the genetic modification and its potential effect on the host plant, will also determine the type and extent of testing.

It is important to recognize that foods are complex mixtures containing substances that may be shown to be either harmful or beneficial to health when subjected to conventional testing methods. While whole foods are not typically assessed for health effects, they are generally presumed to be safe. This means that a food is presumed to be safe unless it contains a poisonous or deleterious substance in an amount shown to make it ordinarily injurious to health [Kotsonis et al., 2001].

It is expected that many substances intentionally introduced or modified in GM foods will be similar to those already present in the food supply. Genetic modification may alter the sources of these substances and their exposure levels. For these substances, the adequacy of data of their history of use in or as foods in terms of chemical structure and levels of dietary intakes will be the major determinants of a testing strategy. In addition, the comparability between the conventional and modified sources in terms of matrix effects or bioavailability may also be relevant.

Whether we are assessing the potential health effects of substances of intentional modification or those of unintentional modification, a reasonable judgement of the adequacy of available data will be made. The need

for additional testing will be determined in proportion to the level of concern and the value of information that will be gained in the protection of population health. To this end, attention will be focused on reasonably significant health concerns and their likelihood, rather than the mere possibility of their occurrence. This is particularly relevant to assessing unintended effects.

The selection of testing strategies and health risk assessment models are intended to account for normally expected variability in sensitivity in the general population, including susceptible subpopulations, such as infants, children, and the elderly. However, it is generally recognized and accepted that the thresholds established from available methods may not be adequate in protecting subpopulations with extreme and distinct vulnerabilities [IOM, 1998; NRC, 2001].

PROPOSED GRADED TESTING OPTIONS

Outlined in this part of the document is a proposed gradation of testing that corresponds to the respective concern levels in the RBCS (detailed in Appendix B) for two categories of health risks:

(1) Potential for toxic and antinutritional effects
(2) Potential for allergenic effects.

For each category of health concerns, the discussion will be preceded by a summary of the proposed classification and testing options (in a box format). A detailed description of the characteristics of the substances that fit into the different concern levels for testing in the RBCS will be provided, as well as an outline of the proposed testing options that correspond to each concern level in the scheme. Also described are the types of testing, the objectives of testing, or the indications/triggers when certain types of testing may be required.

Background information on the proposed approach to classification and testing of toxic/antinutritional unintended effects and allergenic effects is also summarized, as there are distinctly different views on the adequacy of current approaches to the testing of these effects [Freese and Schubert, 2004; König et al., 2004; Cellini et al., 2004; Goodman et al., 2004; Spök et al., 2005].

The proposed graded testing is based on currently accepted testing methodologies. It is built on current guidelines, recommendations, and practices applicable to the testing of food-related chemically defined substances (e.g., food additives and pesticides), as well as complex products (e.g., botanical substances, novel macro-ingredients and complex foods such as infant formulas). Responses received from previous expert consultations were also considered.

The testing options provided here are intended to illustrate the progressively extensive nature of testing among the different concern levels in the RBCS. A system of graded testing can provide a framework for enhancing the transparency in communicating premarket product review decisions. They are not intended to be prescriptive or rigid, as regulatory requirements may vary among jurisdictions. The proposed testing options indicate the *baseline information* that will likely be required in evaluating a GM food in a specific concern level of a particular category of potential hazard. In some cases, if product-relevant data are available from the literature, actual testing of a given GM food may be reduced. In other cases, further testing may be required to resolve issues that are raised in baseline testing. It is also recognized that as new testing methods are developed and validated, the range of testing options will expand and some testing options suggested here may be replaced.

POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS

1- INTENDED EFFECTS

With respect to gene products that are intentionally introduced or modified in GM foods, the approach to testing their potential for toxic and antinutritional effects is similar to that used in the hazard identification of a chemically defined substance.

Testing will normally be conducted on the *purified form of the test substance.* Due to the lack of sufficient amount of the plant-derived protein gene product, the protein for testing may be produced by micro-organisms. In this case, the structural, biochemical and functional equivalence of the microbial substitute to the newly expressed plant protein will have to be demonstrated. Toxicity testing of the protein gene product is typically conducted in one rodent species.

Whole food testing may also be indicated. This is to identify any synergistic effects between multiple introduced gene products, or interactions between gene products and food constituents.

The proposed framework for classifying intended effects with respect to toxic and antinutritional potential will focus on the following three broad classes of substances that correspond to the respective concern levels in the RBCS.

I. Substances (protein and non-protein) that have a history of known or presumed safe consumption from foods and have minimal concerns with respect to expected exposure.

II. 1. Novel protein substances at low expression levels.
2. Non-protein substances that are naturally occurring food constituents.

III.1. Novel protein substances expressed at physiologically significant levels in the modified food.
2. Novel non-protein substances that do not have a history of consumption from foods.

Corresponding to these broad classes of substances are three proposed levels of testing.

I. Minimal testing (beyond testing that provides the core information for classification).

II. Basic general toxicity testing and/or specific assessments.

III.Comprehensive testing.

I. Minimal testing (beyond testing that provides core information for classification)

Types of substances

Substances (protein and non-protein) that have a history of known or presumed safe consumption from foods and have minimal concerns with expected exposure. They include substances that have all of the following characteristics.

1. Naturally occurring food constituents, or other substances that have been safely consumed from foods[1] (e.g., protein constituents of adventitious non-pathogenic bacteria commonly present on fresh produce).
2. No physiologically or toxicologically significant increase in dietary exposure based on theoretical maximum estimates[2].
3. No expected change in chemical structure for non-protein substances[3], or no concern with essential elements of characterization for a protein gene product expressed in an alternate species, genus, or cell type (i.e., no sequence similarity with known mammalian toxins; rapid and complete digestibility using standard assays; biological activity comparable to the source protein with a known safety profile).

4. No concerns with safety or function based on prior knowledge.
5. No unusual degradation metabolites expected at pharmacologically or toxicologically significant levels in the modified plant (e.g. metabolites from the detoxification of a herbicide) and in mammalian metabolism (e.g. biologically active peptides from incomplete digestion of certain types of protein; reactive products of bioactivation).
6. No interactions with adverse health implications expected among gene products and with food constituents[4].
7. Gene products expressed at levels comparable to those that have been previously assessed and approved for food use.

Notes:
1. "Safely consumed" means no deleterious health effects under expected conditions of consumption of the modified food, taking into consideration the function and exposure of the substance or a closely related substance [Codex Alimentarius, 2003]. Expected conditions of consumption of the modified food include considerations of food preparation and effects of processing of ingredients derived from the modified crop. For example, the antinutritional effects of a protease inhibitor can be minimized in a food that is normally consumed in a cooked form. If this constituent is transferred to a different crop and the derived food is normally consumed raw, this criterion will not be met. Similarly, a food constituent may be concentrated during the processing of a particular fraction of the edible part of the crop. The level of this constituent in the resultant ingredient should be considered, in addition to the level expressed in the edible part of the modified crop before processing.

2. No physiologically or toxicologically significant increase in dietary exposure based on theoretical maximum estimates includes: (a) an insignificant increase in dietary exposure relative to the baseline exposure level (a ratio close to 1), (b) a significant increase in new dietary exposure that has a wide margin of exposure (e.g., ADI assessed but not specified), or below an established upper limit (e.g., ADI, tolerance level, or equivalent), or below the threshold of toxicological concern for the chemical class where applicable [Kroes et al., 2004].

3. A non-protein substance (e.g. fatty acid) from a gene source that does *not* have a history of consumption as a food (e.g. algae) but has a chemical structure that is identical to a naturally occurring food constituent with all the characteristics for a level I substance would present low concern with respect to intended effects. The level of concern for unintended effects in the GM food will require separate scrutiny.

4. Interactions between an introduced/modified gene product and endogenous food constituents include those resulting from changes in bioavailability due to matrix effects when a gene product and its intended metabolite are expressed in a different host organism. For example, whether a micronutrient will be bioavailable should be considered when it is introduced to a target crop for the purposes of nutritional enhancement. Similarly, the bioavailability of endogenous nutrients may also be affected by the introduced/modified substance. The criterion of "no interactions" would not be met if a negative effect on bioavailability can reasonably be expected.

Testing

No toxicity or other testing will be required for substances in level I, beyond the testing that has already been conducted to provide the core information for classification. No further testing is required because no toxicologically relevant concerns would arise from substances that are comparable in all aspects of their exposure and nature to components of foods that have a known or presumed safe history of consumption, as defined above[1].

Notes:
1. In the case of proteins expressed in a GM plant where both the plant and the new protein have a history of safe consumption and known biological function in the modified plant, oral toxicity tests might not be required [Codex Alimentarius, 2003; EFSA, 2004].

However, acute oral toxicity testing of a protein gene product introduced in a modified plant has been required or submitted to confirm that the protein is non-toxic, despite evidence for structural, physicochemical, and biological equivalence between the plant-derived protein with the source protein that has a history of consumption from foods [Hérouet et al., 2005; Nair et al., 2002].

II. Basic general toxicity testing and/or specific assessments

Types of substances

1. Novel protein substances at low expression levels[1] that have one or more of the following characteristics.

a) The gene source has no prior history of consumption from foods.

b) There are concerns with one or more elements of protein characterization
- sequence similarity with known mammalian toxins or antinutrients
- incomplete digestibility using standard assays
- biological activity different from the source protein with a known safety profile.

c) There are concerns related to processing or matrix effects[2]
- toxic/antinutritional effects due to differences in methods of preparation or processing between the food derived from the modified crop compared to the food derived from the unmodified crop
- differences in digestibility or bioavailability (matrix effects) between food derived from the modified crop compared to the food derived from the source organism.

Notes.

1. Protein substances at low expression levels include proteins with enzymatic activity and are expressed at low levels (less than 0.1% of the total protein, or less than 1 ppm in a food, or intake of the protein of less than 0.1 mg/kg bw/day [König et al., 2004].

Novel proteins at higher expression levels or are members of protein classes associated with toxic/antinutritional effects or biological activity will be classified as level III for comprehensive testing and detailed hazard characterization. Examples of protein classes to be excluded from level II include: protease inhibitors, ribosome inhibiting proteins, lectins/ hemagglutinins, proteins with antimicrobial, immunomodulatory or neurotoxic effects).

2. Processing and matrix effects of protein substances considered in level II are restricted to proteins at low expression level and for which any toxic/antinutritional effects of the substance in the gene source have been well-characterized. The modified food containing the substance may be deemed unacceptable at the expected exposure level if initial concerns about bioavailability or antinutritional effects are verified by testing.

Testing

Applicable to a) and b)

Repeated dose oral toxicity (28-day, in one rodent species)[1] will be required to identify potential hazards triggered by a source without a history of consumption or by one or more concerns related to protein characterization.

Specific toxicity tests may replace general repeated dose oral toxicity test in determining an acceptable upper limit of intake where specific toxic or antinutritional effects are known.

Applicable to c)

Comparison of digestibility of the food derived from the modified vs. unmodified crop comparing the digestion and absorption of the protein in the native and modified/processed food matrix will be required. Depending the results, additional specific toxicity tests may be required to investigate chronic effects and to establish an ADI, if appropriate.

Genetic toxicity screen (in vitro) is *generally not necessary* unless sequence similarity with a mycotoxin is identified[2].

Notes.

1. It is proposed that novel protein substances at low expression level (except those in specified protein classes) do not require testing that is as extensive as that typically required for novel non-protein substances. The reasons are as follows:

(a) Protein substances, if toxic, generally have acute effects, with few exceptions such as mycotoxins. A repeated dose oral toxicity test of 28-day in rodents for newly expressed protein has been suggested in the EFSA guidance document [2004].

(b) As a general rule, a structural change (e.g. from mutagenesis) in a nontoxic enzymatic protein which improves desired enzymatic function is also unlikely to result in a toxic protein [Pariza and Foster, 1983; Pariza and Johnson, 2001].
(c) Peptide fragments resulting from breakdown of protein are less toxic than intact protein, with notable exceptions (that are excluded from level II as specified) [SAP, 2000].

2. Proteins, including most microbial toxins, are not genotoxic. Some mycotoxins are genotoxic, but they also induce other toxic effects that are easily determined in short-term feeding tests [Pariza and Foster, 1983; Pariza and Johnson, 2001; Sjoblad et al., 1992].

Types of substances

2. Non-protein substances that are naturally occurring food constituents with *either*

a) a limited empirically based toxicity profile but no identifiable cause for concern at the expected increased exposure[1], *or*

b) known toxicity profile[2] and an established ADI (or equivalent) but limited data on expected exposure from foods derived from the modified crop(s).

Notes.

1. "No identifiable cause for concern" for a level II non-protein substance that is a naturally occurring food constituent and that has a limited empirically based toxicity profile includes the following considerations:
(a) not structurally similar to compounds of toxic concern, and
(b) a modest increase in exposure that is comparable to mean intake of population groups with a history of higher intake from foods than the target group [Kruger et al., 2002].

2. A substance for which an ADI (or equivalent) has been assessed or established will be considered as having known toxicity. Limited data on expected exposure from foods derived from one or more modified crop(s) would require detailed dietary assessment.

Testing

Applicable to a)

Absorption/Distribution/Metabolism/Excretion (ADME) study*: to verify whether the substance is metabolized into innocuous metabolites that are similar to common dietary or body constituents.

Repeated oral toxicity (90-day in one rodent species)[1]

Genetic toxicity (in vitro) tests.

Applicable to b)

Detailed dietary exposure assessment using a probabilistic approach is recommended in order to assess the probability of occurrence of intakes of the intentionally added substance in excess of an established upper limit in different population subgroups.

*Not part of minimum testing but can be informative[2].

Notes.

1. Subchronic studies may not be required if there is a very wide margin of exposure (>2000-4000 times the estimated human exposure in 28-day subacute oral toxicity tests [FDA, 2003]), or if there are sufficient data based on metabolic and pharmacokinetic studies to support comparable toxicity profile with a chemically related compound of known toxicity [IPCS, 1987].

2. Extensive testing may be mitigated when a substance has a natural occurrence in food and has a history of human food use if the toxicological data base is adequate for homologous compounds and suggests a low intrinsic toxicity. In such cases, metabolic and pharmacokinetic data alone may be sufficient to make an evaluation of a related compound [IPCS, 1987].

III. Comprehensive testing

Types of substances

1. Novel* protein substances expressed at physiologically significant levels in the modified food.
2. Novel non-protein substances that do not have a history of consumption from foods.

*"Novelty" includes the following considerations.

1. Gene product from a new, synthetic, or extensively modified gene.
2. Structural or functional dissimilarity from existing food constituents.
3. Limited data on mammalian toxicity.
4. Unusual degradation metabolites that can result from the digestion process. They include, for example,
   a) biologically active peptides such as exorphins from casein and gluten.
   b) conversion of plant-derived components into toxic or carcinogenic compounds by digestive enzymes or by enzymes of gut flora are also well documented (e.g., HCN from cyanogenic glycosides and nitrosamines from nitrates). Fermentation products of saccharides that are not readily digested may also require scrutiny for toxic metabolites.
   c) reactive products of bioactivation in mammalian metabolism.
   d) metabolites from the detoxification of a herbicide in plant metabolism.
5. Likely/probable or uncertain interactions with adverse health implications among gene products and with food constituents.

Testing

The following tests are proposed as a minimum[1] to examine potential general toxicological and genotoxic effects, including testing at a sensitive stage of development.

All substances in level III

ADME study
Repeated dose oral toxicity (90-day in one rodent and one non-rodent species)
Genetic toxicity (in vitro)
Two generation reproduction/developmental study
Detailed dietary exposure assessment

Additional tests may be required based on the structure and known characteristics of the substance in question or those of other substances in the same chemical class, as well as the intended use of the modified food. The following list of additional tests is suggested for illustrative purposes only and is not meant to be complete.

All substances in level III

Toxicity testing of whole food[2] repeated dose oral toxicity (90-day in a rodent species) may be indicated if there is the potential for synergistic effects or interactions among gene products or with food constituents.

Non-protein substances

Chronic toxicity: indicated if there are concerns with a compound that bioaccumulates and/or is associated with late-occurring toxicity as suggested by metabolism/subchronic studies.
Carcinogenicity study: indicated by positive results in genetic toxicity test or by structural similarity to known carcinogens.
Bioavailability of the substance in the modified food: indicated for a micronutrient introduced in the modified crop for nutritional enhancement

Macro-substances of nutritional or physiological significance (including protein substances)[3]

Fermentation studies: indicated if incomplete digestion is suspected (e.g., biologically active peptides from casein, gluten etc., saccharides that resist digestion).
Human tolerance: indicated for substances that are not readily digested.
Protein quality study: indicated by changes in amino acid profile of a dietary source of protein.

Growth study in an appropriate animal model and in humans: the modified food or ingredient is a major component of infant formula[4].
Specific metabolic study: indicated if the substance is expected to have a metabolic effect (e.g., a lipid with an unusual fatty acid profile).

Notes.
1. We propose a comprehensive battery of tests for novel non-protein substances and novel macro-substances (including proteins) introduced/modified in GM foods. As with novel substance in other applications, core studies are usually required unless otherwise justified [European Commission, 1997; The Scientific Committee on Food, 2001]. Alternatively, they can be based on considerations of chemical structure and exposure levels [FDA, 2003]. Similarity in metabolism with other chemical analogues with known toxicity may reduce the need for extensive testing [IPCS, 1987].

2. A testing strategy combining modified whole food with varying spiked levels of purified test substance and two control groups (unmodified food and nutritionally equivalent diet alone without whole food) can provide an efficient approach for testing both intended and unintended effects in the same study [König et al., 2004; see Toxicity Testing under Unintended Effects - Level II].

3. For macro-substances of nutritional or physiological relevance, animal toxicity studies are not likely to be universally applicable due to difficulties in maintaining nutritional balance when fed at high doses in animal diet. The type and extent of animal studies required is considered on a case-by-case basis [U.K. Food Standards Agency, 2001]. Typically, a 90-day subchronic study in a rodent species is likely to be the minimum reasonable duration, and may often be the sufficient duration necessary, to provide data adequate for use in evaluating safety or to determine whether there is need for further studies [Howlett et al., 2003]. To compensate for the lack of dose accentuation for macro-substances in toxicity testing, studies of absorption, distribution, metabolism and elimination become more important in animals as well as in humans [Hatton, 1996; IPCS, 1987; LSRO Report, 1999]. If, through metabolism studies, a substance is shown to be completely broken down to substances that are common dietary or body constituents, other toxicity studies may not be necessary [IPCS, 1987].

4. A hierarchical approach to premarket safety assessment and in-market surveillance for infant formulas containing novel ingredients has been proposed, including a two-level assessment approach to assess organ, immunological, and endocrinological systems, and a three-level approach to assess developmental outcomes [IOM, 2004].

2- UNINTENDED EFFECTS

Sources of potential health hazards from unintended effects in GM foods

Some possible mechanisms that may result in unintended effects in plants modified by rDNA methods have been summarized and compared with natural genetic recombination processes that occur in traditional plant breeding [Cellini et al., 2004; Conner and Jacobs, 1999; IOM and NRC, 2004a]. Comparable mechanisms of unintended effects can also be identified in plants modified by other conventional plant breeding approaches using induced mutations (such as mutagenesis by chemical agents or irradiation and somaclonal variations induced by tissue cultures without transgene insertion) [Brar and Jain, 1998; Kaeppler et al., 2000; Lee and Phillips, 1998; van Harten, 1998]. Unintended effects of wide-cross hybridization have been reviewed in few publications [Health and Welfare Canada, 1989; NRC, 2000]. The extent of modification on genome structure and the frequency of occurrence of the specific types of effects may vary among different genetic modification methods and are difficult to quantify. However, it can be concluded that the types of unintended changes are qualitatively similar regardless of the method of genetic modification. These changes are categorized below.

Changes from insertional mutagenesis at the genomic level

- Interruption of sequences of a functional gene
- Chromosomal changes depending on the location of the insertion

Changes in gene expression at the transcription level

- Alterations in expression of transcription-related factors involved in regulatory and signalling pathways
- Activation of silent genes
- Disruption or silencing of active genes
- Epistasis (new interactions between genes at different loci) resulting from chromosomal rearrangements
- Position effects influencing the expression of a gene by its position in the genome
- Epigenetic effects on changes in gene expression without a change in DNA sequence attributable to DNA methylation

Changes at the metabolic level

- Interactions between novel gene products and endogenous enzymes and metabolites
- Alterations in flow through of metabolites in biochemical pathways resulting in changes in the concentrations of metabolites

Unintended effects may be predictable or unpredictable depending on our current knowledge of functional genomics and metabolic network control of the key regulatory points and rate limiting steps in biochemical pathways and transport processes. This type of knowledge varies between crops as well as within the same crop species [ILSI, 2004].

In the development of the RBCS, we examined two particular aspects of concern of unintended effects to determine their relative importance in GM foods and the priority that should be given to their identification in relation to health-related effects.
(1) modified expression of endogenous toxic substances or metabolites in different tissues or at different levels,
(2) generation of new toxic compounds.

Conclusions from this analysis are summarized below.

a) *Change in the level of known toxicants vs. biosynthesis of new toxicants.* There is support for the premise that unintended effects of genetic modification of food crops that have a history of traditional breeding, when they occur, would result in a change in the level of expression of already recognized toxicants in the plant. It is less likely that critical toxicants, inherent in the plant but hitherto unexpressed, will be unintentionally induced by genetic modification [WHO, 1995].

b) *Safety of donor organism.* The safety of donor organism in untargeted transfer of genetic material from plants without a breeding history for food use will require additional scrutiny. In cases where the safety of the donor plant is uncertain, genetic relatedness among plant species can be a useful guide in identifying botanical chemical constituents of concern [Franck-Oberaspach and Keller, 1997; IOM and NRC, 2004b].

c) *Genetic relatedness and degree of uncertainty.* Genetic relatedness between the host and donor organisms is a useful indicator of the predictability of unexpected changes in genetic modification involving two organisms and the likelihood of new dietary exposures [NRC, 2000; EPA, 2001]. Less uncertainty about how the gene will function and the production of new metabolites is expected in plants within the same family that are considered to share a common pool of genetic material by genetic mapping studies [Wink, 2003]. Greater uncertainty is expected when the two organisms are distantly related and when a powerful heterologous gene promoter is used that can affect the extent of control of normal homeostatic feedbacks in the host plant.

d) *Analysis at the molecular vs. metabolic levels.* While molecular analysis can facilitate the prediction of metabolic changes and can provide sensitive molecular surrogate markers of metabolic changes, their correlation with metabolite levels may only be qualitative. The measurements of actual metabolic changes are important in quantifying and characterizing potential health hazards associated with unintended effects. Compositional analysis of basic and judiciously selected constituents in the modified plant can provide a highly sensitive indication of unintended effects of direct health relevance. Importantly, components in foods are what consumers will eat and are what really matters [SAP, 2004]. In concert with observable phenotype and function, compositional analysis can provide a basis for assessing unintended changes in the levels of known antinutrients and inherent plant toxins [Novak and Haslberger, 2000].

Two classes of GM foods are proposed with respect to unintended effects with health relevance.

I. GM foods derived from sources with a history of consumption as food *or* from crops modified with well-characterized transferred/modified genetic elements *and* low potential for unpredictable adverse health effects.

II. GM foods derived from sources without a history of consumption as food, from crops modified with inadequately characterized transferred/modified genetic elements, *or* high potential for unpredictable adverse health effects.

The corresponding levels of testing for these two classes of GM foods are as follows.

I. Minimal testing (beyond testing that provides the core information for classification).

II. Expanded compositional and biochemical analyses, and toxicity testing of whole food where warranted.

In assessing unintended effects, testing will typically be conducted on the *whole food.*

I. Minimal testing (beyond testing that provides the core information for classification).

Types of GM foods

GM foods derived from sources with a history of consumption as food *or* from crops modified with well-characterized transferred/modified genetic elements *and* low potential for unpredictable adverse health effects. These GM foods have all of the following characteristics.

1. a) In uncharacterized gene modification (e.g., induced mutation, conventional wide-cross hybridization): the donor organism (and genetically related organism if the donor organism does not have a history of food use) or the host plant (in the case of induced mutation) is not a source of known toxicant or antinutrient[1].

b) In characterized gene modification (e.g., using rDNA techniques): the source organism is not a source of known toxicants or antinutrients[1], or the transfer of undesirable characteristics of the donor organism to the host plant can be ruled out.

2. Well-characterized function of the introduced/modified gene.

3. No concerns with extensive metabolic perturbations (i.e., modifications with the following properties):
   a) the introduced/modified enzyme has been demonstrated to have narrow substrate specificity in the cellular environment in the host plant, and is unlikely to function in the host plant's metabolism, *or*
   b) complex metabolic engineering is coordinated (e.g., with the use of transcription factors).

4. No concerns with interrupting functional or regulatory genes bordering transgene insertion sites (e.g., through the use of bioinformatics in predicting the likelihood of fusion protein formation[2]).

5. No serious health consequences if the genetic stability of the modified trait is not maintained.

6. No health-relevant significant changes from an appropriate comparator in basic targeted compositional analysis of the modified food[3].

Notes.

1. For the purposes of the RBCS, two characteristics of a natural toxicant/antinutrient are relevant [IFBC, 1990].
   a) any substance that occurs in food as a consequence of biosynthesis in the organism, and
   b) associated with significant toxic effects (irreversible) in humans, domestic animals, or experimental animals or occurring with narrow margins of safety (i.e., at low multiples of approximately 25 or less of ordinary exposures).

2. This criterion may not be applicable to crops modified by untargeted methods (i.e., induced mutation and wide-cross hybridization).

3. As part of the assessment of unintended effects, it is generally recognized that *basic targeted compositional analysis* should include food components of known health significance and other components of predictable changes. This forms part of the core information in classifying GM foods into concern levels with respect to unintended effects.
   a) key nutrients, toxicants, antinutrients, and endogenous allergens of the *host* plant (if known), and of processed fractions, where applicable,
   b) altered constituents predicted from molecular characterization (e.g., fusion protein) and from the biochemical pathways potentially affected,
   c) microbial toxin (if increased disease susceptibility of the modified plant is a concern), and
   d) toxic heavy metals (if increased uptake/accumulation in the modified plant is a concern).

Acceptable levels of the analytes should be compared with established reference values where applicable (such as dietary reference intakes for nutrients relative to the expected dietary exposure/contribution of the modified food; limits established for the relevant class of toxicants, mycotoxins, and heavy metals in the modified or similar foods). Where reference or limit values are not available, comparison will be made with an appropriate comparator grown under similar environmental conditions (control) or other comparable crops in the context of natural variations.

It is expected that in some cases, limited and defined differences from the control may be explicable and further analysis (such as those proposed for level II or III) may not be required before a decision can be made regarding the acceptability of the food. In other cases, the modified food may be considered unacceptable without the need for further testing.

Testing

I. No toxicity or other testing will be required for substances in level I, beyond the testing that has already been conducted to provide the core information for classification provided that

a) there are no differences of health significance between the modified crop and unmodified control from basic targeted compositional analysis, *and/or*

b) limited differences in basic compositional analysis between the modified and unmodified control can be explained.

II. Expanded compositional/biochemical analyses and toxicity testing of whole food where warranted.

Types of GM foods

GM foods derived from sources without a history of consumption as food, from crops modified with inadequately characterized transferred/modified genetic elements, *or* high potential for unpredictable adverse health effects. These GM foods have one or more of the following characteristics.

1. a) In uncharacterized gene modification (e.g., induced mutation, conventional wide-cross hybridization): the donor organism (and genetically related organism if the donor organism does not have a history of food use) or the host plant (in the case of induced mutation) is a source of known toxicant or antinutrient, or this information is uncertain.

   b) In characterized gene modification (e.g., using rDNA techniques): the source organism is a source of known toxicants or antinutrients and the transfer of undesirable characteristics of the donor organism to the host plant cannot be ruled out, *or* the gene is a new, synthetic or extensively modified gene.
2. Function of the introduced/modified gene not well-characterized.
3. Concerns with extensive metabolic perturbations (i.e., modifications with the following properties or there are uncertainties about these properties):
   a) the introduced/modified enzyme has been demonstrated to have broad substrate specificity in the cellular environment in the host plant, and will function or may function in the host plant's metabolism, *or*
   b) complex changes of metabolic engineering are not coordinated (e.g., with the use of transcription factors).
4. Concerns with interrupting functional or regulatory genes bordering transgene insertion sites (e.g., through the use of bioinformatics in predicting the likelihood of fusion protein formation.
5. Serious health consequences if the genetic stability of the modified trait is not maintained.
6. Health-relevant significant (extensive) changes from an appropriate comparator in basic targeted compositional analysis of the modified food.

Testing

Expanded compositional and biochemical analyses are required to identify health-relevant changes in metabolites that may be missed by the current approach to compositional analysis. Changes identified from more extensive compositional analyses can provide useful information in the design of more targeted toxicity testing with better defined sensitivity and power. Expanded compositional analysis consists of three components.

Applicable to GM foods with characteristics (3 and/or 4) and modification involving complex metabolic engineering

1) Precursors and key metabolites in multiple major biosynthetic pathways in the host plant from which toxic secondary metabolites may be derived. To address the question of activating silent genes and cryptic pathways that may lead to the production of toxic/antinutritional substances new in the host plant, substances of concern in genera in the same family phylogenetically related to the host plant should also be assessed and included, where appropriate.

2) Biochemical studies to assess whether novel substances would be produced by the interactions between introduced/modified enzymes and endogenous metabolites. This type of data would be expected to be generated as part of pre-studies in the research and development of the modified crop, particularly in modification involving complex metabolic engineering. Examples of biochemical studies include: supplementation/inhibition studies and enzymatic characterization.

Applicable to GM foods with characteristics (1a) and uncharacterized or untargeted modification

3) Constituents of concern in the *donor* organism (*where the host plant and donor organism are different*), **or the *host* plant** (*where the host plant and donor organism are the same*), including substances of concern in genera

in the same family phylogenetically related to the gene source, should also be assessed. Information on genetically related sources will be important where the safety of the gene source is uncertain and where it does not have a history of food use. If the gene source has a history of consumption as a food with a known allergenic protein, changes in the levels of allergenic protein(s) in the modified food should also be checked (as part of assessing the potential for allergenic effects). The need for this expanded analysis may be reviewed if data on the backcrossing process are adequate in indicating (1) the relative contribution of donor genes in the modified plant in hybridization, or (2) target specificity in induced mutation.

Toxicity testing of whole food[1]

Applicable to GM foods with characteristics (1b, 2, or 6) or significant (extensive) changes in expanded compositional analysis.

Postmarket surveillance[2]

Applicable to GM foods with characteristic (5).

Notes.

1. As noted previously, a testing strategy combining modified whole food with varying spiked levels of the purified test substance and the inclusion of two control groups (fed unmodified food and nutritionally equivalent diet without the whole food) can provide an efficient approach for testing both intended and unintended effects in the same study [König et al., 2004]. However, this strategy does not address the question of sensitivity of testing whole food for unintended effects.

Toxicity tests for unintended effects are generally conducted in one rodent species on the whole food (edible part of the modified crop), unless there are particular concerns about certain fractions processed as food ingredients. Testing the effects of specific unintended, novel compound(s) is probably not practical. First, obtaining and reproducing sufficient quantities of the purified form of the compound(s) for testing is not likely to be feasible, even if they are defined and limited. Second, unless how these compounds are produced in the modified plant is understood, their permanent removal from the modified food is unlikely to be successful. In the context of all available information, a positive toxicity test may serve to eliminate the product from further development. A negative test also requires cautious interpretation, where the ability to detect subtle toxic effects may be limited without the use of sensitive biomarkers.

In practice, toxicokinetic and genotoxicity studies will not be used in testing whole foods with extensive changes. For complex foods, some assurance of the absence of a potential for genotoxicity may be gained by appropriate investigations of bone marrow or peripheral lymphocytes undertaken as additional elements of repeat feeding studies in rodents [Howlett et al., 2003].

2. Postmarket surveillance of GM foods requires the implementation of segregation and identity preservation systems and is not broadly applicable to foods derived from commodity products such as food crops. However, it may have a limited role in specific scenarios where there is built-in commercial incentive for a voluntary segregation system from field to consumer products, such as the one that may exist in the promotion of a health-related trait. Where instability of a health-related trait of a GM food can have serious health consequences, such a product should be subjected to postmarket surveillance. It is recognized that the number of generations to establish stability of inserted traits is not known [SAP, 2004]. While it may be argued that the probability of a commercial GM crop lacking in a claimed benefit being marketed would be low due to liability reasons, this may not be the case for seeds saved at the farm level where adequate quality assurance procedures may not be in place.

POTENTIAL FOR ALLERGENIC EFFECTS

A major challenge in assessing the allergenic potential of GM foods is the lack of validated methods for testing the allergenicity of novel proteins (ability to sensitize individuals who have not been previously exposed). At the present, a protein from an allergenic source would be considered to be highly probable of being allergenic unless proven otherwise [FAO/WHO, 2001].

Few factors have known predictive value for assessing protein allergenicity although several factors have been identified [Huby et al., 2000], including:
(1) presence of individual epitopes on the protein (minimal peptide units within proteins than can recognized by the immune system);
(2) resistance to proteolysis, although this property is not shared by all common major protein allergens. At present, protein stability appears to be the only reliable characteristic being used to characterize known food allergens from normal dietary proteins [Helm, 2001];
(3) post-translational glycosylation, and specifically carbohydrate structures containing an $\alpha(1,3)$-fucosyl residue, and/or $\beta(1,2)$-xylose that may act as a cross-reactive carbohydrate determinant that can bind to IgE from individuals sensitized to unrelated sequences [van Ree et al., 2000];
(4) enzymatic activity that may cause non-specific activation of cells participating in the immune response.

Given the limitations of current methodologies (see also Appendix D), the primary focus of the testing of potential allergenic effects in GM foods has been to assess whether the introduced/modified gene encodes an allergen or a protein sufficiently similar in sequence to a known allergen that it might cause allergic cross-reactions [Metcalfe et al., 1996; Goodman and Leach, 2004].

Recognizing the importance of food matrix in protein structure and allergenic effects, the testing of both the whole food and the purified form of the protein will be an integral part of the assessment of the allergenic potential of GM foods wherever practical. No distinction will be made in classifying and testing intended and unintended effects separately in the proposed classification and testing schemes.

No single test can fully predict the allergenic potential of any new protein. A weight of evidence approach is increasingly favoured over a strict sequential decision tree application of testing strategies in the 2001 report of a Joint FAO/WHO Expert Consultation on Allergenicity of foods Derived from Biotechnology [Codex Alimentarius, 2003; FAO/WHO, 2001; König et al., 2004; Poulsen, 2004]. The factors and criteria underlying the proposed classification and testing are largely based on the 2001 Joint FAO/WHO report and subsequent Codex guidelines using a weight of evidence approach.

Based on the above considerations, two classes of protein are proposed with respect to their potential allergenic activity.

I. Proteins with low likelihood* of allergenic activity.

II. Proteins with moderate or high likelihood* of allergenic activity.

**with respect to amino acid sequence identity with known allergens, protein stability, allergenicity of gene source and structural/functional characteristics.*

The corresponding levels of testing for these two classes of allergenic proteins in GM foods are as follows.

I. Minimal testing (beyond testing that provides the core information for classification).

II. Specific serum screening.

I. Minimal testing (beyond testing that provides core information for classification).

Types of allergenic proteins

Low likelihood of allergenic activity. Proteins in this category have both characteristics (1-2).

1. Amino acid sequence identity with known allergens < 35% over 80 or more amino acids[1].
2. Not resistant to pepsin digestion and not heat stable[2] under standard test conditions.

Notes.

1. IgE cross-reactivity between the newly expressed protein and a known allergen was considered a possibility when there is more than 35% identity in a segment of 80 or more amino acids [FAO/WHO, 2001]. However, cross-reactivity is considered most likely with more than 70% identity and rare below 50% identity [Aalberse, 2000].

2. Stability is not a prerequisite for allergenic proteins. Allergens known to be heat labile include Bet v 1-related proteins and profilins. A protein belonging to these classes or potentially cross-reactive with these classes of protein will be assigned to concern level II.

No cut-off values for stability have been provided in guidance documents on GM food safety assessment. Three general patterns of stability have been observed [Astwood et al., 1996].

a) complete stability for 60 minutes with few or no detectable proteolytic fragments
b) intermediate stability (less than 60 min but more than 30 sec) for the whole protein with observed fragments that were stable for additional periods of time
c) no stability for the whole protein with stable fragments observed for at least 8 min.

The common in vitro pepsin digestion assay protocol used in a multi-laboratory evaluation provides standardized test conditions for the test procedures [Thomas et al., 2004].

Testing

Minimal testing (beyond testing that provides core information for classification[1]).

Notes.

1. Quantification of the level of known allergenic protein(s) in the modified food derived from a crop that is a known allergenic source is included as part of testing for unintended effects (core information for classification). However, the interpretation of the clinical significance of elevated level of allergenic protein(s) in the modified food would be challenging, as current methods are generally qualitative or semi-quantitative. In addition, no validated or useful tests are available in assessing the allergenic activity or allergenicity of a protein that has no prior history of exposure or has low sequence identity with known allergens.

While the FAO/WHO advisory group [2001] and the European Food Safety Authority [2004] recommended broadly targeted serum screening of IgE binding to the introduced protein, using sera from individuals who are allergic to species distantly or broadly related to the source of the gene, there has been no assessment of the predictive value of such an approach [Goodman et al., 2005]. The Codex [2003] did not recommend targeted screening as a routine practice for assessing GM products.

II. Specific serum screening

Types of allergenic proteins

Moderate or high likelihood of allergenic activity. Proteins in this category have one or both characteristics (1-2).

1. Amino acid sequence identity with known allergens ≥ 35% over 80 or more amino acids.
2. Resistant to pepsin digestion and heat stable under standard test conditions.

Additional criteria

For a protein for which evidence for satisfying the specified criteria for level I or II is equivocal, satisfaction of one or more of the following criteria will confirm the assignment of the protein to concern level II.

3. For proteins with a history of exposure: gene source is a known allergenic source.
4. Post-translational modification involving glycosylation with primarily N-linked, specific xylose or fucose substitutions [van Ree et al., 2000].
5. A member of, or belonging to protein families or functional categories commonly associated with allergenicity (specifically prolamin, Bet v 1, cupin, profilin; lipocalins, parvalbumins; seed storage proteins of the 2S albumin type; pathogenesis-related protein families 2, 3, 4, 5, 8, 10, and 14 comprising β-1,3 glucanases, chitinases of classes I and III, lipid transfer proteins) [FAO/WHO, 2001; Hoffmann-Sommergruber, 2002; Jenkins et al., 2005].
6. Proteins with any of characteristics (3-6) at ≥1% of total protein in the modified food [Kimber et al., 1999; Buchanan, 2001].

Testing

Specific serum screening (IgE binding tests)[1] using sera from patients known to be sensitized to the gene source if it is known to be allergenic, or to those known allergenic proteins that share ≥ 35% sequence identity with the introduced/modified protein of interest. This is to assess if an allergenic protein has been transferred to the modified food, or if the allergenic activity of the protein of interest has a moderate to high likelihood of cross-reacting with known allergens.

a) If the gene source is a known allergenic source: test materials should include the purified protein and extracts of the gene source.
b) If the test protein is shown to be resistant to proteolysis under standard test conditions: the residual fragments following pepsin digestion should also be included in the IgE binding test [EFSA, 2004; Oehlschlager et al., 2001].

Notes.
1. Assessment of allergenicity should be based on the matrix/matrices that the novel protein would be introduced into the diet [Helm, 2001]. To avoid possible adjuvant-like effects of nonallergenic components of foods, the protein of interest should be tested in the purified form in order to assess its allergenic effect. For the purpose of assessing the allergenic activity of the introduced/modified protein in the GM food, IgE binding should preferably be conducted on both the purified protein and the food extract derived from the host plant (particularly if it is an allergenic source). Wherever practical, testing of the food extracts should be included in order to compare IgE binding between the food derived from the GM crop and the wild type.

These generalizations may not be applicable
a) if the host plant and/or the source organism is not a known allergenic source to which sera from sensitive individuals may be available;
b) if the protein is at low level in the food. In this case, it may be feasible to test only the protein in the purified form.

If the gene source is a food plant or animal with a history of exposure and is known allergenic source, and sequence identity is with a known allergenic protein from the gene source, the inclusion of a food extract derived from the gene source for comparison can confirm or rule out the transfer of an allergenic protein to the modified food.

For protein without a history of exposure, a conservative approach may be adopted due to limitations in current testing methods with respect to assessing the sensitizing potential (allergenicity) of novel proteins. This means that if, based on defined characteristics, a protein is judged to have a high likelihood of being allergenic (having allergenic activity and/or allergenicity), no further testing would be recommended beyond in vitro serum IgE binding screening tests, even though the probability of a false positive result cannot be ruled out. This conservative approach takes into account that it is both ethically and practically difficult to rule out the probability of false positive results by conducting clinical testing using human subjects (i.e., skin prick and/or double-blind placebo-controlled food challenge). Enhancing the specificity of IgE binding in testing procedure can help to reduce the likelihood of false positive results [Goodman and Leach, 2004].

POTENTIAL LIMITATIONS OF CURRENT METHODS AND FUTURE DEVELOPMENTS

POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS

1- Intended effects

Currently available toxicity testing methods are generally amenable to identifying potential toxic and antinutritional health hazards associated with the intentional introduction or modification of protein gene products and their principal metabolites. Areas requiring special attention include:

1. Low level of expression of protein gene products that may necessitate the use of alternate expression system to synthesize sufficient quantity of the protein for testing.

2. Cytotoxicity and immunotoxicity/immunogenicity of novel proteins that resist digestion.

3. Predictability of protein toxicity using in silico approaches - availability of public databases on toxic proteins, search algorithms and criteria.

4. Moderate increased exposure to substances naturally occurring in foods (history of dietary exposure but limited empirically based toxicity profile) - can the need for toxicity testing be reduced (relative to novel substances without a history of dietary exposure)?

5. More efficient use of animal testing by integrating with in vitro methods that complement in vivo testing.

2- Unintended effects

For the reasons outlined earlier in this document (and in Appendix C), the testing of unintended effects in GM foods remains a major source of contention and uncertainty. Main areas of contention include:

1. Possibility vs. probability of unintended effects with health relevance.

2. Targeted vs. untargeted compositional analysis - usefulness of proposed "expanded targeted compositional analysis".

3. Whole food animal testing - issues of sensitivity; identification of molecular/biochemical biomarkers of effects.

4. Toxicity testing - under what conditions would toxicity testing be most useful? What type of toxicity testing?

5. Molecular characterization as a tool for predicting unintended effects mediated by genome disruptions in the modified plant.

> Current investigations of potential interruption of active genes focus on sequencing regions flanking the transgene insert site. It has been suggested that (1) additional sequences be identified to characterize genes that are distal to the insert site, and (2) the chromosome location of each insert be routinely mapped using Mendelian segregation results or molecular markers [SAP, 2004]. It is also likely that with better knowledge of functional genomics of common crop plants in the future, comparative sequence analysis and gene expression will also be of value [Freese and Schubert, 2004].

POTENTIAL FOR ALLERGENIC EFFECTS

The predictive value of current methods is unknown [Bernstein et al., 2003; Spök et al., 2005]. Current methods appear to be able to identify allergenic proteins that have properties generally associated with known allergens. However, the failure to identify properties associated with common allergens does not provide confidence that the protein is not allergenic. In response to the question whether the strategies currently

proposed for safety evaluation (risk assessment) are adequate to protect sensitive populations, participants at a conference on "Assessment of the Allergenic Potential of Genetically Modified Foods" concluded that "no realistic strategy has been proposed to protect the general population, including sensitive populations" [Germolec et al., 2003]. Major limitations of current strategies include:

1. No generally accepted or validated methods for assessing the allergenicity or sensitizing potential of new proteins in GM foods that do not have a history of exposure.

2. Predictive value of in silico search of amino acid sequence identity with known allergens is not known and will be difficult to assess in human population. Future developments may include searches for epitope sequences and 3-D structural domains, and integration of different protein family classification systems and databases [Gendel, 2004].

3. Resistance to digestibility and heat stability are not predictive of allergenic potential. However, when the information is combined with other factors, it can provide the best indication of a protein's potential for allergenic effects [Helm, 2001].

4. Current methods of serum IgE binding do not necessarily predict clinical significance as they do not differentiate the affinity or avidity of binding [Goodman et al., 2005].

a) At the present, the specificity and reliability of in vitro IgE binding test can be best enhanced by taking steps to improve the quality of testing procedures [Goodman and Leach, 2004].
b) In the future, ex vivo functional tests may be of value in assessing the biological activity of IgE binding. The inclusion of functional tests has been suggested in several guidance documents and reviews [Codex Alimentarius, 2003; EFSA, 2004; FAO/WHO, 2001; Poulsen, 2004]. Examples of functional tests include cell-based assays using human or humanized animal basophils [Dibbern et al., 2003; Ebo et al., 2004; Hamilton and Adkinson, 2004]. However, the value of functional tests such as histamine release in assessing the allergenic potency of allergenic responses to dietary proteins is not generally accepted [Germolec et al., 2003].

5. Methods currently under development for assessing the sensitizing potential of proteins without a history of exposure include:

a) Animal models - It is unlikely that a single animal model will be sufficient to address all the issues concerning prediction of allergenicity to humans [Germolec et al., 2003]. Different animal species and strains of mice may be required as their responses can vary. A few models appear promising but there is no consensus on the criteria for validating an animal model of food allergy, including the selection of end points, response rate, comparability of epitope binding between human IgE and model IgE, and the utility of challenge tests in measuring cellular and humoral sensitization [Westphal and Raybourne, 2004]. While immunogenicity is not necessarily related to allergenicity, parenterally administered challenge tests have been advocated to identify potent and common dietary allergens. This is based on the (reasonable) assumption that antigens with low immunogenicity will less likely induce allergic immune responses [Spök et al., 2005].
b) $Cd4^+$ T-cell epitope identification to predict functional T-cell epitopes in a population of individuals who have not been previously exposed to the protein under study [Harding, 2003; Stickler et al., 2003; 2004].

6. Hypersensitivities other than IgE-mediated allergies are not currently being addressed [Haslberger, 2003; Spök et al., 2005].

7. Postmarket surveillance may have a role in managing potential allergenic risks of certain GM foods, given the challenges of assessing the allergenic potential of novel proteins that do not have a history of exposure. Surveillance can focus on identifiable at-risk groups, including occupational groups exposed to allergenic pollens or grain dust from the modified crop or its processing.

Appendix A
Summary of factors and criteria underlying the assignment of GM foods into concern levels

POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS

1- Intended effects

Factors	*Criteria*
▪ Dietary exposure to gene products[1]	▪ Novelty as a dietary constituent/dietary role
▪ Prior dietary exposure	▪ Theoretical maximum estimates
▪ Expected change	▪ Theoretical maximum estimates
▪ Nature of gene products	▪ Characteristics of major classes of compounds important in GM crops (affecting the type and level of testing)
▪ Structure-activity (focus on proteins)	▪ Elements of protein characterization
▪ Function/activity	▪ Extent of prior knowledge
▪ Degradation products (in plant and mammalian metabolism)	▪ Extent of prior knowledge
▪ Synergisms/interactions	
▪ Prior experience with the gene products introduced/modified	▪ Comparability in expression levels, gene source, gene transfer method and host plant

[1] Gene products include primary and secondary gene products. Primary gene products are the direct gene expression products that are protein in nature. Secondary gene products are the principal metabolite(s) of interest of the introduced/modified protein gene expression products.

2- Unintended effects

Factors	*Criteria*
▪ Safety of host plant and donor/related organism	▪ Extent of prior knowledge
▪ Novelty of introduced/modified gene function or pathway in host plant	▪ Genetic relatedness between donor and host organisms
▪ Enzyme substrate specificity	▪ Biochemical assays
▪ Complexity and interconnectivity of pathways affected by the modification	▪ Biochemical assays
▪ Extent of genomic disruptions in host plant	▪ Molecular characterization and bioinformatics
▪ Health consequences of instability of modified trait	▪ Severity
▪ Comparability in key constituents in edible parts between modified and unmodified crop and/or appropriate comparator	▪ Basic compositional analysis

POTENTIAL FOR ALLERGENIC EFFECTS

Factors	*Criteria*
▪ Donor organism	▪ Extent of prior knowledge and exposure history
▪ Allergenicity	
▪ Gene products	
▪ Exposure history	▪ Extent of prior knowledge
▪ Protein characterization	▪ Amino acid sequence identity with known allergens
	▪ Digestibility and heat stability
	▪ Structural and functional alerts
▪ Host organism	▪ Extent of prior knowledge
▪ Allergenicity	

Appendix B - Proposed risk-based classification scheme for testing genetically modified foods (July 2005)

POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS

1- Intended effects from primary and secondary gene products introduced/modified - applicable to both protein and non-protein substances, unless stated otherwise.

	Criteria for Determining Concern Levels for Testing		
Factors	**I**	**II**	**III**
Dietary exposure to primary and secondary gene products			
1. Prior dietary exposure (history of safe use in relation to food)	a/ Naturally occurring food constituents, or b/Other substances that have been safely consumed from foods *Note criteria for levels II & III.*	*Novel proteins.* a/ Gene source or protein: no prior history of consumption from foods (including dietary proteins from a new, synthetic, or extensively modified gene, or from a novel gene source without prior dietary history)	
		Non-protein substances. b/ Naturally occurring food constituents	*Non-protein substances.* a/No history of consumption from foods
2. Expected change in total dietary exposure (based on theoretical maximum estimates)	a/ No physiologically or toxicologically significant increase in dietary exposure	*Novel proteins.* a/ New dietary exposure *at low expression level*	*Novel proteins.* a/ New dietary exposure *at physiologically significant levels*
		Non-proteins. b/ Modest increase in exposure within the range of high consumption from foods in some population groups (linked to 4a), *or* c/ Limited data on expected exposure (linked to 4b)	*Non-proteins.* b/ Significant increase in exposure, *or* c/ Limited data on expected exposure (and toxicity)
Nature of primary and secondary gene products			
3. Structure-activity characterization	*Proteins expressed in a different species, genus or cell type*: a/ No concerns with all essential elements of protein characterization: ▪ no sequence homology with known toxins ▪ rapid and complete digestion in standard tests ▪ structural and functional similarity to comparator/source protein *Non-proteins*: b/ Structurally and functionally similar to natural food constituents	*Novel proteins.* a/ Concerns with or insufficient data on one or more of the essential elements of protein characterization (listed for level I) *Non-proteins.* b/ No structural or functional similarity to compounds of toxic concern	a/ Structurally or functionally dissimilar to substances that have been widely consumed from foods, or b/ Not well characterized

	Criteria for Determining Concern Levels for Testing		
Factors	**I**	**II**	**III**
	c/ Not applicable (no primary gene product expressed due to gene silencing)		
4. Prior knowledge of function/safety (including food ingredients derived from the modified crop, impurities or by-products of ingredient processing) * Relevant where modified food is prepared/processed differently from a food it replaces	a/ Not implicated in mammalian toxicity or antinutritional effect under expected conditions of consumption of the modified food, or b/ Specificity of biological function or mode of action well-established with no potential for adverse human health effects	*Novel proteins.* a/ Concerns of toxicity or antinutritional effects related to food preparation/ processing methods under expected conditions of consumption of the modified food* *Non-proteins.* b/ Limited empirically based toxicity profile with no identifiable cause for concern, *or* c/ Known toxicity profile and established ADI (or equivalent)	a/ Limited data on mammalian toxicity or antinutritional effect under expected conditions of consumption of the modified food, or b/ Specificity of biological function or mode of action not well-established, or c/ Macro-substances of nutritional or physiological relevance
5. Degradation products			
5.1. Metabolism in the edible part of the modified plant	a/ No unusual metabolites expected at pharmacologically or toxicologically significant levels		a/ Unusual metabolites expected or identified
5.2. In mammalian metabolism	a/ No unusual metabolites expected at pharmacologically or toxicologically significant levels		a/ Unusual metabolites expected or identified
6. Interactions among gene products introduced/modified (in the same or separate transformation events) and/or with food constituents	Potential for synergistic toxicity, anti-nutritional effects, or cumulative effects on biochemical pathways - All substances		
	a/ Not applicable, *or* b/ Not expected based on known functions of the gene products		a/ Likely/probable, *or* b/ Undetermined /unable to assess based on available information
7. Prior experience with gene products introduced/modified	a/ Comparable in expression levels, gene source and transfer method, and host plant		a/ No comparable prior experience
Rules for determining concern levels	Meet criteria for <u>all</u> factors listed.	Meet criteria for <u>all</u> factors listed for the respective category (i.e., novel proteins or non-proteins). For Non-proteins: factors 2 and 4 are linked as follows: (2b and 4b), *or* (2c and 4c).	Meet criteria for <u>any</u> of the factors listed.
Concern level =			

2- Unintended effects in the modified food from pleiotropic and secondary metabolic changes resulting from genetic modification of the host plant

	Criteria for Determining Concern Levels for Testing	
Factors	**I**	**II**
1. Safety of donor (source) or genetically related organisms, or of the host plant (in the case of induced mutation or gene silencing) - as a source of known toxicants, antinutrients at normal consumption levels or patterns by normal consumers	*Uncharacterized gene modification.* a/ Not a source of known toxicant or antinutrient *Characterized gene modification.* b/ Not a source of known toxicant or antinutrient, *or* c/A source - transfer to or expression of undesirable characteristics in the host plant can be ruled out (based on chemical, biochemical or biological assays)	*Uncharacterized gene modification.* a/A source of known toxicant or antinutrient, or b/ Safety of donor organisms uncertain or undetermined *Characterized gene modification.* c/ A source of known toxicant or antinutrient and transfer to or expression of undesirable characteristics in the host plant cannot be ruled out, or
2. Novelty of introduced/ modified gene function or pathway in the host plant (expected degree of regulatory control in the modified plant)	a/ Gene encoding product(s) that have well-characterized function in the modified plant	a/ A new, synthetic gene, or b/ Extensively modified (mutated) gene, or c/ Function of the introduced/modified gene not well-characterized
3. Substrate specificity of gene product (if it has enzymatic activity)	a/ Narrow substrate specificity b/ Not applicable (does not have enzymatic activity)	a/ Broad substrate specificity b/ Uncertain or not determined
4. Complexity and interconnectivity of pathway introduced/modified (as characterized by biochemical or metabolic fate studies in the modified plant)	a/ Introduced/modified gene product unlikely to function in the host plant's metabolism, *or* b/ Coordinated complex metabolic engineering (e.g., with the use of transcription factors)	a/ Introduced/modified gene product function s or may function in the host plant's metabolism, *and/or* b/ Uncoordinated complex metabolic engineering c/ Uncertain or not determined
5. Extent of genomic disruption of host plant and characterization of gene insertion/modification site	a/ Limited and well-characterized (e.g., absence of fusion protein predicted by bioinformatics analysis or confirmed by acceptable transcript or protein detection methods)	a/ Extensive or inadequately characterized (e.g., likelihood formation of fusion protein predicted by informatics analysis and not ruled out by acceptable transcript or protein detection methods) c/ Uncertain or not determined
6. Health consequences of instability of the modified trait‡ ‡Therapeutic health trait is outside the scope of RBCS for foods derived from GM crops	a/ Modified trait not health-related	a/ Modified trait (e.g., reduced allergenicity) has potentially serious health consequences, *or* b/ Nutrition-related trait and the food is a major source of the affected nutrient in the diet

	Criteria for Determining Concern Levels for Testing	
	I	II
7. Basic targeted compositional analysis of modified food†	a/Limited, explicable changes from appropriate comparator	a/Consistent and extensive change from appropriate comparator
Rules for determining concern levels	Meet criteria for <u>all</u> factors listed.	Meet criteria for <u>one or more</u> of the factors listed.
Concern level =		

†Basic targeted compositional analysis includes: (1) key nutrients, toxicants, antinutrients, and endogenous allergens of the *host* plant and of processed fractions, where applicable, (2) other constituents predicted from molecular characterization and biochemical pathways potentially affected, (3) microbial toxin (if increased disease susceptibility of the modified plant is a concern), and (4) toxic heavy metals (if increased uptake/accumulation in the modified plant is a concern).

POTENTIAL FOR ALLERGENIC EFFECTS - *Applicable to protein primary or secondary gene products*

	Criteria for Determining Concern Levels for Testing	
Factors	**I**	**II**
1. Amino acid sequence identity with known allergens	a/ Sequence identity with known allergens < 35% over 80 or more amino acids	a/ Sequence identity with known allergens ≥ 35% over 80 or more amino acids
2. Stability under standard testing conditions	a/ Rapidly digested in pepsin digestion and not heat stable	b/ Resistant to pepsin digestion or heat stable
Additional criteria - For a protein for which evidence for satisfying the specified criteria for level I or II is equivocal, satisfaction of one or more of the following criteria will confirm the assignment of the protein to concern level II.		
Nature of donor organism (gene source)		
3. Allergenicity of donor organism (gene source)		*For proteins or gene source with a history of exposure* a/ Source of allergenicity or gluten-sensitive enteropathy
Nature of primary and secondary gene products		
4. Post-translational modification		a/ Involving glycosylation with primarily N-linked, specific xylose or fucose substitutions
5. Structural or function alerts		a/ Member of or belonging to specific protein families or function categories commonly associated with allergenicity*
6. Abundance		a/ Proteins with any of the characteristics (3-6) at ≥ 1% of total protein in the modified food
Rules for determining concern levels	Meet criteria for all factors listed.	Meet criteria for one or more of the factors listed.
Concern level =		

* Specifically prolamin, Bet v 1, cupin, profilin; lipocalins, parvalbumins; seed storage proteins of the 2S albumin type; pathogenesis-related protein families 2, 3, 4, 5, 8, 10, and 14 comprising β-1,3 glucanases, chitinases of classes I and III, lipid transfer proteins) [FAO/WHO, 2001; Hoffmann-Sommergruber, 2002; Jenkins et al., 2005].

Appendix C
Current approaches to assessing unintended effects in GM crops and derived foods and limitations

Current approaches to assessing unintended effects in crops modified by rDNA techniques have focused on

- molecular characterization
- targeted compositional analysis

1) ***Molecular characterization*** aims to establish that the integrity of the transferred DNA has not been modified as a result of the transformation process. Requirements may differ among jurisdictions but essentially consist of identifying significant DNA sequence alterations to the native gene in the expression cassette used in transformation and the DNA sequence inserted in the modified plant. Data on the size and copy number of all detectable inserts are required, typically determined by Southern blot analysis. Sequence data extending into the host plant genome are required in some jurisdictions to identify insertion into, and interruptions of, known or suspected open reading frames (ORFs or coding regions). Sequencing of flanking regions also serves as a marker for the transgenic event for traceability. If potential chimeric ORFs are identified, bioinformatics and transcript analyses will be required to investigate the possibility of any expression of fusion proteins and the extent of sequence similarity with known toxins or allergens. Stability of the introduced DNA must also be demonstrated.

Limitations
Southern blot analysis, which is a commonly used molecular method as part of the safety assessment of transgenic plants, has been criticized as being an insensitive method in identifying all insertion-site mutations and the extent of genomic disruption [Wilson et al., 2004]. Increasingly other complementary methods (such as PCR or DNA sequencing) are used in addition to Southern blot hybridization.

The molecular characterization methods typically used in bioengineered crops are generally not amenable to assessing changes at the genomic level in plants modified by non rDNA techniques. Other molecular methods used in conventional plant breeding may be useful for safety assessment. At present however, there are few regulatory requirements for safety assessment of conventionally bred food crops and there are no incentives to develop new methods or adapt existing methods for safety assessment of this category of GM crops.

2) ***Targeted compositional analysis*** has been justified for several reasons. First, it has greater sensitivity than animal testing in identifying individual compounds of relevance [ILSI, 2004]. Second, it has been argued that targeted compositional analysis can provide a general metabolic profile beyond those components being analyzed. When compared to unmodified comparator plants grown under the same conditions, compositional analytes are part of a highly integrated and interconnected set of regulated enzymatically catalyzed pathways that should reveal metabolic disruptions if levels of selected metabolites are outside of normal ranges [SAP, 2004]. Third, compositional analysis is applicable to all GM crops regardless of modification method.

Animal testing of complex food products presents considerable technical challenges (e.g., maintaining nutritional balance of the test diet for long duration, low sensitivity without new biomarkers, exaggerated doses, or large sample size) that make interpretation of questionable results difficult [Hattan, 1996]. Toxicological testing of whole GM foods for unintended adverse health effects is therefore not a standard requirement but may be required in specific cases [EFSA, 2004]. Critics judged that the apparent lack of emphasis on toxicological or biochemical tests was anti-scientific [Millstone et al., 1999].

The major criticism of the limitations of targeted compositional analysis in assessing unintended effects in GM foods is arguably the (exclusive) reliance on the concept of comparative safety assessment (or substantial equivalence). The implementation of this concept has left much scope for interpretation, including the standards required to establish "substantial equivalence" and its relevance to health risk potential, as well as the selection of a "conventional counterpart" as a comparator.

A conventional counterpart is deemed to be safe on the basis of experience through its common use as food [Codex Alimentarius, 2003]. There are two problems with using "conventional counterpart" as the basis of comparison without clear guidance. First, the typical commercial lifetime of 5-7 years of new plant varieties that may form part of the pool of conventional foods [Day, 1996] does not meet the standard of "substantial history of consumption" (U.S. GRAS "common use" definition) or that requires a number of generations for establishing

"history of safe use as food" (revision of Health Canada's Guidelines for the Safety Assessment of Novel foods, 2003 draft). Second, crops modified by induced mutations would have concerns with respect to unintended effects similar to those in crops modified by rDNA techniques. Yet crops modified by the former methods have no history of being formally assessed for safety before being marketed as food crops, although the extent of their contribution to commercial breeding programs varies with the crop and is difficult to quantify in developed countries [Ahloowalia et al., 2004; Wilson et al., 2004].

The selection of specific components for targeted compositional analysis is also perceived to be biased and lacking in scientific rigour [Millstone et al., 1999; The Royal Society of Canada, 2001]. To address these criticisms, the OECD, among other organizations, are now developing "minimum lists" to standardize the selection of components of food and feed for compositional analysis. Recommendations to strengthen the application of the substantial equivalence concept were proposed [Novak and Haslberger, 2000]. Untargeted profiling methods have been advocated [Kuiper et al., 2002].

In a recent report [IOM and NRC, 2004], broad application of general profiling techniques is considered to be of limited value in reducing uncertainty associated with unintended effects in the absence of an improved knowledge base of defined chemical food constituents, their natural variations, biological effects, and responses to environmental stress. In contrast, some proponents of general profiling techniques suggested that determinations of perhaps six widely planted cultivars of a particular crop, grown under a variety of conditions, could be sufficient in establishing a baseline for determining a normal distribution of natural variations in an unspecified spectrum of metabolites for that crop [Freese and Schubert, 2004]. However, the validity and utility of such a database and how deviations outside the range of normal variations should be interpreted remain unclear. Premature termination of further development and testing of a product may result due to uncertain interpretation of profiling analysis.

An emphasis on statistical significance in comparing differences metabolites between modified and unmodified crops is problematic [Cellini et al., 2004], given that unintended effects are to be expected in GM crops, although their frequency is unknown.

In the current approach to targeted compositional analysis, the analytes selected do not necessarily represent the breadth of the metabolic pathways that may be affected by changes in the expression of a modified endogenous gene or the expression of a foreign gene. Deviations from normal homeostatic feedbacks of the host plant are known to occur in genetically modified organisms [Hallerman, 2003]. It also does not consider new compounds that can be formed between endogenous metabolites and one or more new enzymes introduced to the host plant. Given the current emphasis on transgenic crops, the role of the donor organism as a potential source of toxic or antinutritional compounds other than the intended gene product has been largely ignored.

Possibility vs. probability of unintended effects relevant to health risks

Against the background of general concerns about unintended effects of genetic modification of crop plants, our limited knowledge about foods that contain naturally occurring toxicants has accentuated the level of concern about potential health risks of GM foods. In particular, the lack of sensitive methods for testing the delayed or chronic effects of food components remains a major source of uncertainty in assessing unintended effects of GM foods [Committee on Comparative Toxicity, 1996, p.36; Domingo, 2000; Freese and Schubert 2004; Kuiper et al., 2001; Millstone et al., 1999; Speijers and van Egmond, 1997].

In terms of health risk assessment, it would be justifiable to focus on

- unintended effects that are meaningful to human and animal health
- health-relevant unintended effects based on probability

An analysis of the factors to be considered in judging the possibility vs. the probability of unintended effects with health relevance is summarized below.

Possibility of unintended effects	Probability of health-related unintended effects
Interference with transcriptional regulators for genes encoding biosynthetic enzymes can "wake up" dormant biochemical pathways.	The ability to synthesize particular classes of secondary metabolites is commonly restricted to selected plant groups [Osbourn et al., 2003]. For example, by nature, cereal grains have relatively few intrinsic toxins, a characteristic also maintained by the domestication process, compared to some other major food crops [Cheeke, 1998, p.87]. The metabolism of alkaloids and many branches of the phenolic pathways are found or are amplified only in specific plant families [Croteau et al., 2000].
Chemical diversity and metabolic flexibility is a common feature of plant metabolism. Redundancy of pathways, broad substrate specificity of enzymes, and the adaptability of plants in using more than one enzyme in catalyzing a similar reaction pose other challenges [Firn and Jones, 2003; LSI, 2004].	The production of secondary metabolites with potent biological activity is likely a rare property of any one molecule [Firn and Jones, 2003]. The vast majority of plant substances are not toxic [IFBC, 1990]. A major class of plant chemicals such as alkaloids with potent toxic effects require highly substrate specific enzymes and are highly compartmentalized in their biosynthesis [Croteau et al., 2000]. Major classes of plant chemicals that have exhibited adverse health effects in domestic animals and in humans and their distribution are reasonably well established [Beier, 1990; Harborne, 1999; Manners, 1996]. The number (209) of food-borne natural toxicants substances that exhibit a wide range of adverse effects from microbial and plant sources constitutes less than one-tenth of 1% of the total number of constituents in food plants and microorganisms that have been identified [IFBC, 1990]. While some natural toxicants have not yet been isolated or structurally identified and the exact number is not known, it is likely not to be a large number, given the very high sensitivity of current capability of analytical chemistry. Even with our incomplete knowledge, the proportion of food-borne toxicants that have been shown to cause harm in humans when consumed in a normal diet can be considered to be low. Further, of these 209 toxicants, only 21 food-born non-microbially derived toxic substances have been identified to be of plant origin (< 0.1% of the total number of food constituents) with 9 of them being biosynthesized by plants and the remaining being pass-through contaminants in honey and milk [IFBC, 1990].

It is concluded that there is support for the premise that unintended effects of genetic modification of food crops that have a history of traditional breeding, when they occur, would result in a change in the level of expression of already recognized toxicants in the plant. It is less likely that critical toxicants, inherent in the plant but hitherto unexpressed, will be unintentionally induced by genetic modification [WHO, 1995].

Appendix D
Testing of allergenic potential of GM foods: current limitations

The assessment of the potential for allergenic effects in GM foods focuses on true food allergens that mediate immediate hypersensitivity reactions through specific immunoglobulin E (IgE) antibodies. Other food allergens that are implicated in cell-mediated, delayed reactions through other classes of immunoglobulins and other immunogenic effects without the involvement of antibodies have not received as much attention. One exception is the assessment of gliadin proteins implicated in gluten-sensitive enteropathy for which the amino acid sequence identity searches can be conducted and immunoglobulin specific ELISAs (enzyme linked immunosorbant assays) have been developed [Ahlstedt et al., 2003]. In an allergenic reaction, which is an interaction between an individual with a genetic (atopic) background and an allergen that is recognized by an IgE, the specificity and affinity of IgE binding to a given allergen can vary widely due to the genetic heterogeneity of the population, as well as the nature, extent, and duration of exposure [Bannon et al., 2003]. Given the nature of an allergenic reaction, allergenic substances in foods are primarily protein in nature although low molecular weight chemicals may be involved in an immune response by covalently binding to proteins to form a hapten-carrier complex [Kroes et al., 2004].

There are two stages in the development of allergic reactions: (1) sensitization, and (2) elicitation of a reaction. Correspondingly, the assessment of potential for allergenic effects in GM foods consists of two elements: (1) assessment of allergenic activity (the propensity of a substance to elicit allergic reactions), and (2) allergenicity (the likelihood of inducing de novo sensitization in a nonallergic individual) [Spök et al., 2005]. This also corresponds to two types of allergenic proteins: incomplete proteins (able to elicit allergic symptoms but do not usually sensitize) and complete proteins (able to sensitize and elicit an allergic reaction) [Aalberse, 2000]. Two pathways of allergic sensitization and routes of exposure can also be distinguished among complete proteins: air-born or occupational allergens that are associated with late-onset allergies, and food-born allergens that tend to trigger allergies in early childhood. Non-dietary routes of exposure to allergens derived from GM crops have not been considered as significant sources of sensitization for the general population.

Alterations in the allergenic potential of GM foods can take several forms [Lehrer and Bannon, 2005; Buchanan, 2001], including:

- Transfer of known allergens from existing food sources to new food sources
- Introduction of novel protein from sources without a history of exposure that may become allergenic in man
- Increase in the levels of endogenous allergens of an existing allergenic food source
- Increase in allergenic activity or allergenicity of known allergens or other proteins (due to increased exposure, post-translational modification, changes affecting protein structure such as folding, or changes in the cellular matrix in a new host)

Several studies and reviews have examined the relative importance of these sources of allergenic risks. The following conclusions may be drawn.

1) At an aggregate level, the likelihood of selecting and transferring a gene encoding an allergen in transgenic modification can be considered small. This is inferred from the observation that despite the vast number of protein substances in nature, most proteins are not allergens [Goodman et al., 2005; Halsberger, 2003; Lehrer, 2004].

2) The proportion of allergenic proteins from foods is also relatively small and they cluster in few protein families, with four families accounting for more than 65% of plant food allergens [Jenkins et al., 2005]. Of particular significance is a general lack of IgE cross-reactivity observed both within and between the other subfamilies.

3) Our understanding of how food matrix or adjuvant factors may affect the allergenicity of a protein is limited [Teuber, 2002], despite growing knowledge about the relationship between protein structure and allergenic potential.

4) The likelihood of increasing the levels and reactivity of endogenous allergens of an allergenic host plant may be considered as a function of general unintended effects.

5) Increased exposure to potential allergens may represent a broader problem that is not limited to GM foods [de Jong et al., 2004].

In silico approach to predicting cross-reactivity with known allergens based on amino acid sequence identity is a principal tool currently being used in assessing allergenic potential of transgenic proteins. Again, the predictive

value of this approach has not been verified and the methodology is evolving [Becker and Reese, 2001; Björklund et al., 2005; Brusic et al., 2003; Goodman et al., 2002; Kleter and Peijnenburg, 2002; Stadler and Stadler, 2003]. This approach is based on assumptions of minimal size of T-cell and B-cell epitopes [Becker, 2001]. However, non-contiguous or conformational epitopes are not identified by current algorithms. In silico approach is also limited by the availability of sequence information of known allergens in public databases and the scientific literature [Codex Alimentarius, 2003].

In addition, while the abundance of the protein in a food appears to play a role, the threshold for a "safe" level of protein with respect to sensitization is not known. It is expected that the induction of allergenic sensitization in a previously naïve individual invariably requires exposure to concentrations of an allergen that are greater than those necessary to elicit a response in a sensitized individual [IPCS, 1999]. However, the levels of allergenic proteins from eggs and cow's milk for sensitizing new born infants through breast milk feeding were estimated to be very low (500-1000 ng) [Hill, 2001]. Reported protein levels in food or the quantity of allergenic food associated with elicitation of objective symptoms in already sensitized individuals also span a wide range. The lowest reported provoking dose appears to be around 100-250 μg peanut protein (equivalent to less than 1 mg whole peanut), which may be considered the lowest observable adverse effect level (LOAEL) [Germolec et al., 2003]. Using a statistical model similar to the concept of "benchmark dose", Bindslev-Jensen et al. [2002] estimated that the threshold dose giving a reaction of one in a million in susceptible patients for four common allergenic foods (egg, cow's milk, soy an peanut flour) to be in the range of 0.7-5 μg.

Two assumptions are inherent in developing a classification and gradation of testing of the allergenic potential of proteins in GM foods within current methodological constraints.
1) A protein that has a low likelihood of having allergenic activity is also less likely to induce sensitization.
2) A protein that can induce sensitization is likely to have allergenic activity. If it has cross-reactivity with another known allergen, it could be predicted by in silico methods even if it is a new protein without a history of prior exposure.

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APPENDIX E

APPENDIX E

EXPERT CONSULTATION 5

Pilot test

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PHASE 3 (Evaluation of RBCS)

Phase 3 of the research was an evaluation phase consisting of a pilot test (expert consultation 5) and a concordance study (expert consultation 6).

5. Pilot test – Assessment of the product profiles and a combined RBCS-product rating form

5.1. Purpose

The purpose of the pilot test was to assess the clarity and ease of use of a combined RBCS-product rating form and profiles of reference GM foods to be included in the concordance study (described in Chapter 9 of the thesis, Volume I).

5.2. Recruitment and selection of experts

Participants from expert consultation 3 who expressed an interest in participating in a preliminary assessment of product classification and other participants from previous consultations who did not request to discontinue contact regarding future phases of the project were contacted. Approval for the recruitment process was included as part of a certificate of ethical approval for Phase 2 (Annex 3-1). Out of 18 potential participants who received an information letter (**Annex 5-1**), nine agreed to participate. Interested individuals received information on their role as research participants (**Annex 5-2**). Six participants returned completed assessment of two assigned products each. One additional participant returned assessment of one of two assigned products. The expertise indicated by the seven participants included: toxicology (4), immunology (2), pharmacology (1), molecular biology (2), plant sciences (2), agricultural sciences (2), food/nutritional sciences (2), agricultural biotechnology (2), analytical sciences (1), health risk assessment (4), and other biological sciences (2). Participants were affiliated with universities (2), governments (2), and public research institutes (3) from five countries in Europe and North America.

5.3. Data collection

Participants were provided with the descriptions of two GM food products and a rating form that outlined the criteria proposed in the RBCS (**Annex 5-3**). They were asked to assign concern levels for each product using the classification scheme and to indicate the applicable criteria for their assignment. They were also asked to identify any ambiguities in product descriptions and classification criteria, and to indicate the length of time required to complete the classification for each product. The product codes can be found in the list of products used in the concordance study included in **Appendix F**. In addition, participants were provided with supplementary information for applying the RBCS. Supplementary information aimed to facilitate a common understanding of the assumptions made in developing the product profiles; objectives, and development process of the scheme; as well as the rationale for the

factors, criteria, and concern levels in the scheme. A revised version of this document provided to participants of the concordance study is included in Appendix F.

5.4. Data analysis

Ease of use of the RBCS

Average length of time to complete the assessment of the first product was 76 minutes (range 45-120 minutes). Average completion time for the second product was lower (56 minutes, range 30-120 minutes). The average completion time was within an expected duration. A shorter time for completing the assessment of a second product suggests that completion time could reduce with repeated use of the scheme.

Product profiles with insufficient or equivocal information

Three participants noted that five products (E, K, L, Q, and R) contained insufficient or equivocal information for 4 factors in Part A (factors 1, 2, 3, and 7.2), 3 factors in Part B (factors 5.2, 5.3, and 6), and 1 factor in Part C (factor 5). The reasons given are summarized below.

Table 5-1. Reasons for rating a product "product information insufficient/equivocal".

Factor	*Comments*
Part A	
Factor 1	Threshold of toxicological concern not applicable to proteins. Edible part of the crop not specified.
Factor 2	Not clear how common or widespread adventitious occurrence is. No information on processing effect.
Factor 3	Rice consumers not described with respect to dietary pattern or region/country. No data on 95th and/or 99th percentile intake.
Factor 7.2	Interactive effects not clear.
Part B	
Factor 5.2	Assumption was made that intake level of a metabolite might be within upper range of normal despite increase in the modified plant.
Factor 5.3	Answer required detailed knowledge of health effects of consumption of large amounts of foods with high content of the metabolite.
Factor 6	No information on lot-to-lot variation.
Part C	
Factor 1	For uncommon allergens, some guidance on frequency would help.
Factor 5	No guidance on what are "common" allergens.

Factors for which a rating could not be made

For a total of 7 products (C, D, E, F, I, J, Q), four participants were unable to rate 5 factors in Part A (1, 2, 4.1, 4.2, and 6), 4 factors in Part B (1.1, 4.1, 6), and 4 factors in Part C (2, 3, 4.2, 4.3). "Classification criteria unclear" was the reason given for factors A.4.1 and A.6 for product F, Factor B.6 for product I, and factor C.3 for product E. "Other" reasons were given for the remaining factors and products.

Table 5-2. Reasons for inability to provide a rating for any factor.

Factor	*Comments*
Part A	
Factor 1	Inclusion of information on threshold for toxicological concern would be helpful.
Factor 2	Not possible to reach fully supported conclusion: while the enzyme from the bacterium has no known history of safe consumption, information on the metabolites formed is concise. Cannot state with certainty that the plant expressed protein has a safe history: the recombinant protein introduced into the plant is not equivalent to the native protein since it is glycosylated.
Factor 4.1/4.2	See comment for factor 2 regarding glycosylation of recombinant protein.
Factor 6	In vitro digestibility of the introduced enzyme not described. The fate of the metabolites during digestion also not known.
Factor 8	Not clear about the purpose of the question/criteria.
Part B	
Factor 1.1	Type of marker gene not elaborated.
Factor 4.1	No information given on ORFs.
Factor 6	No clear indication of adequate quality control. No real data available.
Part C	
Factor 2	No sequence comparison between recombinant and native proteins, presumably because the protein is not known to be an allergen. A distinction should be made that the IgE binding test is not to detect an allergen, but rather, the potential for cross-reactivity. Therefore, even if the protein is not a known allergen, this does not exclude *a priori* that IgE binding in cross-reactivity with other allergens would not occur.
Factor 4.2	Not clear why the criterion refers to lipid transfer protein when the protein introduced is not one.
Factor 4.3	No mention of abundance in product profile.
Product I	Insufficient information about lysine modification in introduction.
Product C	The absence of transgenic protein from the refined oil does not necessarily preclude its

Factor	*Comments*
	presence due to commingling and presence in the seed meal for animal feed.

General comments

Five participants provided general comments, which are summarized as follows. Where there is more than one respondent providing similar comments, the number of respondents is included in brackets.

- Aggregation of ratings - difficult. May become easier with experience or with instructions (2).
- Counter-intuitive wording of criteria is confusing. Intuitively, one would think that "yes" response would mean "no concern", but this is not always the case.
- Use of key words would be helpful, otherwise an unreasonable amount of time would be spent trying to figure out what you mean.
- Some skip patterns are not clear.
- Level III is ill defined.
- Some answers require assumption when there is no clear basis for rating in the information provided.
- The lack of raw data and the use of multiple measurements to get "the feeling" of the GM organisms made it difficult to just use the statements provided in the product profiles.
- Matches of 6 amino acids are irrelevant. Matches of 8 amino acids may also be irrelevant. What matters is a match of > 35% (not < 35%).
- Additional characteristics to be considered for unintended effects:
  - phenotypic and agronomic characteristics, especially if there is an indication that chloroplast function may be affected.
  - other proteins should be included, especially if the recombinant protein is expressed at high levels.
- Compliments, please continue to make it a success.

5.5. Discussion

Comments were received on 13 out of 20 products to be used in the concordance study. No single factor appeared to dominate in the comments received. In several cases, the reasons given for not being able to rate a factor may also be interpreted as not providing sufficient information in the product profiles. In general, this suggests that there were few major problems with the criteria proposed in the RBCS. However, there was the need to improve the description of the aggregation rules. In addition, the appropriateness and clarity of a few criteria were also questioned. The comments received from the pilot test guided the following revisions of materials used in the concordance study.

- Addition of explanatory/interpretive notes in the combined RBCS-product rating form
- Clarification of several criteria.

- Reformatting of aggregation rules.
- Editing of product profiles to improve clarity.

Annex 5-1
Letter to Interested Participants from Previous Consultations - Product Classification

Date

Dear Dr. (name):

The research project on a "risk-based classification scheme for testing genetically modified foods" at the University of Ottawa, in which you participated in previous consultation(s), is now entering phase 3. [Attached is a report on the results of consultation 3 in which you participated. Based on the comments received, the risk-based classification scheme (RBCS) has been simplified.

In the previous consultation conducted as part of the research project, you indicated an interest in applying the proposed classification scheme to a few reference GM food products provided by the researcher. Information on this aspect of the study is provided below].

PARTICIPATION

Participants will be provided with the descriptions of 2 GM food products (4-5 pages each) and a rating form that outlines the criteria based on the proposed risk-based classification scheme. They will be asked to assign concern levels for each product using the classification scheme and to indicate the applicable criteria for their assignment. They will also be asked to identify any ambiguities in product descriptions and classification criteria, and to indicate the length of time required to complete the classification for each product. Supplementary information that will facilitate completion of the evaluations will be provided. I expect that it will take about one hour to evaluate each product. A participant will have about 2 weeks to return the assessments.

CONTACT INFORMATION

I will contact you by phone within the week to discuss your interest and availability for participation and to answer any questions that you may have.

If you prefer to provide a response by e-mail to Eunice Chao (echao097@uottawa.ca), that would be much appreciated. Suggestions for other potential participants are welcome. If you do not wish to be contacted about this study, please advise me that this is your preference. Thank you for your consideration.

Sincerely,

Eunice Chao, MSc
Doctoral candidate
Population Health PhD Program
University of Ottawa

echao097@uottawa.ca

Tel: (613) 562-5800 ext 2367
Fax: (613) 562-5380

McLaughlin Centre for Population Health Risk Assessment
University of Ottawa
1 Stewart Street, Room 318B
Ottawa, Ontario
CANADA K1N 6N5

To participants of previous consultations: the following will replace the text in [ ]

I am seeking experts to participate in phase 3 of the project by applying the proposed classification scheme to 2 reference GM food products provided by the researcher.

Annex 5-2. Project Information for Research Participants - Product Classification

Name of researcher(s): Eunice Chao

Institution, Faculty, Department: University of Ottawa, Population Health PhD Program

Name of supervisor: Dr. Daniel Krewski

Institution, Faculty, Department: University of Ottawa, Faculty of Medicine, Department of Epidemiology and Community Medicine, Institute of Population Health

Project Description

The doctoral research project "A risk-based classification scheme for testing genetically modified foods" is conducted by Eunice Chao of the Population Health PhD Program at the University of Ottawa under the supervision of Dr. Daniel Krewski. The purpose of the research is to develop and evaluate a risk-based classification scheme as guidance for the testing of foods that are derived from crops genetically modified (GM) by a range of methods. The proposed scheme aims to (1) differentiate and classify GM foods based on levels of concern about potential health risks and attendant uncertainty, and (2) guide the application of testing methods for GM foods in proportion to their potential to produce human health risks. The research will be conducted in several phases. You are asked to participate in phase 3 of the project.

Participation

Participants will be provided with the descriptions of 2 GM food products and a rating form that outlines the criteria based on the proposed risk-based classification scheme. They will be asked to assign concern levels for each product using the classification scheme and to indicate the applicable criteria for their assignment. They will also be asked to identify any ambiguities in product descriptions and classification criteria, and to indicate the length of time required to complete the classification for each product. It will take about one hour to evaluate each product. A participant will have about 2 weeks to return the assessments.

Confidentiality and Anonymity

The contents of the completed assessment will be used only for the purposes of developing a risk-based classification scheme for testing genetically modified foods and that a research participant's confidentiality will be respected through the following actions taken by the researcher.

All of the data collected (including written responses and electronic files) will be tabulated and maintained by the researcher in accordance with confidentiality standards for research involving human participants. The data will be accessible only to the researcher in a locked filing cabinet and a password protected personal computer on the premises of the Institute of Population Health, University of Ottawa. Data files will be kept for 5 years after the completion of the thesis.

Anonymity of a participant's individual views and responses will be protected. The rating form will be coded for the purposes of follow-up. While group results of the assessments at the aggregate level will be published, and discussed within the research team, and at conferences, no names will be linked to individual views or responses in data analysis, reports or in presentations.

In order to acknowledge pa ants' contribution to the research project and to make the expert consultation process transparent and cr from a research perspective, the names of individuals and affiliations (the organization where one w or which one represents) will be included in a list as an appendix to the doctoral thesis. Participant: be given the opportunity to indicate their preference as part of their written responses included in the r orm. Only one combined list of participants for all phases of the research will be published without identif ne specific phase to which they made a contribution.

Research participants agr to disclose or publish any aspects of the risk-based classification scheme developed by the researcl ept with the express consent of the researcher.

Voluntary Participation

There is no direct individ efit from participating in the study and from completing the assessments. All costs associated with pa on in the project (long-distance phone calls and postage, where applicable) will be born by the McL Centre for Population Health Risk Assessment. Return postage for mailed responses of participants countries outside Canada will be paid in the form of international postal reply coupons, if requested.

Participation is completel ntary. A participant is free to withdraw from the project at any time, refuse to participate, or decline to a certain questions.

THE RETURN OF COMPL D PRODUCT CLASSIFICATIONS IMPLIES CONSENT.

Any information about the s of a research participant may be addressed to **Protocol Officer for Ethics in Research, University tawa,**

Any questions about the cc ct of the research project may be directed to the researcher or her supervisor at the coordinates listed be

Eunice Chao: **Telephone ber:** **Fax number:**
E-mail add

Daniel Krewski: **Telephone ber:** **Fax number:**
E-mail address.

Address: McLaughlin Centre for Population Health Risk Assessment
Institute of Population Health, University of Ottawa

Annex 5-3. Questionnaire

Product ID: Participant ID:

Annex 5-3

COMBINED RISK-BASED CLASSIFICATION SCHEME - PRODUCT RATING FORM (November 2005)

Instructions for completing product rating.

1) For each of the three parts (A, B, C) of the risk-based classification scheme (RBCS), please indicate for each factor whether the criteria for that factor have been met, by placing a check mark under the appropriate column.

Where there is more than one substance in a product to be assessed, assess each substance. Check the column that corresponds to the highest concern level for each factor.

Increasing concern →

☺ = Yes or not applicable ? = Product information insufficient or equivocal ☻ = No

2) Based on your ratings for the factors in each part of the scheme, please assign a concern level for that part using the aggregation/decision rules that are provided after Part C of the rating form.

3) Please complete the questions on the last page of the rating form. The information you provide will help identify areas for improvement for the classification scheme and the product profiles.

PLEASE RATE THE PRODUCTS BY USING THE INFORMATION AS PROVIDED IN THE PRODUCT PROFILES. The objective of this evaluation is to assess the applicability of the proposed RBCS to the product information given. Space is provided on the last page of the rating form for any comments you may have on the information in the product profiles and/or the classification scheme.

If you have a question about a factor or criterion, please refer to section 4 of "Supplementary Information for Applying the Risk-Based Classification Scheme" in a separate document. Supplementary information on the factors and criteria is provided in the same order as they appear on the rating form for easy reference.

A response date of November 30, 2005 is requested. If you are unable to complete the product evaluations, please advise Eunice Chao (echao097@uottawa.ca) as soon as possible, as it will be necessary to find a replacement to complete the planned product evaluations. Thank you.

Product ID: Participant ID:

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES RESULTING FROM THE PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED - applicable to both protein and non-protein substances, unless stated otherwise.

☺ = Yes or not applicable ? = Product information insufficient or equivocal ☻ = No

Factors and Criteria	☺	?	☻
Factor 1. Expression of gene product(s) in edible part(s) of the modified crop.	☐	☐	☐

Protein and non-protein substances meeting any one of the following criteria (1.1, 1.2, or 1.3) will be classified as Level I. If "no", assess the remaining factors in Part A. If "yes" to criteria (1.1 or 1.2), go to Parts B and C. If "yes" to criteria (1.3), go to "aggregation of ratings for factors" on p. 8 of the rating form.

Criteria

1.1. Substances with NO nutritional role *and* NOT expressed in the modified crop due to intentional gene silencing, *or*

1.2. Total dietary intake of gene product(s) is below the threshold of toxicological concern for the chemical class where applicable, *or*

1.3. Gene products NOT expressed, detected or changed in the edible part(s) of the modified crop
a/ tissue-specific expression NOT involving the edible part(s) of the modified crop, *or*

b/ the edible part is used as a food ingredient in a limited form as a highly refined and well-defined chemical entity (e.g., refined oil from oilseed rape and cottonseed).

Factor 2. Prior history of known or presumed safe consumption from foods.	☐	☐	☐

Criteria

a/ Naturally occurring food constituents, *or*

b/ Other substances that have a history of consumption from foods without reported adverse health effects (e.g., protein constituents of adventitious non-pathogenic bacteria commonly present on fresh produce).

Factor 3. Expected change in total dietary exposure (based on theoretical estimates).	☐	☐	☐

Criteria

Substances with known toxicity concerns

a/ Total dietary intake below established upper limit (ADI, tolerance level, or equivalent) with an adequate margin of safety.

Other substances

b/ Changes within normal variation in dietary intakes.

Factor 4. Prior knowledge of safety/mode of action (including considerations of food preparation/processing methods, e.g., the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

Factor 4.1. Food constituents and substances with a prior history of consumption from foods.	☐	☐	☐

Criteria

Substances with known toxicity concerns

a/ Established upper limit (ADI, tolerance level, or equivalent).

Other substances

b/ Well-established biological function or specificity of mode of action with NO potential for mammalian toxicity, antinutritional, and adverse metabolic effects under normal conditions of consumption, *or*

c/ Limited empirically based toxicity profile *and* simple chemical structure suggestive of low order of oral toxicity.

Factor 4.2. Substantially modified or novel substances (NO or limited prior history of consumption from foods).	☐	☐	☐

Criteria

Pesticidal and enzymatic proteins

a/ NO structural or functional similarity to known proteins with toxic or antinutritional properties relevant to mammals *and* at low expression level.

Non-protein single chemical substances

b/ WITHOUT structural similarity to known mutagen or carcinogen *and* < 0.5 ppm in the total diet.

Factors and Criteria ☺ ? ☻

Other protein and non-protein complex substances
c/ WITHOUT structural similarity to known mutagen or carcinogen *and* < 0.1% diet.

Factors (5-7) are *particularly relevant to substances with prior dietary history (i.e., "yes" for factors (2) and (4.1) for determining concern levels).* *For substances without a dietary history, information on these factors may be limited. To the extent that such information is available, it would also help determine an appropriate concern level for testing.*

Factor 5. Structure-activity characterization. ☐ ☐ ☐

Criteria

Proteins derived from non-plant or non-food source
a/ NO amino acid sequence identity when compared with known mammalian toxins, rapid and complete pepsin digestion (< 10 min) using standard testing conditions, *and* structurally similar and functionally identical to comparator/source protein.

Non-proteins
b/ Structurally similar and functionally identical to natural food constituents.

Factor 6. Digestion and degradation products (based on known function and metabolism of the introduced/ modified gene products). ☐ ☐ ☐

Criteria

In edible parts of modified crop.
a/ NO potentially toxic metabolites expected from catabolism of an introduced/modified substance or of an exogenous chemical (e.g., detoxification of a herbicide or mycotoxin), *and*

In mammalian metabolism from consumption of the gene product.
b/ NO products of digestion or bioactivation at levels of immunological, pharmacological, or toxicological significance.

Factor 7. Synergism and interactions (based on known functions of the introduced/modified gene products).

Factor 7.1. Between gene products introduced/modified (in the same or separate transformation events). ☐ ☐ ☐

Criteria

a/ NO synergistic toxic effects expected.

Factor 7.2. Between gene products introduced/modified and food constituents. ☐ ☐ ☐

Criteria

a/ NO negative effect on bioavailability of food constituents expected.

Factor 8. Prior experience with gene products introduced/modified - NOT part of product rating for the pilot test. Please evaluate this product as a new product.

Criteria

a/ Comparable in expression levels, gene source, transfer method, and host plant, *and*
b/ Approved by at least one national competent authority.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT - applicable to the food derived from the modified crop.

☺ = Yes or not applicable ? = Product information insufficient or equivocal ☻ = No

Factors and Criteria	☺	?	☻
Factor 1. Safety of donor organism (gene source).	☐	☐	☐
Criteria: a/ NOT a source of known toxicant, antinutrient, or pathogenicity/infectivity.			
Assess factor (1.1) or (1.2) if rating for factor (1) is "no" or "product information insufficient or equivocal".			
Factor 1.1. Characterized gene modification (based on nucleic acid techniques).	☐	☐	☐
Criteria a/ Absence of transfer of undesirable characteristics to the host plant is supported by experimental evidence.			
Factor 1.2. Poorly characterized gene modification (based on undirected mutagenesis, somaclonal variation, or wide-cross hybridization).	☐	☐	☐
Criteria a/ Genetically related organisms (in the same genus) are NOT sources of known toxicant or antinutrient, *or* b/ Extent of backcrossing is sufficient to remove large percentage of genetic material from the donor organism.			
Factor 2. Safety of host plant.	☐	☐	☐
Criteria a/ NOT a source of known toxicant or antinutrient.			
Factor 3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).			
Factor 3.1. Knowledge of gene product function.	☐	☐	☐
Criteria a/ Protein gene product(s) have well-characterized function in the modified plant.			
Factor 3.2. Impact of gene product function in the modified plant.	☐	☐	☐
Criteria a/ Expression product of the introduced/modified gene does NOT have a metabolic, regulatory, or signalling function in the modified plant.			
Applicable if gene product has enzymatic function in the modified plant. **Factor 3.3. Substrate specificity of gene product.**	☐	☐	☐
Criteria a/ Narrow substrate specificity (i.e., unlikely to interact with endogenous substrates to form novel compounds).			
Factor 4. Potential for disrupting coding or regulatory DNA sequences in the modified plant (based on bioinformatics and transcript analyses).			
Factor 4.1. In the inserted/modified DNA.	☐	☐	☐
Criteria a/ NO novel open reading frames (ORFs), disruptions of existing ORFs, or functional expression.			
Factor 4.2. In genomic sequences bordering the site of insertion/modification.	☐	☐	☐
Criteria a/ NO novel open reading frames (ORFs), disruptions of existing ORFs, or functional expression.			
Factor 5. Chemical analysis of food derived from the modified plant. ***NOT applicable to substances intended to be changed.***			
Factor 5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents, where available).	☐	☐	☐
Criteria a/ Within acceptable ranges of natural variation, *or* b/ NOT expected to have adverse nutritional, metabolic, or toxicological effects.			
Factor 5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus	☐	☐	☐

Factors and Criteria	☺	?	☹

documents, where available).

Criteria

a/ Within acceptable ranges of natural variation, *or*

b/ NOT expected to have adverse nutritional, metabolic, or toxicological effects.

Assess factor (5.3) if rating for any of the factors (1-4) above is "no" or "product information insufficient or equivocal":

Factor 5.3. Other metabolites/parameters. ☐ ☐ ☐

Criteria

a/ Reasonably justified to be sufficient in scope, *and*

b/ NO unknown compounds or significantly altered levels of endogenous metabolites outside the range of natural variation, *or*

c/ If changes occur, the likelihood of negative health effects can reasonably be justified to be low.

Factor 6. Health consequences of instability of the modified trait. ☐ ☐ ☐

Criteria

a/ Modified trait is NOT intended to provide nutrition or health benefits, *or*

b/ Modified trait is intended to provide nutrition or health benefits, *and*

c/ Adequate quality control is in place to ensure consistency in lot-to-lot expression of the targeted gene product.

Product ID: Participant ID:

PART C - POTENTIAL FOR ALLERGENIC (IgE-MEDIATED) EFFECTS - applicable to protein substances.

☺ = Yes or not applicable ? = Product information insufficient or equivocal ☻ = No

Factors and Criteria	☺	?	☻
Factor 1. Allergenicity of donor organism (gene source).	☐	☐	☐

Criteria

a/ NOT a known source of allergenicity or gluten-sensitive enteropathy.

Protein gene products. ***Assess factors (2) and (3) if rating for factor (1) is "yes" or "product information insufficient or equivocal".***

Factor 2. Potential for protein gene product(s) to cross react with known allergens.	☐	☐	☐

Criteria

a/ Comparison of amino acid sequence identity with known allergens: < 35% over 80 or more amino acids based on a sequential search, *and*

b/ NO identical matches in amino acids in a short sequence (e.g., 6 - 8 contiguous amino acids).

Factor 3. Pepsin digestion (under standard testing conditions).	☐	☐	☐

Criteria

Novel or substantially modified proteins (without prior dietary history)

a/ Rapid and complete digestion with NO stable fragments (< 10 min).

Other proteins (with prior dietary history)

b/ Rapid or moderate digestion with NO stable fragments (< 15 min).

Factor 4. Secondary factors. ***Applicable if the rating for factors (2 or 3) is "no" or "product information insufficient or equivocal".***

Factor 4.1. Structural/functional characteristics.	☐	☐	☐

Criteria

a/ NO post-translational modification involving glycosylation with primarily N-linked, specific xylose or fucose substitutions (applicable to proteins derived from microbial or animal sources), *or*

b/ NO structural or functional relationship to specific protein families or functional categories commonly associated with allergenicity.

Factor 4.2. Heat stability.	☐	☐	☐

Criteria

a/ Heat labile (NOT applicable to lipid transfer proteins).

Factor 4.3. Abundance.	☐	☐	☐

Criteria

Pesticidal and enzymatic proteins

a/ < 0.1% protein.

Other proteins

b/ < 1% total protein.

Factor 5. Allergenicity of host plant.	☐	☐	☐

Criteria

a/ NOT a common source of allergenicity or gluten-sensitive enteropathy, *or*

b/ Level II for Part B, if the host plant is a common source of allergenicity or gluten-sensitive enteropathy.

Aggregation of ratings for factors.

Based on your ratings for individual factors in the previous parts of the scheme, please assign a concern level for each part by following the aggregation/decision rules in the tables on the next page. Select the primary characteristic that applies (e.g., with dietary history, or without dietary history) and determine if the specified factors and criteria for a given level are met.

Where a factor has subsections (i.e., X.X), all subsections must qualify for the rating ("yes/not applicable", "insufficient product information", or "no"), unless specified otherwise.

Example 1 - Part A - for a gene product *with* a dietary history.

Factor	Rating
1.	No
2.	Yes
3.	? (Product information insufficient/equivocal)
4.1	Yes
5.	Yes
6.	Yes
7.	Yes/Not applicable (7.1 = Not applicable - only 1 gene product to be assessed; 7.2 = Yes)

Based on the ratings for the individual factors, the concern level for Part A = II

Example 2 - Part A - for a gene product *with* a dietary history.

Factor	Rating
1.	No
2.	Yes
3.	? (Product information insufficient/equivocal)
4.1	Yes
5.	?
6.	Yes
7.	No (7.1 = Not applicable - only 1 gene product to be assessed; 7.2 = No)

Based on the ratings for the individual factors, the concern level for Part A = III

Example 3 - Part A - for a gene product *without* a dietary history (substantially modified).

Factor	Rating
1.	No
2.	No
3.	?
4.2	Yes
5.	Yes
6.	Yes
7.	Yes/not applicable (7.1 = Not applicable - only 1 gene product to be assessed; 7.2 = Yes)

Based on the ratings for the individual factors, the concern level for Part A = II

Product ID: Participant ID:

Please indicate the concern level for each part by placing a check mark in the applicable row.

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS FROM INTENDED GENE PRODUCTS.

	Yes/Not applicable	Product Information Insufficient/Equivocal	No	Product Rating (check the applicable row)
Level I	1) Negligible dietary exposure			☐
	Only factor (1)	-		
	2) With prior experience			☐
	Only factor (8)	-		
	3) With dietary history			☐
	All applicable factors (2-3, 4.1, 5-7)	Factor (1 or 8)		
Level II	1) With dietary history			☐
	All applicable factors (2, 5-7)	Only factor (3), or (4.1)	None	
	2) Without dietary history			☐
	All applicable factors (4.2, 5-7)	Factor (2), or (3), or both	Only factor (2)	
Level III		None of the above		☐

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN MODIFIED FOOD.

	Yes/Not applicable	Product Information Insufficient/Equivocal	No	Product Rating (check the applicable row)
Level I	1) Negligible expression of intended gene products in edible parts			☐
	Criteria (1.3) for factor (1) in Part A	-		
	2) Characterized gene modification (based on nucleic acid techniques)			☐
	All applicable factors (1 or 1.1 and 2, 3.1, 4.1, 5, 6)	Factor (3.2 or 3.3), or (4.2), or both	Factor (3.2 or 3.3)	
	3) Poorly characterized gene modification (based on undirected mutagenesis, somaclonal variation, or wide-cross hybridization)			☐
	All applicable factors (1 or 1.2 and 2, 3.1, 5, 6)	Factor (3.2 or 3.3), or (4), or both	Factor (3.2 or 3.3)	
Level II		None of the above		☐

PART C - POTENTIAL FOR ALLERGENIC EFFECTS - applicable to protein substances.

	Yes/Not applicable	Product Information Insufficient/Equivocal	No	Product Rating (check the applicable row)
Level I	1) Negligible expression of intended gene products in edible parts			☐
	Criteria (1.3) for factor (1) in Part A	-		
	2) Low identifiable concerns with allergenic activity			☐
	Factors (1, 2, 5) and Factor (4) if applicable		Only factor (3)	
Level II		None of the above		☐

Product ID: Participant ID:

Please provide the following information.

1. Length of time required to complete the classification of this product: _____ minutes.

2. If your rating for this product includes "product information insufficient/equivocal", please identify the relevant factor (e.g., A.4.1) and explain.

3. If you were unable to provide a rating for any factor, please complete the following.

Factor (e.g. A.2.1)	Reasons	Comments
	□ Classification criteria unclear □ None of the criteria applies □ Other (please explain)	
	□ Classification criteria unclear □ None of the criteria applies □ Other (please explain)	
	□ Classification criteria unclear □ None of the criteria applies □ Other (please explain)	
	□ Classification criteria unclear □ None of the criteria applies □ Other (please explain)	
	□ Classification criteria unclear □ None of the criteria applies □ Other (please explain)	

4. General comments (e.g., any comments you may have on the RBCS, product profile, and/or supplementary information).

5. Background and other information.

a) My expertise (check all that apply)

□ Toxicology
□ Immunology
□ Pharmacology
□ Molecular biology
□ Other biological sciences (e.g., microbiology)
□ Plant sciences
□ Agricultural sciences
□ Food/Nutritional sciences
□ Agricultural biotechnology
□ Analytical sciences
□ Health risk assessment
□ Medical/Health sciences
□ Other (please specify)

b) My primary affiliation (check one)

□ Academic □ Government □ Public research institute □ Industry □ Consulting
□ Other (please specify)

c) If you have not already done so, please indicate your preference (yes / no) for acknowledging your contribution to the project.

□ Yes, I agree to have my name and affiliation included in the researcher's thesis
□ No, I do not wish my name and affiliation to be included in the researcher's thesis

THANK YOU FOR YOU PARTICIPATION IN THE STUDY. Please return your completed evaluation to: Eunice Chao (echao097@uottawa.ca).

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APPENDIX F

Annex 6-1

Combined RBCS–Product Rating Form

Product ID: **COMBINED RBCS-PRODUCT RATING FORM** Participant ID:

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES RESULTING FROM THE PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED - applicable to both protein and non-protein substances, unless stated otherwise.

Y = Yes (criterion met) NA = Not applicable No = Criterion not met ? = Product information insufficient or equivocal

Factors and Criteria	Y/NA	No/?	Interpretation /Explanation
Factor A.1. Expression of gene product(s) in edible part(s) of the modified crop.			"Edible parts" refer to those parts of the modified crop that are used in food or ingredient processing for human consumption. A gene product that meets any of the criteria (A.1.1, 1.2, 1.3) for this factor will be classified as Level I, subject to confirmation by chemical analysis on samples that are representative of normal post-harvest handling and processing. This is to ensure that no safety concerns would arise from post-harvest comingling with non-edible parts of the modified crop.
Criteria A.1.1. Substances with NO nutritional role *and* NOT expressed in the modified crop due to intentional gene silencing, *or*			Substances with a nutritional role are macronutrients (protein, fat and carbohydrates), vitamins and minerals. **If a gene product meets this criterion, go to Parts B and C.** Suppressed expression of gene products due to intentional gene silencing may trigger unintended changes, including attendant allergenic effects if the host plant is a source of allergen.
Non-protein substances only A.1.2. Total dietary intake of gene product(s) is below the threshold of toxicological concern for the chemical class where applicable, *or*			Structure-based thresholds of toxicological concern (TTC) have been proposed for non-protein substances [Kroes et al., 2000; Renwick, 2005]. Thresholds apply to aggregate exposure to all sources. Chemicals in structural classes I, II, and III: 1800, 540, and 90 μg/ person per day (600, 180, 30 ppb diet), respectively. Organophosphates: 18 μg per person per day (6 ppb diet). Compounds with structural alerts for genotoxicity (excluding aflatoxin-like, azoxy- and nitroso-compounds): 0.15 μg/ person per day (0.05 ppb diet). The 3 structural classes based on the decision tree approach of Cramer et al. [1978] can be summarized as follows. Class I: Substances of simple chemical structure and efficient modes of metabolism. Class II: Intermediate substances with structures that are less innocuous than class I substances but not do contain structural features suggestive of toxicity like those substances in class III. Class III: Substances of a chemical structure that permits no strong initial presumption of safety or may even suggest significant toxicity or have reactive functional groups. This criterion does *not* apply to protein substances for which no TTC values are available. **If a gene product meets this criterion, go to Parts B and C.**
A.1.3. Gene products NOT expressed, detected or changed in the edible part(s) of the modified			**If a gene product meets part (a) or (b) of this criterion, go to "aggregation of factors" on p. 15-16 of this rating form.**

Factors and Criteria	Y/NA	No/?	Interpretation /Explanation
crop a/ tissue-specific expression NOT involving the edible part(s) of the modified crop, *or*			At present, the application of this criterion may be limited since many of the promoters currently in use are more appropriately referred to as "tissue-enhanced", rather than "tissue-specific", because their expression may not be completely confined to a single tissue or plant part [Potenza et al., 2004].
b/ the edible part is used as a food ingredient in a limited form as a highly refined and well-defined chemical entity (e.g., refined oil from oilseed rape and cottonseed by hot solvent extraction).			A gene product meeting part (b) of this criterion can be considered to be of low concern (Level I) for unintended changes, as well as for allergenic effects where the efficacy of processing in removing protein and other substances has been established. Established specifications for identity and purity of food ingredients are also applicable to food ingredients derived from modified crops. Also, foods or food fractions from GM products that are always processed to remove essentially all of the introduced protein would not pose allergenic risk even if the introduced protein were an allergen [Goodman and Hefle, 2005].
If none of the above criteria is met, check "No".		□ No	**If the rating for factor 1 is "No", assess the remaining factors in Part A.**
Factor A.2. Prior history of consumption from foods.			This factor focuses on assessing whether a substance introduced or modified in the GM crop is completely novel in the food supply, or is a food constituent that has been substantially altered.
Criteria a/ Naturally occurring food constituents, *or*			A substance is considered to be a naturally occurring food constituent (i.e. with a prior history of consumption from foods) when a) a protein substance from a food source is encoded by a modified gene without changes in its amino acid sequence, or an intended change in its amino acid sequence without detrimental changes in its functionalilty. b) a non-protein substance from a food source with minor changes in its structure without detrimental changes in its functionality. c) a protein or non-protein substance from a non-food source that has a similar structure and identical function to known food constituents. Use additional information (A.5) in rating this factor.
b/ Other substances that have a history of consumption from foods without reported adverse health effects.			Examples include: protein constituents of adventitious non-pathogenic bacteria commonly present on fresh produce or at trace levels in some foods; pesticide residues with established upper limits.
If none of the above criteria is met, check "No" or "?".		□ No □ ?	
Factor A.3. Expected change in total dietary exposure (based on theoretical estimates).			As tier 1 screening of expected change in dietary exposure, estimates analogous to theoretical maximum may be used. Depending on the degree of concern or uncertainty about this initial estimate, more refined calculations or modelling can follow as part of further analysis and testing.
Criteria ***Substances with known toxicity concerns*** a/ Total dietary intake below established upper limit (ADI, tolerance level, or equivalent) with an adequate margin of exposure.			Substances with known toxicity concerns are those substances for which upper limit of consumption has been established. If an upper limit is not provided in the product profile, assume no such value is available.

Factors and Criteria	Y/NA	No/?	Interpretation /Explanation
			For this evaluation, an adequate margin of exposure for a naturally occurring toxicant or antinutrient may be considered if it is present in foods at multiples of approximately 25 or more of ordinary exposures [IFBC, 1990] (see also explanation B.1.a).
Other substances b/ Changes within normal variation in dietary intakes.			If no changes in the consumption of the modified *food* are expected, changes within normal variation of a *gene product of interest* may be deduced if the level of the substance in the modified crop is comparable to the level in the unmodified counterpart, or to the level in food types with similar consumption patterns (e.g.., within cereal grains, vegetables, or fruits). For many insecticidal and enzymatic proteins that are intentionally introduced in GM crops, their expression levels are very low (< 0.1% total protein) and the incremental new exposure may be considered insignificant relative to background level of exposure to dietary protein.
If none of the above criteria is met, check "No" or "?".		□ No □ ?	
Factor A.4. Prior knowledge of safety/mode of action.			This factor includes consideration of normal conditions of consumption - see explanation A.4.1(b) below.
Factor A.4.1. Food constituents and substances with a prior dietary of consumption from foods. (with dietary history).	□ NA		**If the rating for factor A.2 above is "Y", assess factor A.4.1. Otherwise, check "NA" and assess factor A.4.2.**
Criteria ***Substances with known toxicity concerns*** a/ Established upper limit (ADI, tolerance level, or equivalent).			Substances with known toxicity concerns - see explanation A.3.a.
Other substances b/ Well-established biological function or specificity of mode of action with NO potential for mammalian toxicity, antinutritional, and adverse metabolic effects under normal conditions of consumption, *or*			Normal conditions of consumption include considerations of food preparation/processing methods, e.g., the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing; the levels and/or bioavailability of the gene product of interest may change as a result of processing.
c/ Limited empirically based toxicity profile *and* simple chemical structure or predictable metabolism suggestive of low order of oral toxicity.			For non-protein substances, simple chemical structure and low order of oral toxicity would be equivalent to structural class I [Cramer et al., 1978] or structure category A (including lipids, simple and complex carbohydrates and their metabolic products [FDA, 2003]. Protein and enzymatic proteins that fit the description A.4.2 (a) below would also meet this criterion. This criterion recognizes that many naturally food constituents have not been experimentally tested for toxicity. In the absence of experimental data, structure-based information will be relied upon as part of tier 1 screening.
If none of the above criteria is met, check "No" or "?".		□ No □ ?	
Factor A.4.2. Substantially modified or novel substances (without dietary history).	□ NA		**If the rating for factor A.2 above is "No" or "?", assess factor A.4.2. Otherwise, check "NA".** Three categories of substances with no or limited dietary history are considered. For this evaluation, a

Factors and Criteria	Y/NA	No/?	Interpretation /Explanation
			combination of structural and exposure criteria for the 3 categories are described below.
Criteria ***Pesticidal and enzymatic proteins*** *a/* NO structural or functional similarity to known proteins with toxic or antinutritional properties relevant to mammals *and* at low expression level.			Pesticidal and enzymatic proteins with toxic or antinutritional properties include, but are not limited to: inhibitors of digestive enzymes, some types of lectins/hemagglutinins/ribosome inactivating proteins, and proteins with amino acid sequence identity with known mammalian toxins (~80% - see A.5.a). A "low level of expression" is to distinguish pesticidal or enzymatic proteins (ranging from 1 ppm or less to 2% of total protein) from other proteins that are introduced or modified typically at a higher level for health-related or other functional purposes [Betz et al., 2000; Peumans and Van Damme, 1996; Heil and Bostock, 2002].
Non-protein single chemical substances *b/* WITHOUT structural similarity to known mutagen or carcinogen *and* < 0.5 ppm in the total diet.			At levels < 0.5 ppm, chronic toxicity and carcinogenicity testing is generally not required for substances without structural similarity to known mutagen or carcinogen (equivalent to structure category B for food additive assessment), unless indicated by findings in shorter term testing [FDA, 2003].
Other protein and non-protein complex substances *c/* WITHOUT structural similarity to known mutagen or carcinogen *and* < 0.1% diet.			The level of < 0.1% of diet was chosen as a reference point for complex substances because substances at concentrations > 0.1% cannot be evaluated in animal testing using the approach based on 100:1 uncertainty factor. It is assumed that tests conducted using a substance at a dietary concentration > 10% in the diet of experimental animals will likely lead to results that are not meaningful, and may require alternative types of testing [LSRO, 1999].
If none of the above criteria is met, check "No" or "?".		□ No □ ?	
Factors (A.5-7) are *particularly relevant to substances with prior dietary history (i.e., "yes" for factors(A.2) and (A.4.1) for determining concern levels).* *For substances without a dietary history, information on these factors may be limited. To the extent that such information is available, it would also help determine an appropriate concern level for testing.*			
Factor A.5. Structure-activity characterization.			This factor focuses on demonstration of structural similarity and functional equivalence with substances that have a history of known or presumed safe history of food use.
Criteria ***Proteins*** *a/* NO amino acid sequence identity when compared with known mammalian toxins, rapid and complete pepsin digestion (< 10 min) using standard testing conditions, *and* structurally similar and functionally identical to comparator/source protein.			There is no generally accepted standard for a level of ***amino acid sequence identity*** with known toxins below which there is no safety concerns. It has been suggested that if a protein has a very high similarity to a protein known to be toxic to mammals (in the vicinity of 80% amino acid sequence identity), then there is reason to believe that the introduced protein may also be toxic. However, the determination of amino acid sequence identity depends on the alignment algorithms used and results may be reported in different ways, making it difficult to assign a numerical level that should trigger more rigorous testing [FIFRA SAP, 2000]. ***Pepsin digestibility*** is an *in vitro* test that is a focus of assessment of the allergenic potential of proteins. General patterns of digestion using standard testing conditions have been observed [Astwood et al., 1996; Metcalfe et al., 1996; Thomas et al., 2004]. However, no cut-off values for pepsin digestion assays have been provided in guidance documents on GM food safety assessment (see also C.3.a). It is generally assumed that proteins which act like dietary proteins that are susceptible to digestion and break down into their constituent amino acids would be unlikely to exert adverse effects [Mendelsohn et al., 2003]. ***Structural similarity and functional equivalence*** to comparator/source protein can be demonstrated by a combination of empirical data, including molecular size, amino acid sequence (particularly of the active site and N-linked sequences), immunoreactivity, bioactivity (e.g., target specificity for a pesticidal protein or

Factors and Criteria	Y/NA	No/?	Interpretation /Explanation
			substrate specificity for a catalytic protein), physical-chemical characteristics (e.g., electrophoretic behaviour, heat stability). Susceptibility to heat activation and sensitivity to proteolysis degradation are not characteristics of known protein toxins.
Non-proteins *b/ Structurally similar and functionally identical to natural food constituents.*			Information on functional properties is not required if there are no concerns with chemical structure. The identity of secondary gene products that are generated by the enzyme introduced or modified in a GM crop can be verified based on biochemical analysis and/or other physical-chemical and functional characteristics of the purified form of the substance.
If none of the above criteria is met, check "No" or "?".		□ No □ ?	
Factor A.6. Digestion and degradation products.			The assessment of this factor focuses on known function and metabolism of the introduced/ modified gene products. The major considerations are explained below.
Criteria In edible parts of modified crop. a/ NO potentially toxic metabolites expected from catabolism of an introduced/modified substance or of an exogenous chemical (e.g., detoxification of a herbicide or mycotoxin), *and* In mammalian metabolism from consumption of the gene product. b/ NO products of digestion or bioactivation at levels of immunological, pharmacological, or toxicological significance.			An assessment of the potential for toxic effects of the degradation products in the modified plant would be necessary if an enzyme that is introduced/modified has a function in the modified plant in metabolizing an exogenous chemical, such as a herbicide or mycotoxin. In mammalian metabolism in general, peptide fragments that result from incomplete breakdown of proteins are less toxic than the intact proteins. Exceptions to this general rule are certain protoxins, but it is extremely unlikely that toxic fragments would arise by chance from digestion of larger (non-toxic) proteins. However, certain peptides can exhibit hormonal activity. Bioactive peptides from enzymatic proteolysis of certain proteins from milk and from some cereals have been identified [FIFRA SAP, 2000]. These bioactive peptides are unlikely to be at levels of immunological or pharmacological concern when they are released from normal digestion of proteins from dietary sources. However, the potential for their release from proteins modified for this purpose should not be ignored.
If none of the above criteria is met, check "No" or "?".		□ No □ ?	
Factor A.7. Synergism and interactions.			The assessment of this factor focuses on interactions between the introduced/modified gene products that are based on known functions with the potential to interact and raise safety concerns.
Factor A.7.1. Between gene products introduced/modified.	□ NA		**This factor is applicable to modified crops containing "stacked genes". For the purpose of this evaluation, if there is only one primary gene product to be assessed in a product, check "NA".** For this evaluation, gene stacking refers to the introduction/modification of more than one gene to alter the same trait in the same or separate transformation events.
Criteria a/ NO synergistic toxic effects expected.			An example of gene stacking where one gene product may amplify the toxic effect of another gene product is the combination of type-1 ribosome inactivating protein (RIP) and lectin. Type-1 RIP is normally non-toxic because it cannot penetrate the brush-border membranes of the gut lumen. When combined with a relatively harmless lectin, RIP can potentially be transported into intestinal cells through lectin-mediated co-endocytosis. As the type-1 RIP reaches the cell cytoplasm it can cause cell death by destroying the protein-synthesis capacity of the cell [Peumans and van Damme, 1996].

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Factors and Criteria	Y/NA	No/?	Interpretation /Explanation
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor A.7.2. Between gene products introduced/modified and food constituents.			
Criteria a/ NO negative effect on bioavailability of food constituents expected.			An example of a GM crop producing a protein gene product that has a negative effect on the bioavailability of a food constituent is avidin (a protein derived from egg white). Avidin is known to bind biotin, which is a nutrient present in foods.
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor A.8. Prior experience with gene products introduced/modified.			**NOT part of product rating for this assessment of the RBCS. Please evaluate this product as a new product.**
Criteria a/ Comparable in expression levels, gene source, transfer method, and host plant, *and* b/ Approved by at least one national competent authority.			

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT - applicable to the food derived from the modified crop.

Y = Yes (criterion met) NA = Not applicable No = Criterion not met ? = Product information insufficient or equivocal

Factors and Criteria	Y/NA	No/?	Interpretation/Explanation
Factor B.1. Safety of donor organism (gene source).	□ NA		**If the donor and host plants are the same species, check "NA". Assess factor B.2. If the rating for factor B.2 is "No" or "?", assess factor B.1.1 or B.1.2.**
Criteria: a/ NOT a source of known toxicant, antinutrient, or pathogenicity/infectivity.			A donor organism is considered a source of known toxicant, antinutrient, or pathogenicity/infectivity when a) the organism has been associated with significant toxic effects in humans, domestic animals, or experimental animals, or occurring with narrow margins of exposure (i.e., at low multiples of approximately 25 or less of ordinary exposures) [IFBC, 1990] b) the causative agent is produced as a consequence of biosynthesis in the organism.
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor B.1.1. Characterized gene modification.	□ NA		**For genetic modification based on nucleic acid techniques, assess factor B.1.1 if the rating for factor B.1 is "No" or "?". Otherwise, check "NA".**
Criteria a/ Absence of transfer of undesirable characteristics to the host plant is supported by experimental evidence.			Absence of transfer of undesirable characteristics from the donor organism can be verified by testing for the known toxicant using an *in vitro* culture system (e.g., by cloning and expressing the DNA segment in a cell culture).
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor B.1.2. Poorly characterized gene modification.	□ NA		**For genetic modification based on undirected mutagenesis, somaclonal variation, or wide-cross hybridization, assess factor B.1.2 if the rating for factor B.1 is "No" or "?". Otherwise, check "NA".**
Criteria a/ Genetically related organisms (in the same genus) are NOT sources of known toxicant or antinutrient, *or*			In poorly characterized gene modification such as that involving wide-cross hybridization with a distantly related organism, prior knowledge of the safety of the organism may be limited. In such a case, information on the presence of natural toxins and antinutrients in related plant species may be used as guidance in identifying classes of compounds most likely to occur in the investigated species [Howlett et al., 2003; IOM and NRC, 2004a].
b/ Extent of backcrossing is sufficient to remove large percentage of genetic material from the donor organism.			The extent of backcrossing can be ascertained using a combination of morphological, cytological, biochemical and molecular markers in characterizing the resultant hybrid or mutant. No generalized level of backcrossing can be set. Depending on the mating design, a parent's contribution can range from very small (less than 1% for the donor parent in backcrossing) to very large (over 99% for the recurrent parent in backcrossing) [NRC, 2000, p. 58]. It would be reasonable to expect that the higher the level of uncertainty about the safety of the gene source, the higher the level of contribution of genetic material in the modified crop should be from the plant variety with an established history as a food crop.
If the criterion is not met, check "No" or "?".		□ No □ ?	

Factors and Criteria	Y/NA	No/?	Interpretation/Explanation
Factor B.2. Safety of host plant.			
Criteria a/ NOT a source of known/significant toxicant or antinutrient.			A host plant is considered a source of known/significant toxicant or antinutrient when: a) a toxicant or antinutrient is present in the edible part of the host plant as a consequence of biosynthesis in the plant, and b) the organism has been associated with significant toxic effects in humans, domestic animals, or experimental animals, or occurring with narrow margins of exposure (i.e., at low multiples of approximately 25 or less of ordinary exposures) [IFBC, 1990] By nature, cereal grains have relatively few intrinsic toxicants, when compared to some other major food crops [Cheeke, 1998]. Many cereal grains may be considered not to contain known toxicants or antinutrients at levels of significance.
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor B.3. Potential for novel/altered patterns of metabolites.			This factor can be assessed using a combination of prior knowledge and biochemical studies of the modified plant.
Factor B.3.1. Knowledge of gene product function.			
Criteria a/ Protein gene product(s) have well-characterized function in the modified plant.			Well-characterized function means that the function can be justified by a combination of a) prior knowledge, b) experimental data, and c) assignment to a protein family provided that the function of at least one member of the protein family has been demonstrated by experimental data and the protein family assignment follows guidelines such as those accepted by the USDA [http://www.aphis.usda.gov /brs/pro_func.html].
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor B.3.2. Impact of gene product function in the modified plant.			
Criteria a/ Expression product of the introduced/modified gene does NOT have a metabolic, regulatory, or signalling function in the modified plant, *or*			Examples of proteins with metabolic, regulatory, or signalling functions include: regulatory proteins, transcription factors, enzymes that cause molecular modification of key proteins (e.g., kinases) or catalyze pathways that are part of the biosynthesis of signalling molecules (e.g., hormones, peptides, small carbohydrates).
b/ Expression of the introduced/modified gene is NOT expected to have a systemic effect on the metabolism of the modified plant.			Examples include the introduction/modification of enzymatic proteins that are known to affect the biosynthesis of proteins or metabolites that have a regulatory or signalling function.
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor B.3.3. Substrate specificity of gene product.	□ NA		**This factor applies if gene product has enzymatic function in the modified plant. Otherwise, check "NA".**

Factors and Criteria	Y/NA	No/?	Interpretation/Explanation
Criteria a/ Narrow substrate specificity (i.e., unlikely to interact with endogenous substrates to form novel compounds).			A variety of studies can provide information on substrate specificity of gene products and detect changes at the metabolic level before developing the transgenic plants by simulating transformation experiments (e.g., supplementation experiments, inhibitor application, heterologous gene expression and enzymological assays with plant protein extracts and recombinant proteins) [Martens et al., 2003].
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor B.4. Potential for disrupting coding or regulatory DNA sequences in the modified plant.			This factor can be assessed using bioinformatics and transcript analyses. Molecular characterization methods typically used in bioengineered crops are generally not amenable to assessing changes in plants modified by non rDNA conventional techniques, for which alternative approaches are emphasized (identifying differences in expressed proteins and/or metabolites).
Factor B.4.1. In the inserted/modified DNA.			
Criteria a/ NO novel open reading frames (ORFs), disruptions of existing ORFs, or functional expression.			For this evaluation, this criterion is met when one of the following elements are described in the product profile. a) Absence of open reading frames (ORF or unknown DNA sequences) in the inserted sequences. b) If an ORF is identified, justification is provided that its transcription or expression is unlikely. Molecular characterization focuses on identifying any newly expressed protein(s), including functional expression of open reading frames (DNA sequences with potential or unknown function). Different approaches can be used for the assessment, including: bioinformatics analysis, analysis of the whole inserted DNA sequence and comparison with the vector/insert sequence; transcript, and/or protein analysis.
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor B.4.2. In genomic sequences bordering the site of insertion/modification.			
Criteria a/ NO novel open reading frames (ORFs), disruptions of existing ORFs, or functional expression.			See B.4.1(a) above.
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor B.5. Chemical analysis of food derived from the modified plant.			**Note: This factor does NOT apply to substances intended to be changed.**
Factor B.5.1. Key nutrients.			Key nutrients are those components in a particular product, which may have a substantial impact in the overall diet. These may be major constituents (fats, proteins, and structural and non-structural carbohydrates) or minor compounds (vitamins and minerals). Data should be provided to justify conclusions. Where available, key nutrients suggested for food use in applicable OECD consensus documents can be used.

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Factors and Criteria	Y/NA	No/?	Interpretation/Explanation
Criteria a/ Within acceptable ranges of natural variation, *or*			There are no generally accepted standards for determining an acceptable range of natural variation, although some authors and regulators have suggested the use of "statistical significance" or "20% difference" relative to an appropriate comparator as a trigger for more detailed case-by-case assessment [Cellini et al., 2004; Nordic Council, 2000].
b/ NOT expected to have adverse nutritional, metabolic, or toxicological effects.			Key food constituents falling outside natural ranges of variability do not necessarily pose a threat to human health. For this evaluation, judgement will be required in determining whether this criterion has been met by considering the justification provided in the product profile.
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor B.5.2. Known toxicants/antinutrients.			Key toxicants and antinutrients are those compounds that are known to have toxicologically relevant or nutritionally adverse effects and that are inherently present in the species, *i.e.*, compounds whose toxic potency and levels may impact human and animal health. Data should be provided to justify conclusions. Where available, key toxicants and antinutrients suggested for food use in applicable OECD consensus documents can be used. Known allergens are considered in Part C of the RBCS.
Criteria a/ Within acceptable ranges of natural variation, *or*			See explanation B.5.1.a.
b/ NOT expected to have adverse nutritional, metabolic, or toxicological effects.			See explanation B.5.1.b.
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor B.5.3. Other metabolites/parameters	□ NA		**Assess factor (B.5.3) if rating for any of the factors (B.2-B.4) above is "No" or "?". Otherwise, check "NA".**
Criteria a/ Reasonably justified to be sufficient in scope, *and* b/ NO unknown compounds or significantly altered levels of endogenous metabolites outside the range of natural variation, *or* c/ If changes occur, the likelihood of negative health effects can reasonably be justified to be low.			Factors to consider in determining whether the scope of analysis of other metabolites is sufficient include: 1) Metabolites associated with the pathways directly affected by the introduced/modified protein; 2) Modified trait affecting susceptibility of the host plant to endophytic infection or heavy metal uptake; 3) Systemic effects if the introduced/modified gene encodes proteins with regulatory, signalling, or catalytic function (see B.3.2 for examples of proteins with these functions); 4) Toxicants associated with organisms genetically related to the gene source and the host plant, particularly if their prior dietary history is limited. For this evaluation, justification should be provided whether the scope of analytes (combined B.5.1-B.5.3) is adequate. Adequacy will be judged in proportion to the number of "No" or "?" ratings for the preceding factors (B.2-B.4). At present, chemical analysis emphasizes key nutrients and known toxicants/antinutrients and metabolites

Factors and Criteria	Y/NA	No/?	Interpretation/Explanation
			that are associated with the pathway(s) being targeted for modification. This approach has generally been assumed to be sufficient for modification that involves a direct gene product that confers the desired trait with presumably minimal interaction with the metabolism of the host plant, or where the safety of the gene source or that of the genetic material transferred is known. With modification that is intended to alter metabolic, regulatory, or signalling function in the host plant, how constituents of the modified plant are selected for analysis requires reexamination. Selected analytes should reflect global changes at the level of metabolites in the edible part(s) that are consumed and provide a highly sensitive indication of unintended effects of direct health relevance.
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor B.6. Health consequences of instability of the modified trait.			
Criteria a/ Modified trait is NOT intended to provide nutrition or health benefits, *or*			For the intended use of the RBCS, this criterion applies to nutrition or health benefits for humans.
b/ *Modified trait is intended to provide nutrition* or health benefits, *and* c/ Adequate quality control is in place to ensure consistency in lot-to-lot expression of the targeted gene product.			For this evaluation, this criterion is considered to be met where one or more of quality control measures are described or are being developed. Where quality control measures are being developed, confirmation of implementation should follow.
If the criterion is not met, check "No" or "?".		□ No □ ?	

PART C - POTENTIAL FOR ALLERGENIC (IgE-MEDIATED) EFFECTS - applicable to protein substances.

Y = Yes (criterion met) NA = Not applicable No = Criterion not met ? = Product information insufficient or equivocal

Factors and Criteria	Y/NA	No/?	Interpretation/Explanation
Factor C.1. Allergenicity of donor organism (gene source).	□ NA		**If the donor and host plants are the same species, check "NA".** At the present, a protein from an allergenic source would be considered to be highly probable of being allergenic unless proven otherwise [FAO/WHO, 2001]. Therefore, if a protein is derived from an allergenic source, it will be considered as Level II for further testing.
Criteria a/ NOT a known source of allergenicity or gluten-sensitive enteropathy.			Known sources of allergenicity include *common* and *less common* sources. Eight foods or food groups are considered *common* sources of food allergens worldwide (accounting over 90% of the documented food allergies): peanuts, soybeans, tree nuts, milk, eggs, fish, crustacean, and wheat. Other sources of allergens have geographical significance. Sesame seeds, celery root and mustard are common sources of food allergens in Europe, in addition to buckwheat in Asia. *Less common* sources can be identified from established databases. For this evaluation, they are identified in product profiles. Four to five cereal grains are known sources of gluten-sensitive enteropathy: wheat, rye, triticale, barley, and possibly oats.
If the criterion is not met, check "No" or "?".		□ No □ ?	
Protein gene products (factors C.2-C.4).	□ NA		**Check "NA" if criterion A.1.1 is met for a gene product (i.e., not expressed due to intentional gene silencing). Assess factor C.5.**
Factor C.2. Potential for protein gene product(s) to cross react with known allergens.			A protein considered to have the potential for cross-reaction will be considered Level II for further testing.
Criteria a/ Comparison of amino acid sequence identity with known allergens: NO sequence identity of ≥35% over 80 or more amino acids based on a sequential search			The Codex Alimentarius Task Force on Foods Derived from Biotechnology [2003] accepted 35% or higher in amino acid sequence identity to an allergen, in a segment of 80 or more amino acids, as a reasonable criterion for considering a protein likely to be allergenic and therefore requiring further testing. However, contrary view exists that cross-reactivity is rare below 50% identity. In most situations cross-reactivity requires more than 70% reactivity [Aalberse, 2000]. We have excluded the criterion for "NO identical matches in amino acids in a short sequence (e.g., 6 - 8 contiguous amino acids" currently required in several international and national guidelines as recent evidence does not support its use [Goodman and Hefle, 2005].
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor C.3. Pepsin digestion (under standard testing conditions).			Assay conditions critical to consistency of results were described in a multi-laboratory evaluation of the assay protocol for proteins with different allergenic activity [Thomas et al., 2004].
Criteria ***Novel or substantially modified proteins*** (without prior dietary history)			No cut-off values for pepsin digestion test have been provided in guidance documents on GM food safety assessment but general patterns of stability have been reported [Astwood et al., 1996; Metcalfe et al., 1996; Thomas et al., 2004].

Factors and Criteria	Y/NA	No/?	Interpretation/Explanation
a/ Rapid and complete digestion with NO stable fragments (< 10 min).			For this evaluation, we propose separate criteria for novel and dietary proteins to take into consideration of potential knowledge gap and experience in assessing this property for novel proteins. a) Each of the currently registered Bt proteins were all degraded in simulated gastric fluid in 0-7 minutes [Mendelsohn et al., 2003]. b) For several known food allergens, protein fragments were observed at 15 min. or longer. Some remained resistant to digestion at 60 min [Astwood et al., 1996;Thomas et al., 2004].
Other proteins (with prior dietary history) b/ Rapid or moderate digestion with NO stable fragments (< 15 min).			See explanation for part (a) of this criterion.
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor C.4. Secondary factors.	□ NA		**If the rating for factor (C.3) is "No" or "?", assess the following secondary factors (C.4.1 –4.3). Otherwise, check "NA".** A protein with moderate digestibility without other factors suggestive of allergenic potential may be considered to be of low probability of being an allergen [FIFRA SAP, 2005]. Therefore, secondary factors (C.4.1-C.4.3) will apply to a protein receiving a "No" or "?" rating for pepsin digestibility.
Factor C.4.1. Structural/functional characteristics.			Given the equivocal nature of the predictive value of these factors, a protein belonging to these classes or potentially being cross-reactive with these classes of protein will be assigned to concern level II only in conjunction with other information that is suggestive of uncertainty or concern.
Criteria a/ NO post-translational modification involving glycosylation with primarily N-linked, specific xylose or fucose substitutions, *or*			Carbohydrate structures most associated with cross-reactivity are those that contain an α(1,3)-fucosyl residue, and/or β(1,2)-xylose and can be bound by IgE from individuals sensitized to unrelated sequences [van Ree et al., 2000].
b/ NO structural or functional relationship to specific protein families or functional categories commonly associated with allergenicity.			Protein families or functional categories commonly associated with allergenicity include: prolamin, Bet v 1, cupin, profilin; lipocalins, parvalbumins; seed storage proteins of the 2S albumin type; pathogenesis-related protein families 2, 3, 4, 5, 8, 10, and 14 comprising β-1,3 glucanases, chitinases of classes I and III, lipid transfer proteins) [FAO/WHO, 2001; Hoffmann-Sommergruber, 2002; Jenkins et al., 2005].
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor C.4.2. Heat stability.			
Criteria a/ Heat labile (NOT applicable to lipid transfer proteins).			This criterion does *not* apply to lipid transfer proteins which are generally heat labile and are allergenic.
If the criterion is not met, check "No" or "?".		□ No □ ?	

Factors and Criteria	Y/NA	No/?	Interpretation/Explanation
Factor C.4.3. Abundance.			
Criteria ***Pesticidal and enzymatic proteins*** a/ < 0.1% protein.			While not all abundant food proteins are significant allergens, a number of potent food allergens (e.g., peanut allergens) are abundant in the allergenic source, often representing more than 1% of the total protein in the allergenic food [Metcalfe et al., 1996]. This contrasts certain novel proteins in GM foods that are typically expressed at very low levels (< 0.1% protein) and are unlikely to become novel allergens.
Other proteins b/ < 1% total protein.			See explanation for part (a) of this criterion.
If the criterion is not met, check "No" or "?".		□ No □ ?	
Factor C.5. Allergenicity of host plant.			This factor focuses on *common* sources of allergens. This takes into consideration the likelihood of unintended effects, the potential health impact at the population level, and the practicality of obtaining an adequate sample size for further testing.
Criteria a/ NOT a common source of allergenicity or gluten-sensitive enteropathy, *or*			See explanation for factor C.1 for "common" food sources of allergens and sources of gluten-sensitive enteropathy.
Host plant is a common source of allergenicity or gluten-sensitive enteropathy b/ Rating for factors B.5.1, B.5.2, *and* B.5.3 is "Y" or "NA".			**If the host plant is a common source of allergenicity or gluten-sensitivity enteropathy, assess if part (b) of this criterion is met.** Concerns for unintended effects in the level or potency of endogenous allergenic proteins would be greater if the host plant is both a common source of allergen and has a high potential for unintended effects as determined based on factors B.5.1-B.5.3 in Part B.
If the criterion is not met, check "No" or "?".		□ No □ ?	

References - please refer to the reference list in "Supplementary Information".

Product ID: **COMBINED RBCS-PRODUCT RATING FORM** **Participant ID:**

Aggregation of factors.

Based on your ratings for individual factors in the previous parts of the scheme, please assign a concern level for each part using the guidance for aggregation in the following tables. Select the primary characteristic that applies (e.g., with dietary history, or without dietary history) and determine if the specified factors and criteria for a given level are met. **Please mark the concern level for each part by placing a circle or a square around the level classification under the appropriate column and include the criterion that applies for each "Y" rating. ¶ If the classification is Level III for Part A, or Level II for Part B or C, please include the ratings for each factor in the column provided.** For Parts B & C, you may use the blank columns to fill in the ratings and criteria.

PART A Factors	Negligible dietary exposure	With prior experience	With dietary history † Applies only to pepsin digestibility of proteins				Without dietary history	¶Not meeting the criteria for Levels I or II
A.1	Y	-	No	No	No	No	No	No
A.2	-	-	Y	Y	Y	Y	No/?	
A.3	-	-	Y	?	Y	Y	Y/?	
A.4.1	-	-	Y	Y	?	Y	NA	
A.4.2	-	-	NA	NA	NA	NA	Y	
A.5	-	-	Y	Y	Y	?†	No/?	
A.6	-	-	Y	Y	Y	Y	Y	
A.7.1	-	-	Y/NA	Y/NA	Y/NA	Y/NA	Y/NA	
A.7.2	-	-	Y	Y	Y	Y	Y	
A.8 (not rated)	-	Y						
Level	I	I	I	II	II	II	II	III

PART B Factors	Negligible expression of intended gene products in edible parts	Characterized gene modification (based on nucleic acid techniques) *Applicable to *any one* of the ratings marked				Poorly characterized gene modification (undirected mutagenesis, somaclonal variation, or wide-cross hybridization) *Applicable to *any one* of the ratings marked					¶ Not meeting the criteria for level I
Criteria 1.3 in Part A	Y	-		-		-		-			-
B.1	-	Y/NA		?/No		Y/NA		?/No			
B.1.1	-	NA		Y		NA		NA			
B.1.2	-	NA		NA		NA		Y			
B.2	-	Y	?/No*	Y	?/No*	Y	?/No*	Y	?/No*		
B.3.1	-	Y	?*	Y	?*	Y	?*	Y	?*		
B.3.2	-	Y	?/No*	Y	?/No*	Y	?/No*	Y	?/No*		
B.3.3	-	Y/NA	?/No*	Y/NA	?/No*	Y/NA	?/No*	Y/NA	?/No*		
B.4.1	-	Y	Y	Y	Y	Y	?*	Y	?*		
B.4.2	-	Y	?*	Y	?*	Y	?/No*	Y	?/No*		
B.5.1	-	Y	Y	Y	Y	Y	Y	Y	Y		
B.5.2	-	Y	Y	Y	Y	Y	Y	Y	Y		
B.5.3	-	NA	Y	NA	Y	Y	Y	Y	Y		
B.6	-	Y	Y	Y	Y	Y	Y	Y	Y		
Level	I	I	I	I	I	I	I	I	I		II

PART C Factors	Negligible expression of intended gene products in edible parts	Low identifiable concerns with allergenic activity			¶ Not meeting the criteria for level I		
Criteria 1.3 in Part A	Y	-			-		
C.1	-	Y/NA	Y				
C.2	-	Y/NA	Y				
C.3	-	Y/NA	?/No				
C.4.1	-	NA	Y				
C.4.2	-	NA	Y				
C.4.3	-	NA	Y				
C.5	-	Y	Y				
Level	I	I	I		II		

Please provide the following information.

1. Length of time required to complete the classification of this product: _____ minutes.

2. If your rating for this product includes "?", or if you are unable to provide a rating for any factor, please complete the following.

Factor (e.g. B.3.1)	Reasons	Comments
	□ Product info insufficient □ Classification criteria unclear □ None of the criteria applies □ Other (please explain)	
	□ Product info insufficient □ Classification criteria unclear □ None of the criteria applies □ Other (please explain)	
	□ Product info insufficient □ Classification criteria unclear □ None of the criteria applies □ Other (please explain)	
	□ Product info insufficient □ Classification criteria unclear □ None of the criteria applies □ Other (please explain)	

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Annex 6-2

Form for Overall Assessment

Participant ID:

RISK-BASED CLASSIFICATION SCHEME FOR TESTING GENETICALLY MODIFIED FOODS

Overall Assessment

1. On a scale of 1-7, please rate the level of ease in applying the scheme.

Very easy — Very difficult

1 2 3 4 5 6 7

Rating:

Please provide reason(s) for your rating.

2. In your opinion, does the scheme clearly differentiate a GM food into a concern level in each of the 3 categories of health effects (i.e., Parts A, B, C of the combined RBCS-Product Rating Form)?

□ Yes □ No

Please explain.

3. Is the Supplementary Information useful?

□ Yes □ No

Please explain.

4. Did you find it necessary to use any assumptions or default decision rules in applying the scheme? (other than those suggested on p.15 of "Supplementary Information").

□ Yes □ No

Please explain.

5. General comments - please provide any comments you may have on the RBCS, product profiles, and/or supplementary information. Suggestions for improvement would be very helpful.

5. Background and other information.

a) My expertise (check all that apply)

□ Toxicology
□ Immunology
□ Pharmacology
□ Molecular biology
□ Other biological sciences (e.g., microbiology, genetics)
□ Plant sciences
□ Agricultural sciences
□ Food/Nutritional sciences
□ Agricultural biotechnology
□ Analytical sciences
□ Health risk assessment
□ Medical/Health sciences
□ Other (please specify)

b) Please indicate your preference (yes / no) for acknowledging your contribution to the project.

□ Yes, I agree to have my name and affiliation included in the researcher's thesis
□ No, I do not wish my name and affiliation to be included in the researcher's thesis

If more than one individual completed the evaluation, please

(i) include the number of individuals completing the evaluation:

(ii)identify all expertise for Q5(a).

If the individuals(s) would prefer to be acknowledged for their contribution to the project, please provide the name(s) of the individual(s) and their affiliation(s).

THANK YOU FOR YOUR PARTICIPATION IN THE STUDY. Please return your completed evaluation (see instructions for product rating) by **February 6, 2006**, to:

Eunice Chao (echao097@uottawa.ca).

Annex 6-3

Supplementary Information

RISK-BASED CLASSIFICATION SCHEME FOR TESTING GENETICALLY MODIFIED FOODS - PHASE 3

Supplementary Information for Applying the Risk-Based Classification Scheme (RBCS) (January 2006)

The following topics are covered in this document.

1. Introduction (p.2)

2. Definition of terms (p.2)

Risk-based classification scheme (RBCS)

3. Background
   - 3.1. Rationale (p.3)
   - 3.2. Context (p.3)
   - 3.3. Development process (p.3)

4. Conceptual framework
   - 4.1. Overview (p.4)
   - 4.2. Information used in classification (p.4)
   - 4.3. Factors relevant to health risk assessment of GM foods (p.5)
     - A. Potential for toxic and antinutritional effects - intended changes (p.5-6)
     - B. Potential for toxic and antinutritional effects - unintended changes (p.6-9)
     - C. Potential for allergenic effects (p.9-12)
   - 4.4. Criteria for differentiating health risk potential of GM foods (p.12)
   - 4.5. Aggregation of factors (p.13)

5. Evaluation of RBCS
   - 5.1. Evaluation components (p.13)
   - 5.2. Development of product profiles (p.13)
     - 5.2.1. Selection of products in the reference panel (p.14)
     - 5.2.2. Sources of information (p.14-15)
     - 5.2.3. Assumptions used in developing product profiles (p.16)

6. Relevance of RBCS for testing GM foods (p.16)

7. References (p.17-20).

Appendix - Product characteristics by concern level
- Part A: Potential for toxic and antinutritional effects - intended changes from the primary and secondary gene products introduced/modified (p.21-23)
- Part B: Potential for toxic and antinutritional effects - unintended changes in the modified food from genetic modification of the host plant (p.23-24)
- Part C: Potential for allergenic effects (p.25-26)

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1. Introduction.

This document is provided to facilitate a common understanding in applying the proposed risk-based classification scheme (RBCS).

The application and evaluation of the RBCS are part of phase 3 of the research project on a *Risk-Based Classification Scheme for Testing Genetically Modified Foods*. The specific components of evaluation are outlined in section 5.1 of this document.

To provide the relevant background information, this document includes an overview of the conceptual framework of the RBCS. It is proposed that foods derived from genetically modified crops (or GM foods) can be differentiated and classified into different concern levels based on predetermined factors and criteria. The rationale for the factors and criteria underlying the assignment of concern levels is described. The factors and criteria are also listed in the combined RBCS-product rating form in an appendix. The characteristics of products in different concern levels in the scheme are summarized. The relevance of the proposed RBCS for GM food testing and risk assessment and management is discussed briefly.

2. Definition of terms.

Certain terms associated with the RBCS may have meanings that are different from typical usage. This brief glossary clarifies the terms that are used.

Genetic modification. A broad definition of genetic modification is adopted for the development of the RBCS. It encompasses all forms of *intentional manipulation* in changing the heritable traits of an organism, including conventional methods (such as induced mutation and wide-cross hybridization of sexually incompatible plants) and more modern methods (such as recombinant nucleic acid technologies) used in plant breeding.

Considerable variation exists in the definition of "genetic modification". Definitions in the science/regulatory policy literature include both broad and more specific definitions. Broad definition refers to the modification of the genotype of an organism by the application of biotechnology, such as gene transfer, or chromosome set manipulation [NRC, 2002]. More specific definition refers to the modification via transgenesis (inserting individual genes from the same or a different species into another individual), or the targeting of specific changes in individual genes or chromosomes within a single species [The Royal Society, 2001]. The working definition proposed here is consistent with Canadian Novel Food Regulations.

Methods of genetic modification. In this document, "conventional" methods of genetic modification refer to intentional manipulation of the genetic make-up of crop plants by wide-cross hybridization (crosses between different species or genera) and induced mutation (including mutagenesis by chemical agents, irradiation, and tissue culture). These methods have evolved in the past several decades. Natural selection without deliberate manipulation of the genes and crosses between different cultivars within the same species have a much longer history. These methods may be referred to as "traditional" methods. More "modern" methods refer to the exchange of genetic material through *in vitro* nucleic acid techniques [Codex Alimentarius, 2003]. These methods are also widely known as genetic engineering.

Gene products. Two categories of gene products may be distinguished: *primary* and *secondary* gene products. *Primary* gene product refers to the protein(s) expressed by the introduced/modified gene, where as *secondary* gene product refers to the principal metabolite(s) of interest of the target/modified enzyme.

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3. Background.

3.1. Rationale.

Public discourse on genetically modified (GM) foods tends to focus on those aspects of the recombinant deoxyribonucleic acid (rDNA) technology that heighten concerns about potential risks of GM foods. Yet a wide range of GM foods exist, including those that are modified by conventional approaches, such as induced mutation and wide-cross hybridization, the safety of which has not been assessed in detail. Also in contention is the likelihood of unintended effects with adverse health consequences in foods derived from crops genetically modified by more modern techniques, compared to those derived from crops modified by conventional techniques. As well, divergent views exist regarding the methods by which unintended effects should be detected and monitored. With advances in genomic technology, boundaries between conventional and modern approaches to genetic modification may become blurred [Keenan and Stemmer, 2002; Madsen et al., 2002]. There is the need for a reliable method to efficiently differentiate GM foods that require more extensive testing and regulatory oversight from those that do not, regardless of the method of modification.

3.2. Context.

- **Basic concept**. The risk-based classification scheme (RBCS) proposed in this research is intended to be a tool for screening GM foods based on levels of concern about potential health risks and attendant uncertainty using defined criteria. In this context, potential health risk is conceptualized as the likelihood of known or potential adverse health effects arising from human exposure to substances that are intentionally introduced or modified in a food crop, and that may be produced unexpectedly as a result of the genetic modification process.
- **Intended uses**. The primary intended use of the RBCS is to guide the application of testing methods in proportion to the level of concern.
- The RBCS is **NOT intended** to be used as a health risk assessment tool *per se*, nor for decision-making regarding the approval or disapproval of a GM food.
- **Focus**. The focus is potential human health risks from dietary exposure to foods derived from GM crops. Crops modified to produce therapeutic substances are outside the scope of the scheme. Also excluded are food uses of GM microorganisms and feed uses of GM crops, as well as the assessment of environmental or ecological risks.
- **Scope**. GM foods covered by the scheme includes those derived from crops genetically modified by a range of methods, including conventional (e.g., induced mutation, tissue culture, interspecific and intergeneric crosses) and modern (e.g., cell fusion, *in vitro* and rDNA) techniques to intentionally change the heritable traits of food crops

While the primary usage of the RBCS is to differentiate GM foods for testing purposes, the RBCS can also function as a flexible screening tool in an iterative process during different stages of research and development of a GM food crop. Product developers can use the RBCS to identify major information gaps for safety assessment to be considered in the early stages of research and development. In addition, the RBCS can also provide a consistent and transparent format for communicating product approval decisions to the interested public.

3.3. Development process.

The proposed scheme was developed based on a review of the scientific literature and national/international guidelines on risk assessment of GM foods and related products. Three expert consultations (using paper-based questionnaires) were conducted on earlier drafts of the proposed scheme at different stages of development. One group interview with toxicologists was also conducted on a draft proposal for graded testing options that correspond to the different concern levels in the scheme. The responses received were taken into account throughout the development phase of the classification scheme.

4. Conceptual framework.

4.1. Overview.

The RBCS comprises a total of seven concern levels in three categories of potential health effects, with 2-3 levels in each of the three categories. They are as follows:

[A] POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - intended changes resulting from the primary and secondary gene products introduced/modified (3 levels).

Level I. Dietary substances (protein and non-protein) that have a history of known or presumed safe consumption from foods and have insignificant changes with respect to expected exposure. They can also be gene products of modification using an established transformation procedure.

Level II. Substances that have limited and defined concerns with respect to *either* expected exposure *or* toxicity profile.

Level III. Substances that do not meet the criteria for level I or II.

[B] POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - unintended changes in the modified food from genetic modification of the host plant (2 levels).

Level I. GM foods with low potential for adverse health effects from unintended changes.

Level II. GM foods with moderate or high potential for adverse health effects from unintended changes.

[C] POTENTIAL FOR ALLERGENIC EFFECTS (2 levels).

Level I. Proteins with low potential for allergenic activity.

Level II. Proteins with moderate or high potential for allergenic activity.

4.2. Information used in classification.

For the classification scheme to be useful as guidance for further testing, the factors and criteria underlying the classification scheme emphasize information normally compiled or generated as part of developing the desired crop. The information should also be easily assessed and can differentiate among GM foods of varying levels of concern.

In developing the RBCS, two types of information are distinguished.

Prerequisite information will be met by all GM crops and will not provide a basis for differentiation. This includes information on genetic and phenotypic stability, data quality, and the intended purpose and performance of the modified trait, currently required in the assessment of GM crops and derived foods in many jurisdictions.

Core information, in contrast, should have discriminating value for classifying GM foods. Core information is a subset of all the information that is typically required in premarket assessment. It provides the basis for the factors and criteria underlying the classification scheme and is based on prior knowledge or basic testing of the modified crop and derived foods.

Core information includes prior knowledge related to:
(a) biology of the host plant, donor organism (and/or genetically related organism), including associated toxicity and allergenicity,
(b) prior history of food use,

(c) function and safety of the introduced/modified gene products and where applicable, their principal metabolites and breakdown products in plant and mammalian metabolism and processing effects.

Core information can also be generated from basic testing or assessment at a screening level, including:
(a) level of expression of gene products and principal metabolites of interest in the edible parts of the modified crops,
(b) expected dietary exposure to the food derived from the modified crop and to the gene products of interest,
(c) structure-activity characterization of the gene products of interest and their breakdown products, where relevant,
(d) basic compositional analysis of the edible parts.

Screening level testing or assessment may be performed as part of an iterative process. The nature and extent of testing may vary with the stages of product development which can result in a decision to terminate or to continue with the development process.

4.3. Factors relevant to health risk assessment of GM crops.

There are two areas of focus in assessing the potential for health risks of a food derived from a GM crop: (1) potential for toxic/antinutritional effects, and (2) potential for allergenic effects.

To differentiate the sources of uncertainty, we classify intended and unintended changes separately for potential toxic/antinutritional effects in the proposed risk-based classification scheme (RBCS). Intended changes refer to the direct effects of primary and secondary gene products. Unintended changes refer to the predictable and unpredictable effects of genetic modification in the food derived from the modified crop. Predictable effects are unintended changes that go beyond the primary expected effect(s) of introducing the target gene(s), but that may be explicable in terms of our current knowledge of plant biology and metabolic pathway integration and interactions. Unpredictable effects are those changes that fall outside our present level of understanding of gene regulation and gene-interactions [Cellini et al., 2004; Kuiper et al., 2001].

Consistent with current international approach to the assessment of the allergenic potential of GM foods, the classification of the potential for allergenic effects in the RBCS will focus on protein substances that mediate immunoglobulin E-linked immediate hypersensitivity reactions. Given the influence of food matrix in allergenic effects, no distinction is made in classifying intended and unintended changes for this category of health effects.

The rationale underlying the factors included in the proposed RBCS for determining concern levels for each of these three categories of health effects are discussed below.

[A] Potential for toxic and antinutritional effects – intended changes.

In the context of the RBCS, the risks posed by a dietary substance of interest are determined by its "exposure" (amount of intake) and "hazard characteristics" (the nature of the substance). Eight factors related to exposure and hazard characteristics that are relevant to a substance that is intentionally modified in a GM crop can be identified.

Exposure
(1) Expression of gene product(s) in edible part(s) of the modified crop
(2) Prior history of consumption from foods
(3) Expected change in total dietary exposure

Hazard characteristics
(4) Prior knowledge of safety/mode of action
- Food constituents and substances with a prior history of consumption from foods
- Substantially modified or novel substances

(5) Structure-activity characterization
(6) Digestion and degradation products

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(7) Synergism and interactions

Other factors
(8) Prior experience with gene products introduced/modified

Exposure
It is expected that many substances intentionally introduced or modified in GM foods will be similar to those already present in the food supply, or have a naturally occurring counterpart in living organisms. Genetic modification may alter the sources of these substances and their exposure levels. Further, depending on the nature of the genetic modification, the extent of dietary exposure may be altered by limiting the tissue of expression in the modified crop.

For many food-derived substances, the discriminating value of the duration and frequency of exposure, as well as the size of the population being exposed, can be expected to be small. These factors are excluded in the RBCS. Similarly, indirect or non-dietary routes of exposure via geobiochemical pathways or dermal and respiratory routes are excluded for consideration given their comparatively minor contribution to total exposure among the general population.

Hazard characteristics
The nature of a substance relates to its inherent properties in inducing an adverse health outcome as a function of its structural and functional characteristics. For some substances with a dietary history, their inherent properties can also be based on prior knowledge. The demonstration of structural similarity and functional equivalence with substances with a prior history of known or presumed safe food use is a key factor. For a gene product *with prior knowledge of safety*, structure-activity characterization aims to assess whether a heterologously expressed protein is structurally similar and functionally identical to the comparator/source protein for which prior knowledge on safety has been established. For a *novel* gene product (e.g., a protein that is encoded by DNA sequences that have been substantially modified with the potential for altered protein structure and function, or is designed using *de novo* synthesis based on an existing protein), this factor provides tier 1 screening that may signal the need for further testing.

In the context of foods as complex mixtures, the effects of food matrix, bioavailability, and processing are also relevant in considering the safety of a substance that is an intrinsic component of the edible part of a modified crop. Additional considerations include its metabolic fate in the modified plant and following ingestion, as well as any synergistic and interactive effects with other intentionally altered substances and endogenous food constituents with the potential for adverse health effects.

We have excluded for general consideration in the RBCS, factors related to gene transfer and viral recombination. Concerns about gene transfer are primarily related to the use of antibiotic resistance genes as selectable markers. This type of concerns can be addressed by employing best practices in the selection of marker genes that encode traits that pose little relevance in human and veterinary medicine. Similarly, concerns about viral recombination in the generation of new viral organisms (with respect to plant health) are also best addressed by sound practices such as avoiding the use of complete viral genes (without the presence of replication origins or gene/sequences that might enhance recombination).

Other factors
Prior experience with the gene products introduced/modified will influence the level of scrutiny required. However, for this evaluation of the RBCS, this factor will be excluded for product rating purposes.

Additional factors may be added to make the scheme flexible for future developments where new factors may be identified and new tests developed or required.

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[B] Potential for toxic and antinutritional effects – unintended changes.

Screening for the potential for unintended changes in GM crops that may result in adverse health effects is a function of assessing the likelihood of their occurrence based on four categories of considerations: their genetic sources, the factors affecting their expression, evidence of their expression, and judgement about the health consequences of instability of the modified trait. Six factors are included the RBCS.

Genetic sources

(1) Safety of donor organism

(2) Safety of host plant

Factors affecting the expression of unexpected substances

(3) Potential for novel/altered patterns of metabolites
- Knowledge of gene product function
- Impact of gene product function in the modified plant
- Substrate specificity of gene product

(4) Potential for disrupting coding or regulatory DNA sequences in the modified plant
- In the inserted/modified DNA
- In DNA sequences bordering the site of insertion/modification

Evidence of unexpected expression of known or novel substances

(5) Chemical analysis of food derived from the modified plant
- Key nutrients
- Known toxicants/antinutrients
- Other metabolites/parameters

(6) Health consequences of instability of the modified trait

Genetic sources

The two main aspects of concern of unintended changes in foods derived from GM crops with the potential for adverse health effects are: (1) modified expression of endogenous toxic substances (or metabolites) in different tissues or at different levels, and (2) generation of new toxic compounds. Therefore, the safety of the host plant and that of the donor organism (in the case of poorly characterized gene modification e.g., wide-cross hybridization) as genetic sources of substances that may have adverse health effects at certain level of expression are major considerations.

Available information tends to support the premise that unintended effects of genetic modification of food crops that have a history of traditional breeding, when they occur, would result in a change in the level of expression of already recognized toxicants in the plant. This is deduced from observations that (1) the ability to synthesize particular classes of secondary metabolites is generally restricted to selected plant groups [Cheeke, 1998, p.87; Osbourn et al., 2003]; (2) certain classes of plant chemicals with potent toxic effects (such as alkaloids) require highly substrate-specific enzymes and are highly compartmentalized in their biosynthesis [Croteau et al., 2000]; (3) the production of secondary metabolites with potent biological activity is likely a rare property of any one molecule despite the vast diversity and metabolic flexibility of plant metabolism [Firn and Jones, 1990]; (4) only 21 food-born non-microbially derived toxic substances have been identified to be of plant origin (< 0.1% of the total number of food constituents identified) and only 9 out of the 21 toxicants are biosynthesized by plants and the remaining being pass-through contaminants in honey and milk [IFBC, 1990].

It is less likely that critical toxicants, inherent in the plant but hitherto unexpressed, will be unintentionally induced by genetic modification [WHO, 1995]. At present however, there is insufficient evidence to consider that the likelihood of its occurrence can be dismissed [Facchini et al., 2004]. Also to be considered is that the introduction of new pathways may not cause metabolic problems or identifiable phenotypic changes [Tattersall et al., 2001]. Therefore, the likelihood of occurrence of novel or naturally occurring constituents in the plant with previously unknown toxicity should be considered.

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Factors affecting unexpected expression of known or novel substances

Unintended changes in GM crops can result from alterations (1) at the genomic level from structural changes to DNA sequences or to chromosomal organization; (2) at the transcription level from changes to gene expression resulting from the disruption, modification or silencing of active genes or the activation of silent genes; and (3) at the metabolic level from interactions between novel gene products and endogenous enzymes and metabolites and alterations in flow through of metabolites in biochemical pathways [Conner and Jacobs, 1999; Cellini et al., 2004; Kuiper, 2001].

Divergent views exist regarding the likelihood of unintended effects with adverse health consequences in foods derived from GM crops and the methods by which these effects should be detected. There is a lack of empirical data for estimating the relative magnitude of the potential for or the probability of health hazard associated with unintended changes in GM foods. While no generally accepted predictive factor(s) that may affect the expression of unexpected substances in GM crops have been identified, certain characteristics at the metabolic and genomic levels are indicative of a higher level of uncertainty and the need for more scrutiny. At the metabolic level, these characteristics relate to the function of the gene product, the likelihood for systemic effects in the modified plant, and the substrate specificity of an enzymatic protein. For example, based on current knowledge, it is generally understood (assumed) that recombinant pesticidal proteins that are introduced into food crops to-date do not interact with the plant's metabolic pathways [Ellis, 2001]. These types of protein are unlikely to induce broad unexpected metabolic effects. At the genomic level, concerns relate to the likelihood of interrupting coding or regulatory sequences that are functionally expressed.

The issue of the relative degree of genetic disruption for different methods of genetic modification was reviewed by a NRC and IOM Committee [2004]. The committee concluded, based on expert judgement, that it is the final product of a given modification, rather than the modification method or process, that is more likely to result in an unintended adverse effect. With the exception of mutation breeding, within the same modification method, the greater the genetic distance between the donor and host organisms, the higher will be the likelihood and the extent of unintended effects. On the other hand, due to the common practice of selecting favourable lines and discarding those exhibiting unwanted phenotypic characteristics as part of the breeding program, the extent of unintended effects in commercialized GM lines is expected to be reduced.

Similarly, no generalization can be made regarding whether the introduction of a novel gene or a homologous gene will have a greater effect on the production of novel/altered patterns of metabolites. The expression of a novel gene (e.g., a gene for a biosynthetic enzyme not found in the host plant species) being controlled by novel regulatory elements may be outside the control of normal homeostatic feedbacks [Hallerman, 2003]. On the other hand, up or down regulation of endogenous or homologous genes can result in the interactions with a greater number of other genes/enzymes in the host, because the introduced gene shares a high degree of functional and sequence similarity with one in the host plant [Kuzma, 2002].

Evidence of unexpected expression of known or novel substances

Past experience with the process used in commercializing crops modified by conventional breeding methods has generally resulted in very few documented cases of unacceptably high levels of known toxicants in modified foods. It has been suggested that foods derived from conventionally bred crops could provide a reference point by which the safety of crops modified by rDNA techniques can be compared. Comparisons can be made at the molecular, metabolic, and phenotypic/morphologic levels. The level on which emphasis is placed can vary, depending on the method of genetic modification. For example, molecular characterization methods typically used in bioengineered crops are generally not amenable to assessing changes in plants modified by non rDNA conventional methods. Alternatively, identifying differences in expressed proteins and/or metabolites assumes greater weight. Ideally, appropriate controls for comparison should include an unmodified counterpart (of isogenic or comparable genetic background) grown and harvested under the same environmental and agronomic conditions.

Challenges exist in applying the comparative approach. Challenges include: appropriateness of using the traditional food supply as the basis for comparison; availability of appropriate comparator(s) (e.g., modified traits involving induced responses); the selection and coverage of key compounds; and interpretation of what would constitute health-relevant differences given the paucity of knowledge of natural variation in the levels of

constituents and their health effects in the context of food matrix in a given food and in the total diet. While its limitations are recognized, the comparative approach has remained the primary paradigm in the safety assessment of genetically engineered crops [EFSA, 2004; NRC and IOM, 2004; Kok and Kuiper, 2003; Kuiper et al., 2002]. Moreover, the debate continues whether the current targeted approach to comparative assessment should be replaced by a broad comparative approach. While a targeted approach has the advantage of focussing on the most likely impacts and of using established analytical methodologies, proponents of a broad approach emphasize the need to recognize that genetic alterations can induce non linear responses. Therefore, integrated systemic changes in the modified plant cannot be predicted and should be addressed using a global analytical strategy.

The assessment of phenotypic alterations is an important part of screening of a plant breeding program regardless of the genetic modification method. Phenotypic stability can be regarded as part of prerequisite information. While phenotypic screening is not a factor in the RBCS, it can be included as part of the justification for the scope of the metabolites to be analyzed.

Health consequences of instability of the modified trait
Although data to support genetic stability of the modified trait is generally required as part of premarket assessment, the duration of maintenance of genetic stability is uncertain [FIFRA SAP, 2004]. For food safety assessment and risk management, priority will be given to those health-related traits that are intended to reduce toxic or allergenic substances in crops for human consumption. It is also reasonable to expect that an identity preservation system will be in place for GM foods developed for their health benefits. Given commercial incentives, such a system can be operational even in jurisdiction where GM food tracing is not mandatory.

[C] Potential for allergenic effects.

The factors underlying the proposed RBCS are largely based on the 2001 Joint FAO/WHO report and subsequent Codex guidelines. Judgment of the potential for allergenic activity from the protein substance introduced or modified in a GM crop is based on the following factors.

(1) Allergenicity of the gene source

Characteristics of the protein substance
(2) Potential to cross react with known allergens
(3) Stability in pepsin digestion
(4) Secondary factors
- Structural/functional characteristics
- Heat stability
- Abundance

(5) Allergenicity of the host plant

The following section provides an overview of the issues relevant to assessing the allergenic potential of GM foods, followed by a brief explanation of the factors included in the RBCS.

An allergenic reaction is an interaction between an individual with a genetic (atopic) background and an allergen that is recognized by an immunoglobulin E (IgE), the specificity and affinity of IgE binding to a given allergen can vary widely due to the genetic heterogeneity of the population, as well as the nature, extent and duration of exposure [Bannon et al., 2003]. Given the nature of an allergenic reaction, allergenic substances in foods are primarily protein in nature although low molecular weight chemicals may be involved in an immune response by covalently binding to proteins to form a hapten-carrier complex [Kroes et al., 2004].

There are two stages in the development of allergic reactions: (1) sensitization, and (2) elicitation of a reaction. Correspondingly, the assessment of potential for allergenic effects in GM foods consists of two elements: (1) assessment of allergenic activity (the propensity of a substance to elicit allergic reactions), and (2) allergenicity (the likelihood of inducing de novo sensitization in a nonallergic individual) [Spök et al., 2005].

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Alterations in the allergenic potential of GM foods can take several forms [Lehrer and Bannon, 2005; Buchanan, 2001; Wal, 2001], including:

- Transfer of known allergens from existing food sources to new food sources
- Introduction of novel protein from sources without a history of exposure that may become allergenic in man
- Increase in the levels of endogenous allergens of an existing allergenic food source
- Increase in allergenic activity or allergenicity of known allergens or other proteins (due to increased exposure, post-translational modification, changes affecting protein structure such as folding, unmasking previously unavailable epitopes, or changes in the cellular matrix in a new host)

Several studies and reviews have examined the relative importance of these sources of allergenic risks. The following conclusions may be drawn.
1) At an aggregate level, the likelihood of selecting and transferring a gene encoding an allergen in transgenic modification can be considered small. This is inferred from the observation that despite the vast number of protein substances in nature, most proteins are not allergens [Goodman et al., 2005; Halsberger, 2003; Lehrer, 2004].
2) The proportion of allergenic proteins from foods is also relatively small and they cluster in few protein families, with four families accounting for more than 65% of plant food allergens [Jenkins et al., 2005]. Of particular significance is a general lack of IgE cross-reactivity observed both within and between the other subfamilies.
3) Our understanding of how food matrix or adjuvant factors may affect the allergenicity of a protein is limited [Teuber, 2002], despite growing knowledge about the relationship between protein structure and allergenic potential.
4) The likelihood of increasing the levels and reactivity of endogenous allergens of an allergenic host plant may be considered as a function of general unintended effects.
5) Increased exposure to potential allergens may represent a broader problem that is not limited to GM foods [de Jong et al., 2004].

Goodman and Hefle [2005] concluded that the primary consideration in assessing the allergenic potential of GM foods is to avoid the transfer of any allergen or protein likely to cause cross-reaction in those already sensitized. A secondary consideration is to avoid introducing a protein with characteristics that are likely to sensitize individuals. A final consideration is whether the transformed crop commonly causes serious food allergies by comparing the relative allergenicity or abundance of endogenous allergens in the GM crop with the unmodified crop. The approach taken in the RBCS is consistent with these considerations.

Two assumptions inherent in the approach to assessing the allergenic potential of proteins in GM foods within current methodological constraints are recognized. (1) A protein that has a low likelihood of having allergenic activity is also less likely to induce sensitization. (2) A protein that can induce sensitization is likely to have allergenic activity. If it has cross-reactivity with another known allergen, it could be predicted by in silico methods even if it is a new protein without a history of prior exposure.

Allergenicity of the gene source
At the present, a protein from an allergenic source would be considered to be highly probable of being allergenic unless proven otherwise [FAO/WHO, 2001].

Potential to cross-react with known allergens
A major challenge in assessing the allergenic potential of GM foods is the lack of validated methods for testing the allergenicity of novel proteins (ability to sensitize individuals who have not been previously exposed). Current approaches focus on assessing the allergenic activity of proteins in GM foods (the propensity to elicit allergic reactions in sensitized individuals). Identifying the presence of individual epitopes (minimal peptide units within proteins than can recognized by the immune system) on the protein of interest that shares sequence identity with known allergens remains a principal objective of the assessment of the allergenic potential of a novel protein.

In silico approach to predicting cross-reactivity with known allergens based on amino acid sequence identity is a principal tool currently being used in assessing allergenic potential of transgenic proteins. Again, the predictive value of this approach has not been verified and the methodology is evolving [Becker and Reese, 2001; Björklund

et al., 2005; Brusic et al., 2003; Goodman et al., 2002; Kleter and Peijnenburg, 2002; Stadler and Stadler, 2003]. This approach is based on assumptions of minimal size of T-cell and B-cell epitopes [Becker, 2001]. However, non-contiguous or conformational epitopes are not identified by current algorithms. In silico approach is also limited by the availability of sequence information of known allergens in public databases and the scientific literature [Codex Alimentarius, 2003].

Stability in pepsin digestion
Susceptibility to proteolysis is more indicative of a low potential for allergenic activity that is dependent on conformational epitopes. This is related to the observation that denaturation and partial digestion of proteins reduces the availability of conformational epitopes more efficiently than linear epitopes [Goodman et al., 2005].

Based on theoretical considerations and assumptions of sequence specificity of pepsin substrate and the minimum peptide size required for eliciting the clinical symptoms of allergy, it appears improbable that the presence of a protease resistant fragment of < 3 kDa (29 amino acids) in the pepsin digestion assay would have the ability to degranulate mast cells [Bannon et al., 2002].

Although the property of digestive stability is not shared by all common major protein allergens, at present, protein stability appears to be the only reliable characteristic being used to characterize known food allergens from normal dietary proteins [Helm, 2001]. While the correlation between resistance to proteolysis and allergenic activity is not absolute and cannot be used alone for defining a potential allergen, the *in vitro* digestibility test confirms that the protein is unstable in the presence of digestive fluids and that it is not unusually persistent in the digestive system. The pepsin digestibility assay is not meant to mimic precisely the fate of protein in *in vivo* conditions.

Secondary factors
Given an overlap of characteristics between allergenic and non-allergenic proteins and methodological limitations, no single factor or test can fully predict the allergenic potential of any new protein. Judgement on a combination of factors reflecting a weight of evidence approach is increasingly favoured over a strict sequential decision tree application of testing strategies in the 2001 report of a Joint FAO/WHO Expert Consultation on Allergenicity of foods Derived from Biotechnology [Codex Alimentarius, 2003; FAO/WHO, 2001; König et al., 2004; Poulsen, 2004]. We propose that additional secondary factors may add weight to the concern that a protein could be allergenic where the evidence on the above (primary) factors is equivocal, particularly that of pepsin digestibility.

Structural/functional characteristics. Post-translational glycosylation, and specifically carbohydrate structures containing an α(1,3)-fucosyl residue, and/or β(1,2)-xylose may act as a cross-reactive carbohydrate determinant capable of binding to IgE from individuals sensitized to unrelated sequences [van Ree et al., 2000]. The role of cross-reactive carbohydrate determinants in IgE recognition versus their ability to elicit allergic reactions may depend on binding affinity and the rest of the protein structure [Goodman et al., 2005; Spök et al., 2005].

There is some overlap with a primary factor outlined previously (the potential to cross react with known allergens) given that both factors are based on criteria related to sequence identity. However, there are limitations to identifying potential cross-reactivity of a protein with known allergens based on linear sequence alone. It has been shown that the level of sequence homology is not always correlated with the conservation of allergenicity [Mills et al, 2003]. Low levels of homology can exist with conservation of allergenicity and vice versa.

Heat stability. Many food allergens are heat stable. However, heat stability is not a prerequisite for allergenic proteins. Some allergens are known to be heat labile, including: Bet v 1-related proteins and profilins.

Abundance. Highly allergenic proteins are often expressed at relatively high levels [FAO/WHO, 2001]. However, it is not possible to define a level of expression below which a protein can be considered safe from an allergenicity point of view. As a factor in assessing the allergenic activity of foods produced through agricultural biotechnology, abundance is likely related both to the probability of sensitization and elicitation although threshold levels for sensitization would be difficult to determine [Goodman et al., 2005; Haslberger, 2003; Taylor and Hefle, 2001].

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However, the criterion for the level of protein in a food as a predictor of its allergenic potential should not be confused with the threshold level for sensitization or elicitation. Threshold level is a function of the dose, which also depends on the quantity of the food consumed. With respect to sensitization, the duration of exposure or the number of repeated exposure may also play a role. It has been suggested that the induction of allergic sensitization in a previously naïve individual almost invariably requires exposure to concentrations of the allergen that are greater than those necessary to elicit a response in a sensitized subject [IPCS, 1999].

Allergenicity of the host plant
The allergenicity of the host plant is of concern with respect to unintended changes in the level or potency of endogenous allergenic proteins, if the host plant is an allergenic source. Practical considerations for testing may restrict the application of this factor to common sources of allergen, as further testing, typically involving serum screening, requires the use of sera from patients known to be sensitized to the protein or the food source of a sufficient sample size [FAO/WHO, 2001]. Unintended adverse changes in endogenous allergenic proteins in common allergenic foods will also have a broader impact at the population level compared to minor allergenic foods.

4.4. Criteria for differentiating health risk potential of GM foods.

The specific criteria for differentiating the health risk potential of GM foods for each of the factors included in the RBCS and the explanations are listed in the combined RBCS-product rating form. Only the common elements underlying the criteria will be outlined here.

Three types of criteria may be distinguished in the RBCS. First, data-based criteria that require little judgement (e.g., whether a gene product is expressed or not). Second, data-derived criteria that require the data to be interpreted using a numeric cut-off value (e.g., above or below amino acid sequence identity of 35%). Third, interpretive criteria that require considerable expert judgement (e.g., determining the adequacy of the scope of analytes). Some criteria may be considered provisional, that can be updated in the future as new evidence or new testing methods become available. In some cases, where quantitative criteria are expected to minimize subjective interpretation, criteria were included by this author based on the best available information in the absence of generally accepted values. The types and strength of evidence that can be used to determine whether criteria are met or not also vary. They range from in silico bioinformatics, theoretical estimates (e.g., dietary exposure), analysis of molecular or chemical structure, analysis of cytological markers and food composition, *in vitro* or *in vivo* functional studies (e.g., pepsin digestibility, enzyme activity, target specificity of pesticidal proteins), and generally accepted knowledge (e.g., biology and safety of donor and host organisms).

All criteria are stated in a manner such that a "yes" rating to a criterion means that the criterion has been met. A low concern level (level I) for a category of potential health effects requires meeting the criteria for all applicable factors (see 4.4 - aggregation). Other ratings are possible: "no" (one or more criteria for a factor is not met), or "uncertain" (information is insufficient or equivocal for rating), except where only a "yes" or "no" rating is required (e.g., whether a gene product is expressed or not). An uncertain rating implies that the uncertainty can be addressed by further testing or assessment. An option for "not applicable" is also provided.

For factors related to both intended and unintended effects, the concepts of familiarity and novelty underlie several criteria for discriminating hazard characteristics or potential (e.g., prior knowledge based on the same or similar substance) and exposure (e.g., history of consumption from food). The concept of familiarity is based on the fact that most GM crops are developed from plants the biology of which is well understood [European Commission, 2003]. Familiarity can be defined as indirect knowledge or experience obtained from similar gene products, plant varieties, or progenitor variety grown under similar conditions and used for similar purposes [NRC, 2000: 73]. Familiarity allows the risk assessor to draw on prior knowledge and experience with the introduction of similar crops or similar structural and functional profiles of substances of interest. In contrast, novelty represents the lack of or limited prior knowledge and will necessarily require that an assessment be made on a less certain basis. Consequently, the greater the novelty, the greater will be the likelihood that the level of testing will be increased [Madsen et al., 2002].

For some factors, the criteria vary depending on the nature of the substance being assessed (intended effects) or the method of genetic modification as a function of available analytical capability to characterize changes at the genomic level (unintended changes). This acknowledges the need to tailor the criteria to more appropriately match the characteristics of the substance or the process.

For factors related to unintended changes, it is expected that complexity of the metabolic pathway or network being modified will increase the potential for unexpected effects [Kuiper et al., 2001; Society of Toxicology, 2003]. Consequently, the level of testing would likely be increased in proportion to the need to reduce uncertainty. However, the increased need for testing does not necessarily mean that a product is more risky.

4.5. Aggregation of factors.

An aggregated concern level is assigned separately for each category of health effects based on ratings for individual factors in each category. The rules for aggregation are displayed in a table format in the combined RBCS-product rating form. The approach to aggregation is developed with the following considerations in mind.

1) All GM foods should be classifiable.

2) The assignment of concern levels should be mutually exclusive. This means that a GM food should be assigned into only level for each category of health effects.

3) The assignment of concern levels should reflect a weight of evidence approach where relevant, without the use of arbitrary numeric weighting values.

An example of the application of this principle is how secondary factors are included in the assignment of concern levels in Part C of the RBCS (potential for allergenic effects). Another example is that the weight placed on comprehensive compositional analysis is relative to whether the other factors are considered equivocal or suggestive of concern in Part B (potential for unintended changes). It can be argued that components in foods are what consumers will eat and are what really matters [FIFRA SAP, 2004].

4) There should be flexibility in more appropriately matching the characteristics of a substance of interest or genetic modification process.

This can be illustrated by varying the criteria to be met between characterized and poorly characterized gene modification with respect to the analysis of DNA sequences in assessing genomic disruption. This difference in requirement allows for current limitations in or acceptance of molecular characterization in the safety assessment of foods derived from crops modified by conventional genetic modification techniques. There are also differences of opinion about the value of complete DNA sequencing including junction regions in different regulatory jurisdictions [EFSA, 2004; FDA Food Advisory Committee, 2003; ILSI, 2004].

5) The assignment of concern level should reflect a gradation in the nature and extent of further testing beyond the screening level testing that provides core information for classification.

5. Evaluation of RBCS

5.1. Evaluation components.

This evaluation of the proposed risk-based classification scheme (RBCS) is part of phase 3 of the project on the development of the scheme as guidance for testing foods derived from genetically modified crops (GM foods). Components of the evaluation include:

a) the applicability of the proposed scheme to a range of GM foods with different characteristics
b) the ability of the scheme to differentiate a GM food into a concern level as defined in the scheme
c) the ease of use of the scheme
d) the degree of concordance of product ratings between two users of the scheme

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5.2. Development of product profiles.

Pilot testing of selected products in a reference panel of products was completed by seven individuals with expertise and experience related to safety assessment of GM crops and derived foods. The products were randomly allocated into groups of 2 products each and assigned to pilot test participants. Results of the pilot test guided the revision of the combined RBCS-product rating form with respect to formatting, inclusion of explanatory notes on the form for easy reference, and clarification of several criteria. Product profiles were also edited to improve clarity.

5.2.1. Selection of products in the reference panel.

The GM food products in the reference panel have been selected to reflect a range of characteristics that are relevant to their classification into concern levels using the proposed risk-based classification scheme. They include existing products and future GM crops at different stages of research and development and for which information at varying levels of detail may be available. Efficacy of the modified trait for the intended purpose and the potential for commercialization were *not* considered in product selection for the reference panel.

5.2.2. Sources of information.

All product information was obtained from public sources, including peer-reviewed scientific literature and product assessment reports on websites of regulatory agencies. For current products already approved or reviewed by at least one country, information was drawn from http://www.agbios.com and supplemented by the scientific literature. The Agbios database provides links to decision documents (in English) of national authorities. Out of over 90 products in the database that were screened, 5 products were included in the reference panel. The 5 selected products represent the spectrum of typical modified traits and crop types in the so-called first generation of GM crops. The information for these products provided in the database was considered sufficient for classification using the RBCS. In some cases, composite information was pooled from similar traits and crop types in developing the product profiles. Current products provide a baseline for assessing the utility of the proposed scheme.

For new or future products, information on trends in GM crop research and development was reviewed, using special reports [e.g., APEC, unpublished; ILSI, 2004; Lheureux et al., 2003; Pew Initiative on Food and Biotechnology, 2001; U.K. AEBC, 2002], field trial database websites[1] [e.g., European Commission Biotechnology and GMOs Information; Information Systems for Biotechnology; United States Regulatory Agencies Unified Biotechnology Website], and scientific papers on future trends [e.g., Cockburn, 2004; Dunwell, 2005]. Based on this information, additional literature searches were conducted in 5 categories of modified traits: pathogen resistance, nutritional/food quality, abiotic stress tolerance, and others (which included: reduction of undesirable substances, modification of crops for the extraction of food or functional ingredients, herbicide tolerance, and non-rDNA modifications). Within each category of modified trait, major strategies for achieving target traits were identified. Where applicable, a minimum of two scientific papers describing the same or similar traits and modification strategies were identified. A "simulated" product profile was developed based on this trait and modification specific information. Other relevant supplementary information from the scientific literature was added as necessary. Drafts of profiles on over 30 new or future products were prepared. Included in the final reference panel were 15 products with sufficient information for constructing meaningful product profiles. They span a range of different characteristics, with a focus on traits related to pathogen resistance and nutrition/food quality. New products are emphasized in order to assess the potential of the proposed scheme to be applied to emerging categories of GM foods. Crops modified by conventional methods were underrepresented in the reference panel. Information obtained from the public literature was limited on key characteristics of these crops for the development of product profiles for evaluation purposes.

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[1] European Commission Biotechnology and GMOs Information [http://gmoinfo.jrc.it]; Information Systems for Biotechnology [http://www.isb.vt.edu]; United States Regulatory Agencies Unified Biotechnology Website [http://usbiotechreg.nbii.gov].

The information provided in product profiles is not intended to be exhaustive but should be sufficient for classification purposes. Only essential "core" information relevant to product classification using the proposed scheme is included in the product profiles in order to keep the information to be reviewed to a reasonable length. It is a subset of the information typically submitted or required in premarket assessment. For example, the use of reasonably well-established marker genes is not included in the product profiles as part of the evaluation.

Excluded from the core information is "prerequisite" information that must be met by all GM crops. As noted previously (section 4.2), such information does not provide a basis for differentiating products into concern levels for testing. In particular, issues of the quality of molecular characterization data have been addressed in other guidance documents [e.g., U.K. ACRE, 2001]. IN APPLYING THE RBCS, ONE ASSUMES THAT THESE PREREQUISITE CRITERIA HAVE BEEN EVALUATED AND ARE MET.

Note also that one factor (prior experience with gene products introduced/modified) in the RBCS is NOT the subject of this evaluation. PLEASE TREAT ALL PRODUCTS THAT MEET THE CRITERION OF PRIOR EXPERIENCE AS NEW PRODUCTS.

Several types of information are required in applying the RBCS. They include dietary exposure, safety, and allergenicity of gene products and of their source organisms, as well as that of the host plants. The following basic reference sources were used in developing these types of information. Additional references used and obtained from databases of general scientific literature are listed in the individual product profiles.

Dietary exposure (food consumption):
- CSFII Analysis of Food Intake Distributions [EAP, 2003].

Amino acid sequence of proteins:
- NCBI Protein Sequence Database [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein]

Safety/toxicity information on specific compounds and microorganisms:
- TOXNET [http ://gateway.nlm.nih.gov/gw/Cmd]
- IPCS Chemical Safety Information [http://www.inchem.org/]
- NIH Guidelines for Research Involving Recombinant DNA Molecules, 2002

Allergens (amino acid sequence identity match using guidelines of the FAO/WHO Codex Alimentarius Commission):
- Structural Database of Allergenic Proteins [http://fermi.utmb.edu/SDAP/index.html]
- Food Allergy Research and Research Program [http://www.farrp.org/]
- Allermatch [http://allermatch.org]

Information on taxonomy, food uses and composition of food crops and plants:
- USDA, ARS, National Genetic Resources Program. *Germplasm Resources Information Network - (GRIN)* [Online
- Database: http://www.ars-grin.gov/cgi-bin/npgs/html/index.pl]
- USDA, ARS, Nutrient Data Laboratory [http://www.nal.usda.gov/fnic/foodcomp/index.html]
- Dr. Duke's Phytochemical and Ethnobotanical databases [http://www.ars-grin.gov/duke/]
- OECD Series on the Safety of Novel Foods and Feeds - Consensus Documents
- OECD (1993) Traditional Crop Breeding Practices

Other types of information required in applying the RBCS are specific to individual products and require product-specific testing. Examples of these types of information include: molecular and compositional data on the modified crop, pepsin digestibility and heat stability of protein gene products. For products that are in the early stages of research and development, these types of information are generally not reported in the public literature, or are of insufficient scope or specificity in applying the RBCS.

5.2.3. Assumptions used in developing product profiles.

To enable product classification using as much information as possible, where information on the required parameters was not available in the public literature, assumptions and default information were used to fill in the gaps. For example, protein substances from food crops or sources not known to be toxigenic would not be considered to have amino acid sequence identity similar to that of known mammalian toxins. Unless the structure of a protein has been identified to be similar to one that is known to be heat stable or resistant to digestion, it would be assumed not to possess these characteristics. In other cases (e.g., molecular, compositional, and protein digestibility data), variations of "model statements" were developed that represent different levels of adequacy of information relative to meeting the criteria in the RBCS. For less critical factors (e.g., heat stability), conclusions may be presented without data-derived supporting evidence.

6. Relevance of RBCS for testing GM foods

A premise underlying the RBCS is that the levels of concern are based on the type and extent of information one has, which then determines the type and extent of testing that will be required to fill the information gaps. The concept of the RBCS as envisioned in this project necessarily has to strike a balance between the usefulness of the tool as guidance for testing and the need for basic information for classification. As explained in previous sections, a certain level of product-specific testing is required in providing core information to achieve meaningful classification. This basic level of testing may be considered as tier 1 testing in a tiered approach. GM foods judged to be of low concern (level I) would be equivalent to have satisfied a basic level of testing without the need for or very minimal further testing. In some cases, the increased need for testing does not necessarily mean that a product is more risky. As a general rule, complex changes at the molecular or metabolic levels will increase the need for more extensive testing because of increased uncertainty, which may not result in adverse health effects.

An early draft of a proposed graded approach to testing that corresponds to the concerns levels in the RBCS was reviewed by a multidisciplinary group of experts in a previous consultation. Areas for improvement were identified and work on revising the proposed approach is continuing. Improvements to be considered include: integrating the testing of intended and unintended toxic and antinutritional effects and identifying testing issues to be addressed without being prescriptive. A further challenge is to develop an approach to testing by using currently available and accepted or validated methods while recognizing their limitations and the ultimate goal of guiding regulatory risk management decisions in the approval of foods derived from GM crops. It is expected that product classifications resulting from the evaluation phase of the project will provide useful examples in developing a graded approach to testing.

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Appendix - Product characteristics by concern level.

The following description illustrates the product characteristics that the concern levels in the RBCS are intended to represent. For the purposes of this evaluation, please use the combined RBCS-Product Rating Form provided and follow the steps in the "Instructions for Completing Product Rating" in determining concern levels for the product profiles to be reviewed.

PART A: POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - intended changes resulting from the primary and secondary gene products introduced/modified.

The factors and criteria are applicable to both protein and non-protein substances, unless stated otherwise.

Level I. Dietary substances that have a history of known or presumed safe consumption from foods and have insignificant changes with respect to expected exposure.

A substance in Level I must meet all applicable criteria for the following six factors.

(1) Has a prior history of known or presumed safe consumption from foods;

(2) Change in total dietary exposure is expected to be within normal variation in dietary intakes. For a substance with known toxicity concern, expected total exposure will not exceed established upper limit of intake;

(3) Has either an established upper limit for intake (for a substance with known toxicity concern), or well-established biological function or specificity of mode of action with no potential for mammalian toxicity, antinutritional, and adverse metabolic effects under normal conditions of consumption (for other substances), taking into consideration comparability in the methods of food processing/preparation between the food derived from the modified crop and that from the gene source;

(4) For a substance with a prior history of known or presumed safe consumption from foods, the counterpart produced in the modified plant must be demonstrated to be structurally similar and functionally identical to the naturally occurring comparator/reference substance on which prior knowledge about safety is based;

(5) No unusual or novel metabolites are expected from proteolytic or catabolic degradation of the gene product(s) in the edible part(s) of the modified plant and from the digestion and metabolism of the substance following ingestion;

(6) No synergistic toxic effects between gene products and no interactions between gene product(s) and food constituents that may negatively affect bioavailability.

Level I includes two additional special categories of foods derived from GM crops.

(1) The gene modification is expected to result in negligible dietary exposure to the intended gene products. This can be the result of intentional gene silencing, tissue-specific expression not affecting the edible part of the modified crop, or the edible part is used as a food ingredient in a limited form as a highly refined and well-defined chemical entity. This special category does not apply to substances with a nutritional role. Confirmation of the absence of gene product(s) in the food is required. A substance at a level below the threshold of toxicological concern will also meet the criteria for Level I.

(2) The gene modification is based on prior experience with respect to gene products introduced/modified, expression levels, gene source, transfer methods, and host plant. This aspect of the RBCS is EXCLUDED from this evaluation.

GM food assessment will focus on the edible part of a crop plant consumed by humans. It is recognized that some food crops have very limited food use for human consumption. If their human food use is restricted only to

the production of highly refined ingredients of well-defined chemical composition (e.g., the only food use of oilseed rape is the production of oil by extraction using a highly refined process), verification of the absence of an intended primary gene product without a nutritional role in the extracted fractions should be sufficient testing for a crop with a limited food use. Currently available standards for the testing and assessment of food ingredients will apply. Similarly, in tissue-specific expression of introduced/modified genes that do not affect the edible parts of a food crop, verification of the absence of intended gene products in the relevant plant parts should be sufficient. In these two specific cases, the application of the RBCS in its entirety would not be necessary.

In the case of intentional silencing, unintended effects will need to be assessed, as well as intended changes in any secondary gene products associated with the modification.

Level II. Substances that have limited and defined concerns with respect to *either* expected exposure *or* toxicity profile.

Two subcategories of Level II substances may be distinguished:
(1) Naturally occurring food constituents (including adventitious substances that have a history of consumption from foods without reported adverse health effects), and
(2) Substantially modified or novel substances.

A naturally occurring food constituent with insufficient or equivocal information on *only one* of the following category of characteristics (without other concerns regarding digestion and degradation products or adverse synergistic and interactive effects with other gene products or food constituents) may be considered as Level II:

- Expected change in total dietary exposure
- Prior knowledge of safety or mode of action
- For protein substances: pepsin digestibility

This means that a naturally occurring food constituent with only one of the following characteristics may be classified as Level II if the only uncertainty is with limited data on expected changes in dietary exposure.

a/ Micro-substance with known toxicity profile and established upper limit for intake.
b/ Micro-substance with limited empirically based toxicity data but no identifiable characteristic for concern.
c/ Macro-substance with no potential health concerns related, but not limited to, alterations in digestibility and/or metabolic effects under typical conditions of consumption.

On the other hand, if the information on these characteristics of a naturally occurring food constituent is limited or uncertain, the information on expected changes in exposure should be considered sufficient. In addition, a protein substance derived from a food source with equivocal information with respect to resistance to proteolysis without other apparent concerns about safety or expected changes in exposure can also be classified as Level II.

A substantially modified or novel substance with a negligible change in or very low expected dietary exposure may also be classified as Level II if it has one of the following characteristics:

a/ For a protein substance: does not have structural-functional similarity to proteins that have known toxic or antinutritional properties.
b/ For a non-protein substance: has simple chemical structure suggestive of a low order of oral toxicity, or physical-chemical properties identical to the native/reference substance with low toxicity concerns.
c/ For other protein and non-protein complex substances: has no structural similarity to known mutagen or carcinogen.

Level III. Substances that do not meet the criteria for level I or II.

A substance that does not meet the criteria for Levels I or II will be classified as Level III for intended changes with a potential for toxic and antinutritional effects.

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This means that a naturally occurring food constituent or a substantially modified or novel substance may have *one or more* of the following characteristics.

(1) More than one of the characteristics described for Level II.

(2) Exceeding an established upper intake level, if known, based on theoretical estimates (necessitating more detailed or probabilistic modelling or discontinuation of product development).

(3) Not meeting one or more of the criteria regarding hazard characteristics: safety or mode of action, structure-activity characterization.

(4) Not meeting criteria regarding digestion and degradation products or adverse synergistic and interactive effects with other gene products or food constituents.

PART B: POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - unintended changes in the modified food from genetic modification of the host plant.

The factors and criteria are applicable to the food derived from the modified crop.

Level I. GM foods with low potential for adverse health effects from unintended changes.

To be classified as Level I, a GM food will meet applicable criteria in four groupings comprising a total of six factors. The four groupings of criteria are:
(1) Genetic sources: the safety of a/the donor organism, and b/the host plant.
(2) Factors affecting the unexpected expression of known or novel substances: potential for a/ novel/altered patterns of metabolites, and b/disrupting coding or regulatory DNA sequences in the modified plant.
(3) Evidence of unexpected expression of known or novel substances: a/ key nutrients, b/ known toxicants and antinutrients, c/ other metabolites and parameters.
(4) Health consequences of instability of the modified trait.

Applicable criteria vary between two subcategories of GM products.
(a) Characterized gene modification based on nucleic acid techniques.
(b) Poorly characterized gene modification including undirected mutagenesis, somaclonal variation, or wide-cross hybridization.

(1) For both characterized and poorly characterized gene modifications, the safety of genetic sources must be demonstrated by prior knowledge or in combination with empirical data
- for crops modified by rDNA techniques: evidence of transfer of only the target gene
- for crops modified by conventional techniques: evidence of a greater level of genomic contribution from the host plant with an established history as a food crop in proportion to concerns about the safety of the donor organism

(2) With respect to the potential for novel/altered patterns of metabolites and disruption of coding or regulatory DNA sequences in the host plant, a judgement of low likelihood can made based on a combination of prior knowledge and experimental data on biochemical and molecular characterization.

a/ Prior knowledge and biochemical characterization will demonstrate
  i/ the function of the protein gene product(s) in the modified plant,
  ii/ that the gene product has no metabolic, regulatory, or signalling function in the host plant, and
  iii/ low substrate specificity if the protein has an enzymatic function.
b/ Molecular characterization will demonstrate the absence of open reading frames (potentially active genes with an unknown function) and their functional expression
  i/ in the inserted/modified DNA, and
  ii/ preferably, also in the sequences bordering the site of insertion/modification.

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The relative weight placed on molecular characterization can vary depend on the nature of the genetic modification, given the recognition that molecular characterization methods typically used in bioengineered crops are generally not amenable to assessing genomic changes in plants modified by non rDNA conventional techniques, for which alternative analyses at the level of metabolites are emphasized. Even for bioengineered crops, comparative sequence analysis may not always be possible due to limited genomic databases for the crop species in question at the present [EFSA, 2004].

(3) With respect to evidence of unexpected expression of known or novel substances, for both characterized and poorly characterized gene modifications, chemical analysis of the food derived from the modified plant must demonstrate that

a/ The levels of key nutrients and known toxicants/antinutrients are within acceptable ranges of natural variation, or are within limits that are unlikely to have adverse nutritional, metabolic, or toxicological effects.

b/ In addition, if the information on other factors described in (2) is limited or missing, the scope of nutrients, toxicants and antinutrients analyzed in (3a) and any additional metabolites/parameters analyzed should be reasonably justified to be adequate and should demonstrate that

- no unknown compounds or significantly altered levels of endogenous metabolites are outside the range of natural variation, or
- if changes occur, the likelihood of negative health effects can be justified to be low.

For characterized gene modification with limited information on the potential for interruption in genomic sequences bordering the site of insertion/modification, and for poorly characterized gene modifications, satisfying information requirement for criteria (3b) is essential.

(4) Health consequences of instability of the modified trait.

Where the modified trait is intended to provide nutrition or health benefits for humans, adequate quality control is in place to ensure consistency in lot-to-lot expression of the targeted gene product. This requirement is applicable irrespective of the method of genetic modification.

Level II. GM foods with moderate or high potential for adverse health effects from unintended changes.

A GM food that fails to meet any of the applicable criteria for Level I will be classified as Level II for further testing with respect to the potential for unintended changes with toxic and antinutritional effects.

This means that a food derived from a crop modified by rDNA technique in Level II may have *one or more* of the following characteristics.

(1) The transfer of uncharacterized genetic material from the gene source cannot be ruled out, where knowledge about its safety is limited.

(2) The levels of endogenous nutrients, toxic or antinutritional substances being outside the range of natural variation and their potential for adverse human health effects is uncertain.

(3) The adequacy of the scope of metabolites/parameters analyzed is not justified, or the health relevance of altered levels of analytes is uncertain, where one or more of the following characteristics are present.

a/ The host plant is a source of known or significant toxicant or antinutrient associated with adverse health effects in humans or animals, or is present with narrow margins of exposure.

b/ There is uncertainty about the function of the gene product, its likely metabolic impact in the modified plant, and substrate specificity if the protein has an enzymatic function.

c/ The potential exists for open reading frames (unknown DNA sequences) to be expressed or functional.

(4) For modified traits that are intentionally introduced to provide nutrition or health benefits for humans, quality control measures to ensure consistency in the level of compounds introduced/modified are limited.

Similar characteristics also apply to a food derived from a crop modified by conventional techniques, especially as they relate to characteristics (2), (3), and (4). Information is likely to be limited for characteristic (3c). In addition, where the safety of the donor plant is uncertain, evidence may be considered equivocal in supporting that sufficient backcrossing has been conducted to remove large percentage of genetic material from the donor organism.

PART C: POTENTIAL FOR ALLERGENIC EFFECTS

The factors and criteria are applicable to protein substances.

Level I. Proteins with low potential for allergenic activity.

A protein substance is classified as Level I when criteria for all of the following factors are met.

(1) The donor organism (gene source) is not a known source of allergenicity or gluten-sensitive enteropathy.

(2) The likelihood of the protein gene product to cross-react with known allergens is low, as determined by recommended criteria for amino acid sequence comparison with known allergens.

(3) The protein is completely degraded within a specified duration in pepsin digestion under standard testing conditions.

Where information on pepsin digestibility is equivocal for eligibility for Level I, criteria for the following additional factors will be met.

a/ The protein does not have structural/functional characteristics that are similar to specific categories in certain protein families that have a high proportion of allergenic proteins, or are suggestive of allergenic property (e.g., specific post-translation glycosylation patterns for heterologously expressed proteins.

b/ The protein is not heat stable, except for lipid transfer proteins that are generally heat labile.

c/ The protein is present in the food at a low level (< 0.1% protein for pesticidal and enzymatic proteins; < 1% protein for other types of protein).

(4)The food is derived from a modified plant that is not a common source of allergenicity or gluten-sensitive enteropathy. If the food is derived from a common allergenic source, it should have characteristics corresponding to having a low potential for unintended changes (based on assessment in Part B).

Level II. Proteins with moderate or high potential for allergenic activity.

A GM food containing a protein that fails to meet any of the applicable criteria for Level I will be classified as Level II for further testing with respect to the potential for allergenic effects.

This means that a protein substance in Level II may have *one or more* of the following characteristics.

(1) The donor organism (gene source) is a known source of allergenicity or gluten-sensitive enteropathy.

(2) The protein gene product is likely to cross-react with known allergens based on amino acid sequence identity with known allergens.

(3) If the protein is moderately to highly resistant to pepsin digestion, it has one or more of the following structural-functional characteristics.

a/ The protein has structural/functional characteristics that are similar to specific categories in certain protein families that have a high proportion of allergenic proteins, or are suggestive of allergenic property (e.g., specific post-translation glycosylation patterns for heterologously expressed proteins);

b/ The protein is heat stable (excluding lipid transfer proteins that are generally allergenic and heat labile);

c/ The protein is present in the food at a relatively high level ($\geq$ 0.1% protein for pesticidal and enzymatic proteins; $\geq$ 1% protein for other types of protein).

(4) The food is derived from a modified plant that is a common source of allergenicity or gluten-sensitive enteropathy and has characteristics corresponding to having a high potential for unintended changes (based on assessment in Part B).

Annex 6-4

Product Profiles – 20 Products

A. Tomato - Polyamines 1

B. Maize - Zein 7

C. Canola - Carotenes 13

D. Rice - Lactoferrin 20

E. Rice - Trehalose 25

F. Rice - Mutant dhps 30

G. Soybean - Fatty acids 35

H. Rice - Chemical mutagenesis 40

I. Maize - CI-2 45

J. Alfalfa - cryIC 51

K. Maize - HT 56

L. Soybean - Isoflavones 61

M. Potato - GNA 67

N. Maize - Amarantin 73

O. Tomato - Accd 78

P. Potato - Cry & GNA 83

Q. Tomato - NPR1 90

R. Rice - Ferritin 96

S. Potato - Cry 102

T. Potato - Somatic hybrid 107

PRODUCT PROFILE

Modified trait	Increased polyamines (spermidine and spermine) in modifying fruit ripening and vine life.
Intended food use of modified crop (host plant)	Tomato (*Lycopersicon esculentum*) is cultivated primarily for human consumption, either fresh or processed.
Gene (source) / Promoter (source)	S-adenosylmethionine decarboxylase (*ySAMdc*) gene from yeast (*Saccharomyces cerevisiae*) under the ripening-regulated E8 promoter for fruit-specific expression.
Transformation method	Agrobacterium-based transformation.
Metabolic reactions / pathways	Spermidine, spermine, and putrescine are the polyamines (PA) most frequently found in plants and other organisms. Spermidine is formed from putrescine (derived from basic amino acids, ornithine or arginine), a reaction catalyzed by SAM decarboxylase using decarboxylated SAM as an aminopropyl-group donor. The conversion of spermidine to spermine is catalyzed by spermine synthase. SAM decarboxylase is a rate-limiting step in polyamine biosynthesis. S-adenosylmethionine (SAM) is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. ACC is an immediate precursor of ethylene, a promoter of fruit ripening. Thus both ethylene and polyamine biosynthetic pathways use SAM as substrate and produce methylthioadenosine (MTA) as a by-product.
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	S-adenosylmethionine decarboxylase (ySAMDC).
Secondary gene products	Polyamines: spermidine and spermine.

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop for food.

ySAMDC: Less than 0.4% of total fruit protein, or < 50 μg/g, fresh weight in fruit at peak level during ripening.

Spermidine and spermine: Levels (nmol/g) increased gradually during ripening to 200 (spermidine) and 10 (spermine), compared to < 100 and 1-2 in control fruit, respectively.

A.2. Prior history of known or presumed safe consumption from foods.

ySAMDC: an enzyme from yeast with prior dietary exposure.

Spermidine and spermine: Polyamines are present in common foods, with typically higher contents of spermidine compared to spermine in foods of plant origin. The highest contents of all polyamines are identified in cheeses, mainly in ripened types. Fruits and vegetables, with the exception of broccoli and cauliflower, are generally low in all polyamines [Eliassen et al., 2002].

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: No change in the food use of the modified tomato is expected.

ySAMDC: Not expected to result in meaningful change in consumption relative to total dietary protein. Protein intake from tomatoes contributes < 0.7% of adult human dietary reference values.

Spermidine and spermine: Limited data suggest that estimated amounts of daily ingested polyamines (including putrescine, cadaverine, spermidine and spermine) range between 100 and 500 μmol. [Eliassen et al., 2002]. Optimal levels of dietary intake are not known [Moinard et al., 2005]. Average consumption of tomato is expected to contribute < 1% to total polyamine intake (from unmodified tomato), and < 2% (from modified tomato).

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

ySAMDC: No safety concern based on prior knowledge, given the level introduced in tomato, and yeast having prior exposure in the diet.

Spermidine and spermine: Oral acute and subacute toxicity of the individual polyamines has been determined in Wistar rats. For putrescine, spermidine and spermine, respectively: acute toxicity was reported to be 2000, 600 and 600 mg/kg body weight; NOAEL: 180, 83 and 19 mg/kg body weight [cited in Kalač and Krauoavá, 2005]. While extreme dietary intakes of polyamines approaching these levels are unlikely, potentially harmful effects of high polyamine intakes cannot be dismissed.

Dietary polyamines have both beneficial and harmful effects, but present knowledge is too sparse to provide recommendations on intakes. Polyamines are essential for the growth, maintenance and function of normal cells. They are metabolized into a large number of derivatives by the action of various oxidases and acetyl-transferases. Terminal catabolism of polyamines is oxidative deamination, essentially in the intestinal mucosa, the liver and kidneys. Both dietary and endogenous polyamines (formed from methionine and arginine) participate in these functions.

High concentrations of polyamines can have toxic effects resulting from the formation of hydrogen peroxide and related oxidative stress during the catabolism of polyamines. Intestinal enterocytes are also exposed to high concentrations of polyamines post-prandially. Polyamines are known to be involved in both normal, adaptive and neoplastic epithelial cell proliferation in the gut, and are preferentially taken up by tumours and tissues with high demands for polyamines.

Even though only a limited fraction of dietary polyamines are absorbed from the intestinal tract, they can be metabolized during the passage through the intestinal wall. Since a significant amount of polyamines required for proliferation and differentiation is provided by the diet, there could be a need for restricting the dietary intake of polyamines and intestinal bacterial generation of polyamines in order to reduce the amount of polyamines available for neoplastic cell growth [Eliassen et al., 2002; Kalač and Krauoavá, 2005; Milovic, 2001; Moinard et al., 2005].

A.5. Structure-activity characterization.

Amino acid sequence identity with mammalian toxins.
ySAMDC: No amino acid sequence identity with mammalian toxins is expected.

Pepsin digestion (under standard testing conditions).
ySAMDC: The protein was rapidly and completely digested in less than 1 min.

Structural/functional comparability with comparator/source protein or non-protein substance.
ySAMDC: Comparison of the plant-derived with the wild-type enzyme indicates that the two proteins did not differ in their substrate affinities. Based on electrophoretic mobility and Western blot analysis, it was determined that the plant-derived enzyme has the predicted molecular weight and immunogenicity as the wild-type enzyme.

Spermidine and spermine: Structurally similar to known standards.

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A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
ySAMDC: No unusual breakdown products of protein in the modified plant are expected - no unusual protein fragments were identified by Western blot analysis.

Spermidine and spermine: No concerns with altered catabolism would be expected from a moderate increase in spermidine and spermine in tomato derived from modified vs. control crop. However, it is recognized that the metabolism of these compounds is complex. Both spermidine and spermine are subjected to oxidative deamination by diamine oxidases forming aminoaldehydes, hydrogen peroxide and ammonia. In addition, both the terminal amino groups of spermine are oxidized to a dialdehyde. Another class of enzymes, polyamine oxidases, also catabolize both spermidine and spermine, forming compounds (aminoaldehydes, hydrogen peroxide) similar to the reaction catalyzed by diamine oxidases.

Polyamines are precursors of various important alkaloids, and diamine oxidases participate in the biosynthesis of alkaloids containing a heterocyclic ring [Crozier et al., 2000].

In mammalian metabolism from consumption of the gene product.
ySAMDC: No concerns with ingested protein that is rapidly digested.

Spermidine and spermine: No unusual metabolism of polyamines from the ingestion of modified tomato is expected. See also description in A.4. Oxidative deaminations of polyamines are confined to those cell types that are rich in diamine oxidase. In addition, the interconversion cycle (catalyzed by polyamine oxidase and spermidineN^1-acetyltransferase) appears to be the general system of polyamine regulation in nearly all tissues of the vertebrate organism with the constitutively expressed polyamine oxidases. The importance of the various processes (uptake and release) and reactions (*de novo* synthesis and degradation) depends on the cell type and physiological and pathological situation [Seiler, 2004].

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

Not applicable.

A.7.2. Between gene products introduced/modified and food constituents.

No unusual interactions with food constituents are expected from a moderate increase in polyamines in modified tomato. The degree of absorption of dietary polyamines is partly determined by the presence of other amines that compete with putrescine for the degrading enzyme diamine oxidase.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

Yeast has a history of dietary consumption and is not known to be a source of toxicants or antinutrients under typical conditions of consumption.

Applicable if the safety of donor organism is not known, or if the donor organism is a source of known toxicant or antinutrient.
B.1.1. Characterized gene modification.

B.1.2. *Poorly characterized gene modification*.

B.2. Safety of host plant.

Tomato belongs to the Solanaceae plant family that encompasses a large number of toxic species. Tomato is a source of glycoalkaloids, including alpha-tomatine, which is the primary toxic agent in tomatoes. Alpha-tomatine is present at higher concentration in green tomatoes (870 μg/g) compared to 450 and 360 μg/g in yellow and red tomatoes, respectively. Consumption of green tomato plants by pigs resulted in illness or death. Small amounts can also be found in the ripe fruit of certain tomato varieties close to the wild form (e.g., "tomadoses" in the Antilles). The alkaloid can be easily bred out. Alpha-tomatine and tomatidine (aglycone form of tomatine) have not been tested in oral toxicity or cancer bioassays other than intravenous injections in mice in which tomatidine was slightly more toxic than the parent glycoside [Beier, 1990].

Other glycoalkaloids include solanine and chaconine. Antinutrients such as lectins and oxalate can also be present. The alkaloids cause membrane disruption in the digestive system and have anticholinesterase activity on the central nervous system. They have also been shown to be potentially teratogenic. Recent studies have shown that many food plants in the Solanaceae also contain significant levels of calystegines, polyhydroxytropane alkaloids that are potent inhibitors of glucosidases [Asano et al., 1997]. These alkaloids may also be involved in digestive disturbances through inhibition of gut enzymes. Antinutrients such as lectins and oxalate can also be present.

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

The introduced enzyme increases polyamine content in the modified crop, as expected.

B.3.2. Impact of gene product function in the modified plant.

Polyamines are precursors of various important alkaloids, and diamine oxidases participate in the biosynthesis of alkaloids containing a heterocyclic ring [Crozier et al., 2000]. As described under "Metabolic reactions and pathways", the polyamine and ethylene biosynthetic pathways are linked, with SAM as the common substrate. Ethylene is a plant hormone that affects many metabolic process in the plant.

Applicable if gene product has an enzymatic function in the host plant.
B.3.3. Substrate specificity of gene product.

B.4. Potential for disrupting coding/regulatory DNA sequences in host plant.

B.4.1. In the inserted/modified DNA.

Southern hybridization and RT-PCR (reverse transcriptase-polymerase chain reaction) revealed integration of one copy of transgene at a single genetic locus with an incomplete inverted repeat.

Comparison of DNA insert and pre-insertion plant DNA (conducted using a combination of the Universal Genome Walker kit and PCR amplification) revealed rearrangements, truncations, and sequence repeats at the insertion site, which is common in transformation of this type. However, bioinformatics analysis and PCR assays did not suggest the presence of open reading frames in the inserted sequences.

B.4.2. In genomic sequences bordering the site of insertion/modification.

Bioinformatics and sequence analyses support that the sequences flanking the insertion regions (about 500 bp) correspond to plant DNA and no unknown DNA sequences were identified.

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B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Analysis included proximates, amino acid and fatty acid profiles, as well as vitamin C and folic acid.

Levels in the modified crop and unmodified control are comparable.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

Known toxicants and antinutrients were analyzed, including: alpha-tomatine, solanine, chaconine, lectins and oxalate.

As expected, the tomatine levels decreased from immature to mature tomatoes in both modified and unmodified control, although the initial levels in the immature fruit was about 20% higher in the modified fruit compared with that in the control fruit, but the levels were within those reported in the literature (49-900 μg/g fresh fruit).

B.5.3. Other metabolites/parameters.

Metabolites related to the target metabolic pathway were analyzed.

Lycopene was increased by 2- to 3-fold compared with the wild-type red ripe fruit, independent of fruit firmness. Ethylene production was also consistently higher throughout ripening in the transgenic crop. How plants regulate the biosynthesis of lycopene is not well understood. Increased accumulation of carotenoids such as lycopene in tomato fruit is related to the differentiation of chloroplasts into chromoplasts. This differentiation is a developmental process that is accompanied by redirected and regulated gene transcription and translation that involves genes controlling carotenoid biosynthesis in an ethylene-independent process. It is suggested that the accumulation of polyamines may cause qualitative changes in transcriptional activity and may affect ethylene receptors or one or more components of the ethylene signal-transduction pathway. Increased production of ethylene is also compatible with published data showing a higher rate of ethylene production in fruit from plants transformed with an antisense endogenous E8 gene. However, the data from this transformation event did not reveal reduced levels of E8 transcripts. In the absence of significant phenotypic alterations, the changes in ethylene synthesis observed are unlikely to have detrimental implications for plant metabolism or changes in other metabolites with relevance for human health.

Acidity of the tomato fruit was also assessed as it is an important quality for food safety in the thermal processing of tomatoes. No difference in pH were found between modified and control tomatoes.

B.6. Health consequences of instability of the modified trait.

The modified trait is not an intended health trait. However, polyamines affected by the intended change do have health relevance. While changes in their levels from tomato derived from this modified crop do not appear to be of concern, there is uncertainty in the optimal intake of polyamines.

PART C - POTENTIAL FOR ALLERGENIC (IgE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN SUBSTANCES.

C.1. Allergenicity of donor organism (gene source).

Baker's or brewer's yeast (*Saccharomyces cerevisiae*) is not a common source of allergen. However, it has been implicated in adverse reactions to yeast-leavened or fermented products and in cross-reactivity in atopic patients.

Protein gene products (factors C.2-C.4).

C.2. Potential for protein gene product(s) to cross react with known allergens.

ySAMDC: No matches of greater than eight consecutive amino acids with known allergens were identified, nor were overall sequence identities of > 35% found.

C.3. Pepsin digestion (under standard testing conditions).

As noted in A.5.2, the protein was rapidly and completely digested in less than 1 min.

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factor (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

C.4.3. Abundance.

C.5. Allergenicity of host plant.

Tomato is not a common source of allergens, but several glycoproteins are known to be allergenic, including: profilin, beta-fructofuranosidase (including clones 1 and 2), lipid transfer protein, anther specific LAT52 protein, thaumatin-like protein NP24. The latter two proteins are non-IUIS* allergens.

* International Union of Immunological Societies.

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PRODUCT PROFILE

Modified trait	Enhanced lysine level in maize for feed.
Intended food use of modified crop (host plant)	Little whole kernel or processed field corn or maize (*Zea mays*) is consumed by humans worldwide when compared to maize-based food ingredients. Maize-based ingredients have wide applications in foods and include starch, sweeteners, flaking grits, flour, and oils from wet or dry milling processing.
Gene (source) / Promoter (source)	An α-zein gene from maize (*Zea mays*) in the antisense orientation was introduced under the control of the γ-zein A promoter.
Transformation method	Microprojectile bombardment mediated transformation.
Metabolic reactions / pathways	The genetic modification aims to increase lysine level in maize by target reduction of 19 kDa α-zein proteins that are almost completely devoid of the amino acid. Studies of mutant lines of corn suggest complex molecular and biochemical control of zein genes and gene products, possibly coordinated by a transcription factor [Huang et al., 2004; Azevedo, 2002; Azevedo et al., 2004]. This involves altered distribution of the endosperm proteins and increased accumulation of soluble lysine in the endosperm, which is mediated by altered level of the activity of enzymes involved in the biosynthesis of aspartate-derived amino acids (e.g. aspartate kinase) and lysine catabolism (lysine 2-oxoglutarate reductase and saccharopine dehydrogenase). Other enzymes in the aspartate pathway, particularly dihydrodipicolinate synthase, the first enzyme in lysine synthesis, may also be implicated.
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	Reduced expression of endogenous 19 kDa α-zein proteins - NOT assessed for intended effects as there are no toxicological/antinutritional concerns. Potential for unintended changes (including allergenic effects) is relevant and will be assessed in Parts B and C (factor C.5).
Secondary gene products	There are safety concerns of amino acids at high levels of intakes. The intended change in lysine content will be assessed, although it is not a reaction product of the protein modified.

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop for food.

A reduced level of the reduction of 19-kDa α-zeins was confirmed by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and MALDI-TOF MS (matrix-assisted laser desorption ionisation time-of-flight mass spectrometry). Transgenic kernels had increases in lysine (4.2 % protein), compared with < 3% typical in unmodified maize.

A.2. Prior history of known or presumed safe consumption from foods.

Lysine is an amino acid that is abundant in many protein-containing foods.

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: No changes in the consumption of maize are expected. It will replace unmodified maize in the same uses.

Lysine: There are also no significant changes in lysine intakes expected among individuals consuming a diet consisting of mixed protein sources, of which maize is not a major cereal source. Depending on the importance of maize in the diet, the increase in lysine can range between 0.002% - 0.02% of the diet. This assumes 100% maize to have enhanced lysine level, which is a gross over-estimate. No undesirable effects are expected at the projected increased lysine intake, which is within the normal range of variation in amino acid content in the diet.
Lysine (typically < 3% protein) is a limiting amino acid in unmodified maize, compared to FAO's recommended concentration of 5.5% for human nutrition. The potential may exist for enhanced dietary protein quality for individuals consuming a maize-based diet, but is unlikely to be of nutritional significance for western populations consuming a varied diet.

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

There are no safety concerns with the extent of changes expected in the endogenous proteins, enzymes, and amino acids from consumption of foods and of the modified corn. There is also no evidence that amino acids derived from usual or even high intakes of protein from foodstuffs present any risk [IOM, 2005]. In addition, very few adverse effects of L-lysine in the purified form in humans or animals after high, mostly acute doses have been reported. The data are not sufficient for a dose-response assessment and the derivation of an upper intake level for apparently healthy humans.

A.5. Structure-activity characterization.

Amino acid sequence identity with mammalian toxins.
Not applicable - no novel protein expressed.

Pepsin digestion (under standard testing conditions).
Not applicable - no novel protein expressed.

Structural/functional comparability with comparator/source protein or non-protein substance.
No changes in the chemical structure of lysine are expected in the modified maize given the mode of the genetic modification and the metabolic reactions involved. This was confirmed by the expected structure of the lysine identified in the modified crop. As well, no unusual structures of other amino acids were detected in the modified maize compared to standards.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
No unusual breakdown products of protein or lysine in the modified plant are expected from changes in the level of expression of endogenous α-zein protein. Very high level of lysine accumulation can have deleterious developmental effects on crops but they were not observed in the modified maize.

In mammalian metabolism from consumption of the gene product.
No changes in the metabolism of the ingested maize proteins are expected.

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

Not applicable - suppressed gene expression.

A.7.2. Between gene products introduced/modified and food constituents.

No interactions between altered levels of zein proteins and amino acids with other food constituents are expected at the altered levels in the modified maize.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

Same as host plant - see B.2.

Applicable if the safety of donor organism is not known, or if the donor organism is a source of known toxicant or antinutrient.

B.1.1. Characterized gene modification.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Maize has a long history of consumption as food and is not a source of significant toxicants or antinutritional factors. Low levels of trypsin and chymotrypsin inhibitors are not considered nutritionally significant. Highly variable levels (0-1000 ppm) of DIMBOA (2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one) have been reported in green- and root-tissues of maize seedlings, with its glycoside form being the most prominent. The deglycosylated form, DIMBOA is toxic to insects; data on effects on humans and animals are limited. Other metabolites include:
a) Furfural: moderate acute toxicity and oral genotoxic carcinogen of low potency; partly removed by heating.
b) Ferulic acid and p-coumaric acid: natural pesticide against insects and fungi. As phenolic acids and weak anti-oxidants, their effects (positive and negative) on mutagenic substances have been reported.

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

The altered α-zein is a storage protein in maize.

B.3.2. Impact of gene product function in the modified plant.

Alpha-zein protein is not generally known to have a direct regulatory, metabolic, or signalling function in the modified maize. However, its altered expression is expected to affect complex molecular and biochemical interactions, as noted under "Metabolic reactions/pathways" on p.1. The mechanism behind the transgenic reduction of specific classes of zein proteins resulting in amino acid composition changes is still unclear [Huang et al., 2004]. Complex control is also likely to be involved in the catabolism of storage proteins. Several amino acids of the aspartic acid pathway (including threonine and methionine) can be affected.
In addition, starch quality is known to be affected in mutant maize low in α-zein protein and the genetic background of the crop may also play a role [Huang et al., 2004].
In addition, greater susceptibility to pathogens has been reported in high lysine maize [Azevedo, 2002].

Applicable if gene product has an enzymatic function in the host plant.
B.3.3. Substrate specificity of gene product.

Not applicable - α-zein protein is not an enzymatic protein.

B.4. Potential for disrupting coding/regulatory DNA sequences in host plant.

B.4.1. In the inserted/modified DNA.

Southern hybridization and RT-PCR (reverse transcriptase-polymerase chain reaction) revealed integration of one copy of transgene at a single genetic locus with an incomplete inverted repeat.

Comparison of DNA insert and pre-insertion plant DNA (conducted using a combination of the Universal Genome Walker kit and PCR amplification) revealed rearrangements at the insertion site, including plant DNA deletion, duplication, and truncation. These rearrangements are common in transformation using recombinant plasmids that carry vector backbone sequences and have no impact on the expression of the target proteins.

Bioinformatics analysis does not suggest the presence of unknown DNA sequences in the inserted sequences.

B.4.2. In genomic sequences bordering the site of insertion/modification.

Sequence analysis supports that the sequences flanking the insertion regions (about 500 bp) correspond to plant DNA.
Bioinformatics analysis does not suggest the presence of unknown DNA sequences in the border integration sequences.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Analysis included proximates, amino acid, and fatty acid profiles, as well as several minerals and vitamins, as suggested in the OECD consensus document for food use of maize. Protein analysis included isolation of all categories of zein proteins and other classes of proteins (albumins, globulins, and glutelins).

Levels of most nutrients analyzed in the modified and unmodified control are comparable, except for amino acids and the types of proteins. No statistically significant difference in the amount of total grain protein was found between the modified and unmodified crop. This supports the hypothesis that an increase in other seed proteins compensates for the reduction in 19-kDa α-zeins. However, changes in other categories of zein proteins and other classes of proteins were observed. In particular, the phylogenetically distanced 27-Dka γ-zein was unexpectedly reduced while the much more closely related 22-kDa α-zein remained unaffected or apparently increased. Corresponding changes in other amino acid levels were also observed: increases in tryptophan and methionine (amino acids that are deficient in the 19 kDa α-zeins), as well as aspartic acid (precursor for the synthesis of essential amino acids), and decreases in proline and leucine, which are abundant in the 19-kDa α-zeins.

Changes in lysine and other amino acids are expected, based on amino acid composition of zein proteins in maize and what is known about mutant varieties of maize that are high in lysine. Increased levels of lysine and tryptophan (4.2 and 1.0% protein, respectively) are comparable to those observed in mutant corn (opaque-2) produced in the Quality Protein Maize program. The observed changes in amino acids are expected with an improved protein quality scoring pattern. This was achieved without a significant change in total protein content.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

Phytic acid and raffinose were analyzed, as suggested in the OECD consensus document for food use of maize. No differences were noted in their levels in the modified and unmodified control.

Analysis of DIMBOA was not recommended because of the high variability of its levels among maize varieties, and the fragmentary knowledge of its toxicology.

B.5.3. Other metabolites/parameters.

Analytes included furfural, ferulic acid, and p-coumaric acid, which are notable secondary metabolites in maize, as described in the OECD consensus document.

To monitor the potential for endophytic fungal infection, analysis of mycotoxins and ergot alkaloids (produced by some endophytic fungi) was conducted. No increase in mycotoxin levels was found relative to control.

The scope of analysis in B.5.1-B.5.3 was considered adequate, which included detailed analysis of different classes of proteins. Altered patterns of amino acids and proteins are expected based on knowledge of mutant maize high in lysine, although not all specific changes were predicted. Protein quality was improved as expected, with no deleterious changes in the levels of other key nutrients, toxicants, and antinutrients.

B.6. Health consequences of instability of the modified trait.

The primary purpose of the modified trait is for the nutritional improvement of animal feed. The modified maize can potentially provide nutritional benefit to population groups for whom maize is a major cereal food in their diet.

PART C - POTENTIAL FOR ALLERGENIC (IGE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN SUBSTANCES.

C.1. Allergenicity of donor organism (gene source).

Same as host plant - see factor 5 below.

Protein gene products (factors C.2-C.4).

Not applicable - no heterologous protein introduced or modified.

C.2. Potential for protein gene product(s) to cross react with known allergens.

C.3. Pepsin digestion (under standard testing conditions).

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factor (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

C.4.3. Abundance.

C.5. Allergenicity of host plant.

Maize is not a common source of food allergen, but case reports of skin, gastrointestinal and respiratory symptoms have been reported. Allergenic protein(s) identified include: Zea m 1 (beta-expansin 1b) and Zea m 14 (lipid transfer protein; pathogenesis related protein PR14).

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PRODUCT PROFILE

Modified trait	Increased carotenoids (primarily α-and β-carotene) in the mature seeds of *Brassica napus* (canola).
Intended food use of modified crop (host plant)	Only the highly refined seed oil will be used as food. The modified oil can be blended with unmodified oil in varying ratios to provide the desired level of β-carotene and other carotenoids for different food applications. It can also be a source of food colorants.
Gene (source) / Promoter (source)	Bacterial phytoene synthase (*crtB*) gene from *Erwinia uredovora* was introduced under the control of the seed-specific napin promoter from *Brassica rapa,* fused with a pea small subunit transit peptide sequence of ribulose-biphosphate carboxylase to target the gene to the plastid.
Transformation method	Agrobacterium-mediated transformation.
Metabolic reactions / pathways	Phytoene synthase is the enzyme that catalyzes the first committed step in carotenoid biosynthesis and is one of the enzymes that has been proposed to regulate flux through the pathway. Phytoene synthase catalyzes the conversion from geranlygeranyl diphosphate to phytoene, which undergoes desaturation and isomerization in the formation of lycopene. Two branch pathways of cyclization follow, which result in the formation of β-carotene (via γ-carotene) and α-carotene (via δ-carotene). α-and β-carotenes are further converted to xanthophylls, with lutein and zeaxanthin being of high interest due to their putative health benefits.
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	Phytoene synthase (CRTB).
Secondary gene products	β- and α-carotenes of the carotenoid biosynthetic pathways, as described above.

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop for food.

Phytoene synthase: Level was not quantified in the seed, as the highly abundant seed storage protein cruciferin interferes with the migration of phytoene synthase in western blot analysis. The protein was not detectable in the oil fraction.
Carotenoids: Up to 50-fold increase in carotenoids (measured by HPLC) in the seed; α-and β-carotenes are the predominant carotenoids elevated. Average μg/g fresh weight: total carotenoids - from 3 to 1600; β-carotene - from 3 to 600; α-carotene - from not detectable to 300.
Refined seed oil has twice the concentration of carotenoids found in seeds.

A.2. Prior history of known or presumed safe consumption from foods.

Phytoene synthase: Plant phytoene synthase is an endogenous enzyme present in food crops. The bacterial enzyme inserted in the modified crop has low sequence identity (27-33%) with the enzyme found in common food crops (e.g., maize, oats, sunflower). Given that the enzyme was not detected in the oil fraction, the issue of prior dietary exposure was not considered important with respect to classification using the proposed scheme.

Carotenoids: It is estimated that natural food sources provide varying levels of carotenoids (median per person per day): β-carotene 2-5 mg; α-carotene 50 μg; β-cryptoxanthin 39 μg; lutein and zeaxanthin 1.4 - 2 mg; lycopene 0.8-5 mg [IOM, 2002]. In developed countries, 70-90% of carotenoid intake comes from fruit and vegetable consumption that provides about 25-35% of the total retinol intake [van den Berg et al., 2000]. Many factors affect estimates of dietary intake of carotenoids because of natural variations among plant varieties. Fruit ripeness and the high variability in intra- and inter-individual intakes, as well as post-harvest storage, food preparation and processing, food matrix, bioavailability, and inconsistencies in food composition tables and databases can all affect intake estimates.

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: Depending on the food application, the modified oil can replace part of other edible oils with a similar fatty acid composition. The modified oil has a distinct colour characteristic of its carotene content and may limit its applications.

Phytoene synthase: Not expected to change given that there are no detectable levels in the oil fraction.

Carotenoids: Canola oil is not a major source of carotenoids in the diet. If the current food usage of canola oil is maintained and the modified oil is blended with other oils to achieve a target level of 6 mg β-carotene/g refined oil, β-carotene intake by individuals at the 90th percentile (vegetable oil consumers only) could increase by approximately 5-10 mg/day (based on 36-78% of total canola oil production in Canada for food uses in the last decade; 13 mg/day if canola oil makes up 100% of edible oils). For individuals with low intakes of fruits and vegetables, modified canola oil could be a useful source of β-carotene. For consumers with high vegetable oil intakes, this could provide up to 2-3 times the level of β-carotene (3-6 mg/day) from the recommended number of servings of fruits and vegetables per day.

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

Phytoene synthase: Not a health concern when rapidly digested. As noted, since the protein is not detected in the oil fraction, safety concern for humans would be negligible since the oil fraction is the only food form of rapeseed for human consumption.

Carotenoids: There is no evidence that high intake of carotenoids from food sources could be harmful. Concerns relate primarily to supplementation using the purified form of these compounds.

Until recently, β-carotene was considered to show no toxicity in humans. Hypercarotenaemia has not been associated with adverse effects other than reversible yellowing of the skin. Long term oral β-carotene therapy up to 300 mg/day has shown no toxic effects in individuals with erythropoietic protoporphyria. Vitamin A toxicity does not occur as the metabolic conversion is regulated by vitamin A status. There have been no reports of reproductive toxicity or teratogenicity associated with high β-carotene intake. While β-carotene is generally considered not to be genotoxic or carcinogenic based on classical toxicity studies [van den Berg et al., 2000; UK EVM, 2003], the EU Scientific Committee on Food [2000] expressed an opinion that there are no good experimental studies especially addressing the genotoxicity of β-carotene *in vivo*. Positive results obtained in a limited study demonstrating micronucleus induction *in vitro* by synthetic β-carotene should be viewed with caution, given the pro-oxidant activity of the substance.

Establishment of safe or tolerable upper level for β-carotene supplementation has been limited by the lack of dose-response data [EU SCF, 2000]. The isomeric form also appears important in the genotoxicity or antigenotoxicity of β-carotene. Given the very small difference between the levels that may confer health benefits (up to 10 mg/d, mainly from natural sources) and those that may produce adverse effects in smokers in the general population (20 mg/day) in the Alpha Tocopheral and Beta Carotene Cancer (ATBC) study, the use of β-carotene supplement should be regarded cautiously [EU SCF, 2000]. Based on the ATBC study [1994], the UK EVM [2003] recommended, using an uncertainty factor of 3 for an extrapolation from a LOAEL to a NOAEL, that supplemental intake of β-carotene not exceed 7 mg/day for non-smokers in the general population. This precautionary recommendation is given due to the

lack of understanding of the mechanism for the promotion of lung tumours. The dose at which supplemental β-carotene promotes carcinogenesis in smokers and other at-risk groups (e.g., workers exposed to asbestos) is unknown.

For the other carotenoids, limited toxicity data are available on the purified substance [Kruger et al., 2002; McClain and Bausch, 2003].

A.5. Structure-activity characterization.

Phytoene synthase: no analysis was conducted due to its absence in the oil fraction.

Amino acid sequence identity with mammalian toxins.

Pepsin digestion (under standard testing conditions).

Structural/functional comparability with comparator/source protein or non-protein substance.
Carotenoids: Carotenoids were assayed using HPLC. The structures of β- and α-carotenes were identified by comparing retention times and spectra of sample peaks to those of known standards.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
Phytoene synthase: No unusual breakdown products were expected in the seed and other parts of the modified plant. Also, no unusual protein fragments were identified by western blot analysis.

Carotenoids: Little information is available on the turnover of carotenoids in plants. Catabolic profiles of these compounds at high expression levels are not known.

Several oxidative cleavage reactions of carotenoids under normal metabolism have been reported. Scission of epoxy-carotenoids such as violaxanthin and neoxanthin initiates the synthesis of abscisic acid (a plant hormone). Other oxidative reactions which break the polyene chain include: crocetin (resulting from the cleavage between C-7/8 and C-7'/8') and bixin (resulting from the cleavage between C-5/6 and C-5'/6'). Non-enzymatic oxidation products (e.g., 1,2-epoxyphytoene and a range of hydroxyphytoenes) have also been identified in the presence of phytoene desaturase inhibitors. In chlorophyll-sensitized photo-oxidation of β-carotene the major products are β-carotene-5,6-epoxide and β-carotene-5,8-endoperoxide [van den Berg et al., 2000].

In mammalian metabolism from consumption of the gene product.
Phytoene synthase: Not a concern - no detectable level in oil.

Carotenoids: No unusual metabolites are expected from the ingestion and metabolism of carotenoids from modified oil.

Normally, a proportion of absorbed β-carotene is converted to retinol primarily within intestinal mucosal cells. Unaltered β-carotene is transported via the lymph to the plasma where it is associated with lipoproteins. Tissue uptake and distribution are not well characterized [UK EVM, 2003]. The structure of β-carotene makes it susceptible to oxidation. Several enzymatic and non-enzymatic pathways of β-carotene degradation have been identified, including those mediated by stimulated neutrophils. Many of the carotenoid breakdown products are aldehydes, carbonyls and epoxides, which are believed to be the chemical basis of the potential toxicity of these compounds, including the impairment of mitochondrial functions. A balance between the antioxidant and pro-oxidant activity of carotenoids is believed to be important in their health effects. High partial oxygen pressure and a high number of inflammatory cells appear to be risk factors for the adverse health effects of β-carotene, particularly in lung tissue [Siems et al., 2005]. It has been suggested that maintaining a complex system of interacting antioxidants is critical to their functioning and in reducing their adverse health effects.

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

Not applicable.

A.7.2. Between gene products introduced/modified and food constituents.

No interactions between endogenous food constituents and carotenoids supplied by the modified oil are expected. However, it is recognized that the bioavailability of carotenoids is dependent on the processing of the food, the presence of dietary lipids, and the isomeric form of the carotenoid. Cross-interference among carotenoids during absorption and transport also needs to be taken into account.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

Erwinia uredovora: not known to be pathogenic to humans.

Applicable if the safety of donor organism is not known, or if the donor organism is a source of known toxicant or antinutrient.

B.1.1. Characterized gene modification.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Brassica napus (canola): Rapeseed is known to contain erucic acid in its oil; acceptable level in low erucic oil is 2% of total fatty acid. Erucic acid has been associated with myocardial lesions in rats. High erucic acid rapeseed oil has been reported to cause abnormal hepatic and renal changes in experimental animals. Chlorophyll-related pigments may induce phototoxicity. Other toxic and antinutritional constituents in the meal fraction include: glucosinolates (with a standard for maximum at 30 μmoles/g); tannins, sinapine, and phytic acid.

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

The introduction of bacterial phytoene synthase has the expected effect on accumulation of carotenoid compounds. The bacterial enzyme with low homology but functional identity to a plant enzyme is believed to prevent co-suppression and to permit overexpression of the key enzyme in the endogenous pathway.

B.3.2. Impact of gene product function in the modified plant.

The target metabolic pathway and the linked terpenoid synthetic pathways are complex.

The central role of carotenoids in plant development and adaptation would suggest that their synthesis is coordinated with other developmental processes. However, the regulation of carotenoid biosynthesis at the gene and enzyme level is poorly understood [Römer and Fraser, 2005; Fraser and Bramley, 2004]. No regulatory genes involved in carotenoid formation have been isolated. It is expected that multiple control points and both transcriptional and post-transcriptional regulation are likely, given the branching nature of the pathways involving the formation of isoprenoid precursors. The carotenoid biosynthetic pathway is localized in the plastid and intricately connected to several isoprenoid forming pathways, including tocopherols and gibberellins (plant hormones). It derives its precursors from both mevalonate

and non-mevalonate pathways. Intermediate metabolites of the carotenoid biosynthetic pathway are also precursors to abscisic acid.

Applicable if gene product has an enzymatic function in the host plant.
B.3.3. Substrate specificity of gene product.

In vitro biochemical characterization suggests substrate specificity of phytoene synthase of higher plants. There is limited information on enzyme characteristic *in vivo*.

B.4. Potential for disrupting coding/regulatory DNA sequences in host plant.

B.4.1. In the inserted/modified DNA.

Southern hybridization and RT-PCR (reverse transcriptase-polymerase chain reaction) revealed integration of one copy of transgene at a single genetic locus with an incomplete inverted repeat.

Comparison of DNA insert and pre-insertion plant DNA (conducted using a combination of the Universal Genome Walker kit and PCR amplification) revealed rearrangements at the insertion site, including plant DNA deletion and duplication. These rearrangements are common in transformation using recombinant plasmids that carry vector backbone sequences and have no impact on the expression of the target protein. This phenomenon has been observed previously in *Agrobacterium* transformation.

One open reading frame (potentially active gene) coding for a complete protein was found. The sequence was identified by BLAST N analysis (which determines DNA sequence homologies in public databases) to come from *Echerishia coli* (source of the marker gene). However, transcript and protein analyses revealed no functional expression of this sequence.

B.4.2. *In genomic sequences bordering the site of insertion/modification.*

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Key nutrients and related compounds analyzed in rapeseed oil included: fatty acid profile and tocopherols and sterols, as suggested in the OECD Consensus Document for the food use of oilseed rape.

The fatty acyl composition was altered with the transgenic seeds having a higher percentage of the oleic acid component (from 58% to 66%) and a decreased percentage of the linoleic acid and linolenic acid components (from 18% to 15%, and 12% to 8%, respectively). Electronic microscopy also revealed changes in membrane structure of plastids in the modified line.

Additionally, levels of sterols (which are derived from non-plastidal isoprene units) remained essentially the same, while tocopheral levels decreased by 50% in the mature seed (principally in gamma tocopheral) compared to unmodified controls. In the transgenic seeds, tocopherols failed to rise during the developmental stages compared to the control. This is not unexpected, given that the substrate for phytoene synthase is also a precursor for tocopherols.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

The primary toxicant in rapeseed oil is erucic acid. A limit of 2% erucic acid for low erucic acid rapeseed oil has been established in the Codex Standard for Named Vegetable Oils.

Erucic acid level in the modified oil was below this limit.

B.5.3. Other metabolites/parameters.

Chlorophyllides and pheophorbides were analyzed. These are pigments that may be phototoxic. No changes compared to the levels in the unmodified control were detected.

The substrate for phytoene synthase is also a precursor for tocopherols and chlorophylls in the plastids. The effects of the modification on these compounds and related compounds associated with the isoprenoid pathway were examined. As noted in B.5.1, the levels of tocopherols in the transgenic crop failed to rise in later developmental stages compared to normal seeds. Changes in fatty acid levels were also detected, as well as major differences in cell membrane structure of the plastids.

B.6. Health consequences of instability of the modified trait.

The modified trait is intended to alter a nutritional attribute of oil derived from the modified crop. Detection of potential contamination of unmodified crop is aided by the visually distinguishable appearance of the seeds and oil.

PART C - POTENTIAL FOR ALLERGENIC (IgE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN SUBSTANCES.

C.1. Allergenicity of donor organism (gene source).

Erwinia uredovora is not a known source of allergens.

Protein gene products (factors C.2-C.4).

As noted above, due to the absence of the protein in the highly refined oil fraction, no further analysis was conducted on the enzyme.

C.2. Potential for protein gene product(s) to cross react with known allergens.

C.3. Pepsin digestion (under standard testing conditions).

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factor (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

C.4.3. Abundance.

C.5. Allergenicity of host plant.

Oilseed rape is not a common source of food allergens. Oil from rapeseed is not a known source of allergens.

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PRODUCT PROFILE

Modified trait	Introduction of lactoferrin (a human milk protein).
Intended food use of modified crop (host plant)	Rice (*Oryza sativa*) for the production of rice-based infant formula and other infant foods. Lactoferrin can also be extracted for pharmaceutical applications.
Gene (source) / Promoter (source)	Synthetic codon optimized human lactoferrin gene (rHLf) was introduced under the control of rice grain specific glutelin promoter.
Transformation method	Microprojectile bombardment.
Metabolic reactions / pathways	Lactoferrin is not expected to function in the metabolic pathway of the modified crop.
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	Recombinant human lactoferrin (rHLf). Two perspectives are relevant in evaluating lactoferrin in modified rice: 1. Concern for contamination of the modified rice in the general food supply. 2. Concern for the modified rice as a major ingredient in infant formula as a sole source of nutrition.
Secondary gene products	Not applicable - Lactoferrin is not expected to function as an enzyme in the modified plant.

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop.

Level of expression of lactoferrin in modified rice: up to 20-50% of soluble protein (0.5-1.0 % by weight of the rice grain).

A.2. Prior history of known or presumed safe consumption from foods.

Human lactoferrin is abundant in human milk with a wide range of levels (80-1375 mg/dL or 8.0-90 % protein), depending on gestational age and time post-partum.

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: The modified rice is not intended for general consumption. The following estimate was prepared to assess the potential level of exposure to lactoferrin-modified rice in the event of cross-contamination.

Consumption of lactoferrin from rice: Using rice consumption level at the 90th percentile for consumers only, up to 3 g of lactoferrin may be consumed per day (assuming 100% modified rice, which is a gross over-estimate in the event of modified rice contaminating the general rice supply) based on the upper range of expression level of lactoferrin in rice.

Level of rice in infant formula: lactoferrin modified rice will be added to infant formula at a level that provides lactoferrin at a concentration comparable to the average level in human milk (400-500 mg/dL).

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

Human lactoferrin is an 80 kDa iron-binding glycoprotein belonging to the transferrin family. Several antimicrobial activities have been reported, which are attributed to its iron-binding properties, as well as to a direct interaction with the protein or lactoferrin-derived peptides.

Several human health concerns about the pharmaceutical role of lactoferrin have been raised [Farnaud and Evans, 2003; Orsi, 2004; Weinberg, 2001]:
- promoting the growth of specific pathogens (including *Helicobacter pylori*, members of the bacterial family *Neisseriaceae* and *Trichomonas* protozoa) that are able to acquire iron from lactoferrin or neutralizing the efficacy of bactericidal agents against these microbes
- stimulating antibody production in susceptible individuals who are at risk of developing autoimmune reactions to milk proteins.

Information on the level of orally administered lactoferrin that will promote the growth of some harmful bacteria at different tissues or sites in the body is lacking. It is expected that *H. pylori* would be directly exposed to ingested rHLf. In *in vitro* susceptibility tests, no strains of *H. pylori* were sensitive to low concentrations of the protein. Inhibition occurs between 10-40 μM, depending on the strain. In another study, ingestion of 1.25 g or 6 g of rHLf over a 24-h period by 6 adult individuals with *H. pylori* infection were ineffective in clearing the infection. No adverse effects were observed.

Lactoferrin is not a documented cow's milk allergen. Although there is extensive literature documenting the presence of anti-lactoferrin autoantibodies in various autoimmune diseases, there is no evidence that these antibodies play any role in the pathology of these diseases. Other factors would need to be present in the development of a harmful autoimmune response [FDA, 2001].

A.5. Structure-activity characterization.

Amino acid sequence identity with mammalian toxins.
No amino acid sequence identity with mammalian toxins is expected.

Pepsin digestion (under standard testing conditions).
Both rHLf and human lactoferrin (HLf) are resistant to pepsin digestion with similar degradation profiles measured as DT_{90} (time until 90% decay).

Structural/functional comparability with comparator/source protein or non-protein substance.
Biochemical and physical characterization showed that rHLf is identical to human lactoferrin (HLf) in isoelectric point, iron-binding capacity, and N-terminal sequence. Both are heat stable. Functional properties were also similar with respect to antimicrobial activity against a human pathogen, and receptor binding activity assessed by a human small intestinal cell line. On the basis of SDS-PAGE, rHLf has a higher molecular weight than HLf.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
The maintenance of high expression level of rHLf in the modified rice was achieved by the use of a signal peptide and is hypothesized to be due to the deposition of the recombinant protein into protein bodies and protected from protease digestion. No unusual breakdown products of protein in the modified plant are expected.

In mammalian metabolism from consumption of the gene product.
No unusual metabolic breakdown products of ingested protein are expected.

Lactoferricin is an antimicrobial peptide derived from the amino-terminal region of lactoferrin from mammalian pepsin digestion. The extent to which this occurs in the human digestive tract is not known. In infants fed human milk, lactoferrin has been shown to be partially hydrolyzed or to pass through the infant gut intact. It is known that digestive enzymes may not function at maximum efficiency during early childhood. The resistance of lactoferrin to digestion is an important factor in the beneficial biological activity of human milk in infant nutrition.

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

Not applicable.

A.7.2. Between gene products introduced/modified and food constituents.

No interactions between rHLf and food constituents that would affect nutrient bioavailability are expected based on its known function. Given the comparability of iron binding capacity between recombinant and native human lactoferrin, no interference with iron absorption is expected from rHLf.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

Human milk is an established source of lactoferrin for infant nutrition.

Applicable if the safety of donor organism is not known or if the donor organism is a source of known toxicant or antinutrient.

B.1.1. Characterized gene modification.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Rice is a well-established food crop with no known toxicants in the form customarily consumed. The bran fraction contains antinutrients (phytic acid, trypsin inhibitor, lectins).

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

rHLf is not expected to function in the metabolic pathway of the modified crop.

B.3.2. Impact of gene product function in the modified plant.

rHLf is not expected to have any metabolic, regulatory, or signalling function in the metabolism of the modified crop.

Applicable if gene product has an enzymatic function in the modified plant.
B.3.3. Substrate specificity of gene product.

B.4. Potential for disrupting coding/regulatory DNA sequences in host plant.

B.4.1. In the inserted/modified DNA.

The HLf gene copy number in transgenic rice plants was estimated to be from 1 to 10, based on Southern blot analysis. Transgenic lines with single copy integration of the transgene were selected for development. Where rearrangement of inserted sequences with truncation was identified, bioinformatics analysis and PCR assays did not suggest the presence of unknown DNA sequences in the inserted sequences.

B.4.2. In genomic sequences bordering the site of insertion/modification.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Analysis included proximates, amylase, amino acid and fatty acid profiles, as well as selected minerals and vitamins, as suggested in the OECD consensus document for food use of rice. Levels in the modified and unmodified control are comparable.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents, where applicable).

Analytes included trypsin inhibitor, lectin, and phytic acid, as suggested in the OECD consensus document for food use of rice. Some differences were note but levels were within ranges of natural variation reported in the literature. The levels of trypsin inhibitor and lectin were below the limit of detection.

B.5.3. Other metabolites/parameters.

Not analyzed - metabolic pathways of modified plant not expected to be modified.

B.6. Health consequences of instability of the modified trait.

The modified trait is intended to introduce a component to rice for health-related purpose. Master seedbank and working seedbank are being developed to ensure consistent lot-to-lot growth of the plant and expression of the target gene products.

PART C - POTENTIAL FOR ALLERGENIC (IGE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN SUBSTANCES.

C.1. Allergenicity of donor organism (gene source).

Human milk is an established source of lactoferrin for infant nutrition - not known to be an allergenic source.

Protein gene products (factors C.2-C.4).

C.2. Potential for protein gene product(s)/ to cross react with known allergens.

The synthetic codon optimized human lactoferrin is not expected to share amino acid sequence identity with known allergens. Lactoferrin in their naturally occurring forms in cow's milk and human milk is not a known allergen. In addition, no overall sequence identity of ≥35% was found when compared with known allergens, nor were matches for ≥8 amino acids detected.

C.3. Pepsin digestion (under standard testing conditions).

As noted in A.5, both recombinant and native HLf are resistant to pepsin digestion.

C.4. Secondary factors (C.4.1-4.3). ***Applicable if information on factor (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

Compared with HLf, the higher molecular weight of rHLf is likely due to glycosylation. Further analysis identified the carbohydrate moiety to be dominant (42.5%) N-linked glycans $Man_3FucXylGlcNAc_2$.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

As noted in A.5, both recombinant and native HLfs are heat stable.

C.4.3. Abundance (applicable to storage proteins).

Level of expression of lactoferrin in modified rice: up to 20-50% of soluble protein (0.5-1.0 % by weight of the rice grain).

C.5. Allergenicity of host plant.

Rice is a less common allergenic source in population for whom rice is not a major component of the diet. Allergenic reaction (atopic dermatitis) to a 16 kD globulin fraction has been reported in Japan. Allergenic proteins (IUIS*) identified included: Ory s 1.

* International Union of Immunological Societies.

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PRODUCT PROFILE

Modified trait	Tolerance to abiotic stresses.
Intended food use of modified crop (host plant)	Rice (*Oryza sativa*), polished or brown, is used for food in various forms. In limited amounts, rice is also used to manufacture starch.
Gene (source) / Promoter (source)	A fusion gene including the coding regions of the *otsA and otsB* genes from *Escherichia coli* was introduced under the control of an abscisic acid-inducible promoter coupled with a minimal rice actin 1 promoter. The fusion gene approach was taken so that only a single transformation is required and a higher net catalytic efficiency can be achieved for trehalose formation. Separately, the two trehalose biosynthetic genes (*otsA and otsB*) encode trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphtase (TPP), respectively.
Transformation method	Agrobacterium-mediated transformation.
Metabolic reactions / pathways	Trehalose is synthesized in a two-step process: trehalose-6-phosphate is first formed from UDP-glucose and glucose-6-phosphate in a reaction catalyzed by trehalose-6-phosphate synthase (TPS). Trehalose-6-phosphate is then converted to trehalose by trehalose-6-phosphtase (TPP).
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	A bifunctional fusion enzyme trehalose-6-phosphate synthase/ phosphatase (TPSP).
Secondary gene products	Trehalose, a non-reducing disaccharide formed by a 1,1 linkage of two D-glucose molecules.

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop for food.

TPSP: Integration of the TPSP transgene was confirmed by DNA blot hybridization analysis

Trehalose: Depending on growth conditions, the transgenic rice plants accumulate trehalose at levels 3-10 times that of the non-transgenic controls, with peak levels remaining below 1 mg/g fresh weight.

A.2. Prior history of known or presumed safe consumption from foods.

TPSP: Adventitious dietary exposure to the two separate native microbial proteins (TPS and TPP) is likely. Trehalose biosynthetic enzymes are also present in yeast and other higher plants, including wheat [El-Bashati et al., 2005; Muller et al., 1995; Wingler, 2002] and other foods in which trehalose has been identified. Based on available information (A.5.3), there appears to be structural and functional similarity between the wild-type and modified enzymes.

Trehalose: Trehalose exists naturally in plants, animals and microorganisms, and has long been consumed by humans as a component of mushrooms, baker's and brewer's yeasts, seaweeds, and lobsters.

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: No change in the food use of modified rice is expected. Modified rice will replace unmodified rice in the same uses.

TPSP: Novel exposure to the fusion protein (estimated to be < 0.1% protein, dry weight basis) is not expected to be biologically significant relative to current exposure to the two native component proteins.

Trehalose: Modified rice could provide an additional 300 mg of trehalose to the diet of rice consumers only at the 90th percentile intake level in North America (assuming 100% modified rice). This expected change is considered to be of no physiological significance, when compared to the normal variations in disaccharide and carbohydrate intake and the level considered safe for consumption (see section below).

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

TPSP: Enzymes are unlikely to be toxic. Components of the fusion protein present in some foods are not known to have adverse health effects. It is unlikely that the fusion protein would create new concerns (see A. 5.3).

Trehalose: Trehalose is a nonreducing disaccharide of glucose. A published review of eight toxicity studies (in doses up to 10% of the diet) revealed no consistent treatment-related, dose dependent adverse effects of trehalose in doses up to 10% of the diet. These studies provided data supporting doses up to 50 g to be safe for human consumption. This included a consideration of the physiologic ability of humans to digest the disaccharide. Considerations were also given to individuals with carbohydrate-related metabolic disorders. Based on studies using trehalase activity assays or trehalose absorption tests, it was estimated that the proportion of individuals with low trehalase activity is well under 1% among Caucasian population [Richards et al., 2002].

A.5. Structure-activity characterization.

Amino acid sequence identity with mammalian toxins.
TPSP: No amino acid sequence identity with known mammalian protein toxins was identified with the two component enzymes.

Pepsin digestion (under standard testing conditions).
TPSP: Rapid digestion in simulated gastric fluid - no stable protein fragments observed by 10 min.

Structural/functional comparability with comparator/source protein or non-protein substance.
TPSP: Structural similarity between the fusion enzyme and its component enzymes was inferred from comparability in thermal stability and expected molecular weight based on electrophoretic mobility on SDS-PAGE. In a comparison of the enzymatic properties of TPSP with an equimolar mixture of TPS and TPP, the Km values of the fusion enzyme was lower; however the k_{cat} values were similar. This means a gain in catalytic efficiency in catalyzing sequential reactions. These data support that the functional integrity of the fusion enzyme was not only maintained, but enhanced.

Trehalose: The identity of trehalose in the plant extracts was confirmed by incubating samples with porcine-kidney derived trehalase enzyme.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
TPSP: No unusual breakdown products of protein in the modified plant are expected.

Trehalose: Although the degradation of trehalose in the modified rice has not been studied nor has the activity of trehalase been reported in rice, the enzyme trehalase is expected to be widespread in plants to prevent trehalose accumulation that can have detrimental effects on plant metabolism [El-Bashati et al., 2005; Muller et al., 1995; Wingler, 2002]. No unusual breakdown products of the disaccharide is expected.

In mammalian metabolism from consumption of the gene product.
TPSP: No concerns with ingested protein that is rapidly digested.

Trehalose: Metabolically, trehalose is hydrolyzed to glucose in a manner similar to the digestion of other disaccharides. The hydrolysis of trehalose is catalyzed by the enzyme trehalase.

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

No synergistic effects of relevance to mammalian toxicity is expected based on the known mode of action of the proteins introduced.

A.7.2. Between gene products introduced/modified and food constituents.

No interactions between TPSP and trehalose with food constituents that would affect nutrient bioavailability are expected based on their known structures and functions.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

A non-pathogenic strain (K-12) of *E. coli* was used. It was derived from a safe lineage, as demonstrated to be free from the synthesis of toxins or other unsafe metabolites under culture conditions by repeated assessment.

Applicable if the safety of donor organism is not known, or if the donor organism is a source of known toxicant or antinutrient.
B.1.1. Characterized gene modification.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Rice is a well-established food crop with no known toxicants in the form customarily consumed. The bran fraction contains antinutrients (phytic acid, trypsin inhibitor, lectins).

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

TPSP: The fusion enzyme resulted in increased level of trehalose, as expected.

Trehalose: has an osmoprotectant role in stabilizing dehydrated enzymes, proteins and lipid membranes, as well as in protecting biological structures from damage during desiccation. It is also associated with elevated capacity for photosynthesis under both stress and non-stress conditions, consistent with a suggested role in modulating sugar sensing and carbohydrate metabolism. At high concentrations, trehalose can have detrimental effects on the regulation of plant metabolism. Its precursor is also likely to be involved in the regulation of developmental and metabolic processes in plants [Wingler, 2002].

B.3.2. Impact of gene product function in the modified plant.

Both the enzyme and trehalose appear to have a role in modulating plant metabolism, as noted above.

Applicable if gene product has an enzymatic function in the host plant.
B.3.3. Substrate specificity of gene product.

Enzyme kinetic studies support that the catalytic efficiency of the fusion enzyme was enhanced compared to its component enzymes without changes in substrate specificity.

B.4. Potential for disrupting coding/regulatory DNA sequences in host plant.

B.4.1. In the inserted/modified DNA.

Transgenic lines exhibiting integration of one copy of transgene at a single genetic locus were selected. Southern hybridization and RT-PCR (reverse transcriptase-polymerase chain reaction) revealed incomplete repeat sequences.

Comparison of DNA insert before and after introduction into plant DNA (conducted using PCR amplification) revealed rearrangements at the insertion site, including DNA deletion and duplication. These rearrangements are common in transformation using recombinant plasmids that carry vector backbone sequences. Several partial insert gene fragments were judged non-functional using Northern blot analysis. No hybridization signals were observed when RNA was probed using gene-specific sequence. Bioinformatics analysis and PCR assays do not suggest the presence of open reading frames in the inserted sequences.

B.4.2. In genomic sequences bordering the site of insertion/modification.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Compositional analysis of the modified food included parameters suggested in the OECD Consensus Document for food use of rice: proximate analysis, individual amino acids, fatty acids, and selected vitamins.

Significant changes were found with several parameters compared with isogenic parental line, but were within natural variation reported in the literature.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

Amylase, and phytic acid were analyzed, in addition to trypsin inhibitor and lectin. For all the varieties tested, the levels of trypsin inhibitor and lectins were below the threshold of detection.

Significant changes were also found with several parameters compared with isogenic parental line, but were within natural variation reported in the literature.

B.5.3. Other metabolites/parameters.

Metabolic responses to abiotic stress are complex and are expected to induce changes in metabolite levels. For example, the relationship between salt stress and plant mineral content is complex and the links poorly understood. Therefore, metabolites associated with stress response were analyzed.

The sodium content of transgenic plant shoots was only 30-35% of the non-transformed plant. This could be caused by a growth dilution because of the much faster growth rate of the transgenic plants under salt stress. Alternatively, trehalose might have played a direct or indirect role in maintaining ion selectivity and, thus facilitating cellular sodium exclusion. Other changes in ion concentrations were also noted (e.g., decreased potassium and calcium in the roots of salt-stressed non-transgenic plant; increased calcium in shoot and root in salt-stressed non-transgenic).

B.6. Health consequences of instability of the modified trait.

Modified trait is not intended to affect human health.

PART C - POTENTIAL FOR ALLERGENIC (IgE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN SUBSTANCES.

C.1. Allergenicity of donor organism (gene source).

E. coli - not known to be allergenic; transfer of allergenic protein from source organism unlikely.

Protein gene products (factors C.2-C.4).

C.2. Potential for protein gene product(s) to cross react with known allergens.

No amino acid sequence identity with known allergens was identified based on the criterion of no more than 35% sequence identity over 80 amino acid segments.

C.3. Pepsin digestion (under standard testing conditions).

Rapid digestion in simulated gastric fluid - no stable protein fragments observed by 10 min.

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factors (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

C.4.3. Abundance.

C.5. Allergenicity of host plant.

Rice is a less common allergenic source in population for whom rice is not a major component of the diet. Allergenic reaction (atopic dermatitis) to a 16 kD globulin fraction has been reported in Japan. Allergenic proteins (IUIS*) identified included: Ory s 1.

* International Union of Immunological Societies.

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PRODUCT PROFILE

Modified trait	Enhanced lysine level in rice for human nutrition.
Intended food use of modified crop (host plant)	Rice (*Oryza sativa*), polished or brown, is used for food in various forms. Relatively little rice is consumed as prepared products although a variety is available in the market, in particular in Asia. In limited amounts, rice is also used to manufacture starch.
Gene (source) / Promoter (source)	Site-directed mutagenesis of maize *dhps* cDNA was applied, using synthetic oligonucleotide, to achieve a point mutation of C to T at nucleotide 536 in order to substitute Ala to Val at amino acid residue 166. The mutant cDNA was sequenced and the designed point mutation confirmed. The *dhps* gene was ligated to a CaMV 35S promoter for constitutive expression. Also included was a maize chloroplast transit peptide, which would direct the Dihydrodipicolinate synthase (DHPS) to the organelle where the aspartate family pathway is located.
Transformation method	Agrobacterium-mediated transformation.
Metabolic reactions / pathways	The modification aims to enhance lysine level in rice by introducing a modified gene which encodes an enzyme in the biosynthesis of lysine that is lysine-feedback-insensitive. Dihydrodipicolinate synthase (DHPS) catalyzes the first reaction in the branched biosynthetic pathway from aspartate to lysine. DHPS with reduced sensitivity to lysine inhibition allows increased accumulation of the amino acid.
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	Modified DHPS.
Secondary gene products	Increased level of lysine.

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop for food.

DHPS: DHPS activity in the leaves and developing seed was about 2.7-4.2- fold higher than in the wild-type plant. Expression level was not quantified.

Lysine: A 2.5-fold increase in free lysine in the leaves, immature and mature seeds (5.0-7.2% protein in modified crop compared to 1.8% protein in wild-type).

A.2. Prior history of known or presumed safe consumption from foods.

DHPS: Based on available information (A.5.3), the modified DHPS appears to be comparable to the wild-type protein in terms of functionality, except for the intended change. Prior exposure to other lysine-insensitive DHPS (e.g. in *E. coli*) is also likely [Shaver et al., 1996].

Lysine: Lysine is a common constituent in protein-containing foods. Lysine in major cereal grains range from 0.3-0.5% of the grain (2-4% of total protein).

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: No major changes in food use of the modified crop are expected. Modified rice will replace unmodified rice in the same uses.

DHPS: enzymes are generally expressed at very low level (< 0.01% total protein) in plants. No dietary change of biological significance is expected relative to baseline exposure.

Lysine: No changes in rice consumption are expected. Assuming rice is 100% modified (which is highly over-estimated), based on 90th percentile rice intake for consumers only in North America, the increase in lysine in modified rice is expected to increase lysine intake in the range of 0.7 - 1.2 g /day (0.02 - 0.04% of the diet), which represents less than 2% of average protein intake.

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

DHPS: The wild-type DHPS is a constituent of maize with a function in lysine metabolism in the plant. As a general rule, a structural change in a nontoxic enzymatic protein which improves desired enzymatic function is unlikely to result in a toxic protein [Pariza and Foster, 1983; Pariza and Johnson, 2001].

Lysine: The level of increase in lysine in the diet is expected to be within normal variation in amino acids from a variety of protein-containing foods with no nutritional or toxicological concerns.

A.5. Structure-activity characterization.

Amino acid sequence identity with mammalian toxins.
DHPS: No amino acid sequence identity with known mammalian toxins was expected or identified.

Pepsin digestion (under standard testing conditions).
DHPS: The protein was rapidly and completely digested in less than 1 min.

Structural/functional comparability with comparator/source protein or non-protein substance.
DHPS: Initial comparisons of modified with wild-type DHPS indicated that the two proteins did not differ significantly in their substrate affinities, except for lysine.

Lysine: Lysine synthesized in the modified crop was structurally identical to that widely consumed in foods.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
No unusual breakdown products of protein or lysine in the modified plant are expected.

In mammalian metabolism from consumption of the gene product.
No concerns with ingested protein that is rapidly digested.

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

Not applicable.

A.7.2. Between gene products introduced/modified and food constituents.

Based on their known function and expressed levels, no interactions are expected between modified DHPS and altered levels of lysine and food constituents that would affect nutrient bioavailability.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

Maize has a long history of consumption as food and does not contain significant toxicants or antinutritional factors.

Low levels of trypsin and chymotrypsin inhibitors are not considered nutritionally significant. Highly variable levels (0-1000 ppm) of DIMBOA (2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one) have been reported in green- and root-tissues of maize seedlings, with its glycoside form being the most prominent. The deglycosylated form, DIMBOA is toxic to insects; data on effects on humans and animals are limited. Other metabolites include:
a) Furfural: moderate acute toxicity and oral genotoxic carcinogen of low potency; partly removed by heating.
b) Ferulic acid and p-coumaric acid: natural pesticide against insects and fungi. As phenolic acids and weak anti-oxidants, their effects (positive and negative) on mutagenic substances have been reported. No adverse health effects have been associated with the consumption of maize in animals or humans.

Applicable if the safety of donor organism is not known, or if the donor organism is a source of known toxicant or antinutrient.
B.1.1. Characterized gene modification.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Rice has a long history of consumption as food. The form customarily consumed does not contain significant toxicants. The bran fraction contains antinutritional factors.

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

The increase in lysine level in the modified rice indicated that the mutant DHPS functions as expected with reduced sensitivity to lysine inhibition.

B.3.2. Impact of gene product function in the modified plant.

DHPS catalyzes the first reaction in the branched biosynthetic pathway from aspartate to lysine.

Applicable if gene product has an enzymatic function in the host plant.
B.3.3. Substrate specificity of gene product.

The point mutation in the DHPS enzyme had the expected effect of reducing its sensitivity to lysine inhibition.

B.4. Potential for disrupting coding/regulatory DNA sequences in host plant.

B.4.1. In the inserted/modified DNA.

Southern blot analysis indicated that one copy of the transgene was inserted at one site in the rice genome with the expected molecular size.

4.2. In genomic sequences bordering the site of insertion/modification.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Analysis included proximates, amylase, amino acid and fatty acid profiles, as well as selected minerals and vitamins, as suggested in the OECD consensus document for food use of rice. Except for the expected increase in lysine, changes in other aspartate-derived amino acids were judged to be within normal variation.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

A number of antinutrients including trypsin inhibitor, alpha-amylase subtilisin inhibitor, lectins, and phytin are typically concentrated in the bran fraction. Their levels are below detection in the transgenic rice.

B.5.3. Other metabolites/parameters.

No other metabolites were analyzed. Except for the expected change in lysine levels, no changes in other nutrients or known toxicants were observed. This suggests low level of concern about unintended effects.

B.6. Health consequences of instability of the modified trait.

The modified trait is intended to affect a nutritional attribute of rice for human consumption. Methods are being developed to facilitate rapid field testing of lysine. Master seedbank and working seedbank are also being developed to ensure consistent lot-to-lot growth of the plant and expression of the target gene products.

PART C - POTENTIAL FOR ALLERGENIC (IGE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN SUBSTANCES.

C.1. Allergenicity of donor organism (gene source).

Maize is not a common source of food allergen, but case reports of skin, gastrointestinal and respiratory symptoms have been reported. Allergenic protein(s) identified include: Zea m 1 (beta-expansin 1b) and Zea m 14 (lipid transfer protein; pathogenesis related protein PR14).

Protein gene products (factors C.2-C.4).

C.2. Potential for protein gene product(s) to cross react with known allergens.

No matches of greater than eight consecutive amino acids were identified, nor were overall sequence identities of > 35% found.

C.3. Pepsin digestion (under standard testing conditions).

The protein was rapidly and completely digested in less than 1 min, as noted in A.5.

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factors (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

C.4.3. Abundance.

C.5. Allergenicity of host plant.

Rice is a less common allergenic source in population for whom rice is not a major component of the diet. Allergenic reaction (atopic dermatitis) to a 16 kD globulin fraction has been reported in Japan. Allergenic proteins (IUIS*) identified included: Ory s 1.

* International Union of Immunological Societies.

References.

Lee SL (2001) Constitutive and seed-specific expression of a maize lysine-feedback-insensitive dihydrodipicolinate synthase gene leads to increased free lysine levels in rice seeds. Molecular Breeding 8, 75-84.

Pariza MW and Foster EM (1983) Determining the safety of enzymes used in food processing. Journal of Food Protection 46(5), 453-468.

Pariza MW and Johnson EA (2001) Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regulatory Toxicology and Pharmacology 33, 173-186.

Shaver JM (1996) Single amino acid substitutions eliminate lysine inhibition of maize dihydrodipicolinate synthase. Proceedings of the National Academy of Sciences U.S.A. 93, 1962-1966.

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PRODUCT PROFILE

Modified trait	Change in fatty acid profile - increase in oleic acid level.
Intended food use of modified crop (host plant)	Soybean (*Glycine max L.*) is primarily used for the production of oils (about 94% of soy food ingredients), protein fractions, and dietary fibre. Isolated soy protein is used in the production of infant formula and other food products as a source of amino acids. Soy-based foods are estimated to make up between 0.8% to 1.5% of a child's (age 1-6) and an infant's diet, respectively. For infants, about 80% of the soy-based food is expected to come from oil, and 20% from soy flour (flake) products. The modified oil has improved functional properties compared to conventional soybean oil or partially hydrogenated soybean oil and will fit into food applications where a highly stable oil is required, such as in fat frying operations. Oil derived from the modified soybean can replace other high oleic acid oils such as olive or canola oil, or high polyunsaturated oils such as corn oil.
Gene (source) / Promoter (source)	A copy of the a soybean fatty acid desaturase gene (GmFad2-1) was introduced which was under the control of a seed-specific promoter from soybean.
Transformation method	Biolistic transformation.
Metabolic reactions / pathways	The introduction of a second copy of the GmFad2-1 gene suppressed the expression of both copies of the gene. This target gene encodes an enzyme (FAD) that converts oleic acid to linoleic acid. Reduced conversion of oleic acid to linoleic acid (18:1 to 18:2) results in accumulation of oleic acid. The conversion from oleic to linoleic acid is the first step in the biosynthesis of polysaturated fatty acids in plants.
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	Silenced expression of fatty acid δ-12 desaturase (FAD) - NOT assessed for intended effects; potential for unintended changes related to toxic/antinutritional and allergenic effects remains relevant.
Secondary gene products	The following assessment pertains to the potential for altered intakes of oleic and linoleic acids, and for unintended effects of suppressed expression of the FAD enzyme.

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop.

Suppressed expression of the target gene was confirmed using Northern blot analysis - no detectable level of GmFad2-a mRNA. Expression of high levels of oleic acid (80% in the oil from modified soybean, compared to 23% in unmodified counterpart) confirmed the intended suppression of the target gene.

A.2. Prior history of known or presumed safe consumption from foods.

There is an extensive history of dietary exposure to oleic and linoleic acids which are endogenous components of soybean and other foods.

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: Modified soybean oil is expected to replace, in part, other oils high in oleic acid. Because of its improved stability, it can also replace other oils in certain application for functional purposes.

High oleic acid soybean oil contains 10% saturated fats, >80% oleic acid, approximately 2% linoleic acid and 3.5% linolenic acid. This compares with 9-17% saturated fatty acid, 19-34% oleic acid, 48-60% linoleic acid, and 2-10% linolenic acid in unmodified soybean oil.

Dietary assessment using data for British adults determined that the use of high oleic soybean oil may lower the intake of dietary linoleic acid (by an absolute maximum of 29%), increase the intake of oleic acid (by 19% at best), and result in a small reduction (5%) in the intake of saturated fats. These changes would generally be applicable to most other Western countries. It was concluded that the change in the ratios of these fatty acids is unlikely to have an impact on public health in terms of cardiovascular disease.

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

The fatty acids which are altered in levels in the modified soybean are not known to be toxic at variable levels normally present in the food supply and consumed.

A.5. Structure-activity characterization. (Factors 5.1-5.2 applicable to protein substances only).

Amino acid sequence identity with mammalian toxins.
Not applicable.

Pepsin digestion (under standard testing conditions).
Not applicable.

Structural/functional comparability with comparator/source protein or non-protein substance.
Fatty acids: structurally identical to oleic and linoleic acids widely consumed in current food supply.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
Biosynthetic pathway of fatty acids is well-established in the host plant. No unusual breakdown products in the modified plant are expected.

In mammalian metabolism from consumption of the gene product.
No unusual metabolic breakdown products are expected from ingestion of the fatty acids.

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

Not applicable.

A.7.2. Between gene products introduced/modified and food constituents.

None expected based on known metabolism of fatty acids consumed at typical dietary levels.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

Donor organism and host plant are the same - see B.2 below.

Applicable if the safety of donor organism is not known or if the donor organism is a source of known toxicant or antinutrient.

B.1.1. Characterized gene modification.

The complete sequence of the DNA insert used in the genetic modification was characterized. There is no evidence of other unidentified DNA sequence being transferred to the host plant.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Soybean belongs to an extremely large legume family, Fabaceae, which encompasses numerous genera that are toxic. Substances of relevance in soybean include:
Antinutrients: trypsin inhibitors, lectins – both subject to degradation upon heating; stachyose and raffinose, and phytic acid. Non-ruminants are particularly susceptible to the adverse effects of trypsin inhibitors in raw soybeans. Other antinutrients, with the exception of phytic acid, do not appear to be of practical significance in animal nutrition where soybeans and soybean meals are a major protein source [Cheeke, 1998].
Bioactive substances: phytoestrogens (isoflavones).

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

The function of endogenous FAD in the host plant is well established. It catalyzes the conversion of oleic acid to linoleic acid.

B.3.2. Impact of gene product function in the modified plant.

FAD has an enzymatic function in the conversion of oleic acid to linoleic acid. This reaction is the first step in the biosynthesis of polysaturated fatty acids in plants and is part of a complex pathway in primary metabolism.

Applicable if gene product has an enzymatic function in the modified plant.

B.3.3. Substrate specificity of gene product.

B.4. Potential for disrupting coding/regulatory DNA sequences in the modified plant.

B.4.1. In the inserted/modified DNA.

Southern blot analysis identified at one locus, 2 intact copies of the GmFad2-1 expression cassette. Gene fragments (possibly from the marker gene for selecting bacteria transformed with recombinant plasmid DNAs) were identified on another locus, but were found to be non-functional using Northern, Western, and amino acid analyses .

B.4.2. In genomic sequences bordering the site of insertion/modification.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Compositional analysis of the modified food included parameters suggested in the OECD Consensus Document for food use of soybean: proximate analysis, individual amino acids, fatty acids, and vitamins, including tocopherols.

No significant changes in the content of total protein, minerals, and vitamins, including tocopheral were identified. Changes were noted in the levels of accumulation of two important seed storage proteins, glycinin (increased) and beta-conglycinin (decreased). The profiles of other storage proteins were similar to those of the parental lines. Comparable variations in the levels of seed storage proteins were also noted in some commercial soybean lines. Trace amounts (0.5%) of a linoleic acid 9,15 isomer were detected. Although absent in unmodified soybean oil, this isomer can be found in other fats and oils. Its level in the oil fraction of modified soybean was comparable to that in butterfat, and considerably lower than that in partially hydrogenated vegetable oils (1-3%).

B.5.2. Known toxicants/antinutrients *(as suggested for food use in applicable OECD consensus documents).*

Analytes included phytic acid, trypsin inhibitor, and lectins, in addition to stachyose and raffinose.

Levels of glycitein and lectin were elevated. These changes were within the range of variation in some commercial soybean lines. No changes in the levels of other antinutritional factors were found.

B.5.3. Other metabolites/parameters.

Isoflavone content was analyzed (total daidzein, total genistein, total glycitein). There were no significant differences between the parental and modified soybean in total daidzein or genistein content, which was also within the literature reported ranges for soybeans. Slightly elevated total glycitein level was detected in the modified line but it was within the literature reported range.

Changes in the levels of two storage proteins noted in B.5.1 prompted a detailed assessment of the potential for the formation of new proteins. The absence of new proteins was confirmed by detailed Northern blots and protein analysis (immunoblots). This, in conjunction with the lack of phenotypic alterations in the modified plant, would suggest that the scope of metabolites analyzed should be sufficient to identify any unintended changes with potential health relevance.

B.6. Health consequences of instability of the modified trait.

The modified trait is intended to alter a nutritional attribute.

PART C - POTENTIAL FOR ALLERGENIC (IgE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN SUBSTANCES.

C.1. Allergenicity of donor organism (gene source).

Donor organism and host plant are the same – see B.5 below.

Protein gene products (factors C.2-C.4).

NOT applicable as no new protein was expressed.

C.2. Potential for protein gene product(s) to cross react with known allergens.

C.3. Pepsin digestion (under standard testing conditions).

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factor (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C4.1. Structural/functional characteristics.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

C.4.3. Abundance.

C.5. Allergenicity of host plant.

Soybean is a commonly allergenic food, containing several antigenic proteins. Several gastrointestinal reactions have been attributed to the 7S and 11S globulins (α- and α'-subunits of β-conglycinin and A and B subunits of glycinin, respectively) in soybean proteins that comprise about 85% of total protein. As note d in B.5.1, changes in the levels of these protein fractions were noted.

References.

Cheeke PR (1998) Natural Toxicants in Feeds, Forages, and Poisonous Plants. Danville, IL.: Interstate Publishers, Inc.

PRODUCT PROFILE

Modified trait	Tolerance to herbicide (imidzolinone).
Intended food use of modified crop (host plant)	Rice (*Oryza sativa*), polished or brown, is used for food in various forms. In limited amounts, rice is also used to manufacture starch.
Gene (source) / Promoter (source)	Host plant and donor organism are the same. Promoter: Not applicable.
Transformation method	Chemically induced seed mutagenesis using ethyl methanesulfonate (EMS) and traditional cross-breeding with commercial varieties.
Metabolic reactions / pathways	A single point mutation within the gene encoding acetolactate synthase (ALS) resulted in an enzyme that is insensitive to the herbicide but maintains its catalytic function. ALS enzyme catalyzes the first reaction in the biosynthesis of branched-chain amino acids: the conversion of pyruvate to *acetolactate* in the synthesis of leucine, and the condensation of alpha-keto-butyrate and pyruvate to *acetohydroxybutyrate* in the synthesis of isoleucine. This mutation restores the production of essential amino acids and protein in the plant.
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	Modified acetolactate synthase (ALS), also known as acetohydroxy acid synthase (AHAS).
Secondary gene products	*Acetolactate, acetohydroxybutyrate and related metabolites*: NOT assessed - they are endogenous metabolites in the modified plant. No safety concern is expected from restoring amino acid biosynthesis in the modified crop that exhibits normal phenotype.

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop.

The level of expression of ALS within plant tissue is generally low and ~0.001% in rice seed. This level of expression was not altered in the modified crop.

A.2. Prior history of known or presumed safe consumption from foods.

There is a substantial history of consumption of rice, including endogenous constituents such as unmodified ALS. The modified enzyme restores biosynthesis of branched-chain amino acids in the modified crop – unlikely to induce new safety concerns.

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: No major changes in food use of the modified crop are expected. Modified rice will replace unmodified rice in the same uses.

Modified ALS: Expression level of the modified ALS is comparable to typical level in rice seed (~0.001% of total protein). No change in total dietary exposure is expected.

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

Plant enzymatic protein of this type is not known to be toxic. Limited and well-characterized modification of the enzyme that restores branched-chain amino acid biosynthesis in the modified plant is unlikely to induce new safety concerns. Enzymes studies comparing the mutant and wild-type enzymes from rice revealed no significant change in enzyme function in the mutant enzyme, other than the selected insensitivity to herbicide toxicity.

A.5. Structure-activity characterization.

Amino acid sequence identity with mammalian toxins.
Unmodified form of the enzyme has no amino acid sequence identity with known protein toxins.

Pepsin digestion (under standard testing conditions).
Modified ALS: Completely degraded within 5 minutes.

Structural/functional comparability with comparator/source protein or non-protein substance.
Modified ALS: Had expected molecular weight based on assessment of electrophoretic mobility on SDS-PAGE. It was also rapidly inactivated by heat (1 minute at 100°C).

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
No unusual breakdown products of protein in the modified plant are expected - no unusual protein fragments were identified by Western blot analysis.

In mammalian metabolism from consumption of the gene product.
No unusual breakdown products are expected from ingested protein that is rapidly digested.

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

Not applicable.

A.7.2. Between gene products introduced/modified and food constituents.

None expected base on the digestibility of the modified ALS enzyme.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

Same as host plant - see B.2 below.

Applicable if the safety of donor organism is not known or if the donor organism is a source of known toxicant or antinutrient.

B.1.1. Characterized gene modification.

B.1.2. Poorly characterized gene modification.

Not applicable - see B.2.

B.2. Safety of host plant.

Rice is a well-established food crop with no known toxicants in the form customarily consumed. The bran fraction contains antinutrients (phytic acid, trypsin inhibitor, lectins).

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

3.1. Knowledge of gene product function.

ALS has well-established function in the plant. The modified ALS restores branched-chain amino acid biosynthesis as expected.

B.3.2. Impact of gene product function in the modified plant.

ALS is an enzymatic protein.

Applicable if gene product has an enzymatic function in the modified plant.

B.3.3. Substrate specificity of gene product.

Modified ALS restores branched-chain amino acid biosynthesis as expected. Enzyme studies of the mutant and wild-type enzymes from rice revealed no significant change in enzyme function in the mutant enzyme, other than the selected insensitivity to herbicide toxicity.

B.4. Potential for disrupting coding/regulatory DNA sequences in the modified plant.

B.4.1. In the inserted/modified DNA.

EMS as a chemical mutagenic agent is known to induce chromosome aberrations and high frequencies of base-pair substitutions, almost exclusively of the transition type at the G-C site [van Harten, 1998]. The likelihood is considered very small for the disrupted or mutated sequence to cause altered qualities to the protein with health risk implications. This is based on the knowledge that base exchange is a natural process that frequently occurs in natural situations with unchanged or reduced functions rather than enhanced or new functions.

B.4.2. In genomic sequences bordering the site of insertion/modification.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Compositional analysis of the modified food included parameters suggested in the OECD Consensus Document for food use of rice: proximate analysis, amino acids (including valine, leucine or isoleucine), fatty acids. No differences between the herbicide-tolerant lines and conventional commercial lines were detected.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

Amylase and phytic acid were analyzed, in addition to trypsin inhibitor and lectin. For all the varieties tested, the levels of trypsin inhibitor and lectins were below the threshold of detection. No differences between the herbicide-tolerant lines and conventional commercial lines were detected.

B.5.3. Other metabolites/parameters.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and high pressure liquid chromatography (HPLC) of protein extracts from modified and unmodified rice varieties did not indicate the expression of new proteins, or altered expression of existing proteins. This, together with findings in the analysis of amino acids and other metabolites in B.5.1. and B.5.2, would support that protein metabolism was not affected by the chemical mutagen and that no other metabolic changes of human health significance are expected.

B.6. Health consequences of instability of the modified trait.

Modified trait is not intended to affect human health.

PART C - POTENTIAL FOR ALLERGENIC (IGE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN SUBSTANCES.

C.1. Allergenicity of donor organism (gene source).

Donor organism and host plant are the same. See C.5 below.

Protein gene products (factors C.2-C.4).

C.2. Potential for protein gene product(s) to cross react with known allergens.

Unmodified form of the enzyme has no amino acid sequence identity with known protein allergens. No amino acid sequence identity of > 35% was identified over sequential 80 amino acid segments.

C.3. Pepsin digestion (under standard testing conditions).

As noted in A.5, modified ALS was completely degraded within 5 minutes under standard testing conditions.

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factor (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

Based on enzyme studies comparing modified and unmodified enzymes, the single amino acid change within the native ALS enzyme resulted in only an expected change in its function. This change is not anticipated to have an effect on the potential allergenicity of this protein, when viewed in the context of other data related to its rapid digestion and susceptibility to heat treatment - see C.4.2.

C.4.2. Heat stability (NOT applicable to lipid transfer protein).

As noted in A.5, modified ALS was not heat stable.

C.4.3. Abundance.

The enzymatic protein is expressed at very low level in rice seed (~0.001%), as explained in A.1.

C.5. Allergenicity of host plant.

Rice is a less common allergenic source in a population for whom rice is not a major component of the diet. Allergenic reaction (atopic dermatitis) is associated with a 16 kDa protein, which is a member of the alpha-amylase/trypsin inhibitor family of proteins. Other allergenic proteins in the 14-15 kDa range, present in the albumin plus globulin protein fraction of the rice endosperm, have also been reported.

References.

van Harten, AM (1998). Mutation Breeding. Theory and Practical Applications. Cambridge, UK: Cambridge University Press.

PRODUCT PROFILE

Modified trait	Lysine enrichment for nutritional enhancement of feed.
Intended food use of modified crop (host plant)	Little whole kernel or processed maize or field corn (*Zea mays*) is consumed by humans worldwide when compared to maize-based food ingredients. Maize-based ingredients have wide applications in food and include starch, sweeteners, flaking grits, flour, and oils from wet or dry milling processing.
Gene (source) / Promoter (source)	A mutant gene from barley (*Hordeum vulgare L.*) encoding chymotrypsin inhibitor-2 (CI-2) was engineered with multiple lysine substitutions and introduced under the control of the endosperm-specific barley-derived hordein promoter. Lysine substitutions were engineered at the surface residues and residue positions where a number of different side chains were accepted and where it would be more likely to tolerate substitutions. The modification was based on the three-dimensional structure of the protein, calculated solvent accessible surface areas of the side chains and homologous sequence data.
Transformation method	Microprojectile bombardment.
Metabolic reactions / pathways	The introduction of a gene that encodes for a lysine-enhanced protein under the control of an endosperm-specific storage protein promoter is expected to increase lysine content in the target tissue of the modified crop.
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	CI-2 protein.
Secondary gene products	The intended change in lysine content in the modified crop will be assessed, although it is not a product of the mutant gene.

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop for food.

CI-2 protein: CI protein was expressed at 10% of total seed protein.
The introduction of the CI protein resulted in an increase in total protein in the maize grain to 14% from 9%.

Lysine: Lysine content doubled to 0.50% from 0.25% of the grain. However, the level of lysine as % total protein only increased slightly at about 3%, comparable to lysine content in wild type maize.

A.2. Prior history of known or presumed safe consumption from foods.

CI-2 protein: The protein introduced in the host plant appeared to be structurally comparable to the wild-type protein derived from barley based on the parameters analyzed (see A.5).

Lysine: Lysine is a common constituent in protein-containing foods. Lysine in major cereal grains range from 0.3-0.5% of the grain (2-4% of total protein).

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: No changes in the consumption of the modified maize are expected. It will replace unmodified maize in the same uses.

CI-2 protein: Quantitative data are limited on the concentration of the native CI-2 protein in barley and of similar proteins in other cereal grains and food sources, but their levels are expected to be much lower than the level of the modified protein expressed in maize. Depending on the level of consumption of maize, CI protein can contribute between 0.5 - 5 g protein to the diet (0.02-0.2% of diet). This represents 1 - 10% of typical protein intake for adults, assuming 100% lysine-enhanced maize, which is a gross over-estimate.

Lysine: The following assessment assumes that the modified CI-2 protein is bioavailable upon digestion.

No physiologically significant change in lysine or protein intake in the total diet is expected, given that the lysine and protein content of the modified grain is comparable to that in other cereal grains. In the context of normal variation in food consumption, changes in the levels of lysine and protein as a result of consuming the modified maize would be negligible among individuals consuming a diet consisting of mixed protein sources, of which maize is not a major cereal source. The potential may exist for enhanced dietary protein quality for individuals consuming a maize-based diet. No undesirable effects are expected at the projected increased lysine intake, which is within the normal range of variation in amino acid content in the diet.

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

The native protein is a food constituent with a history of consumption from food. It is believed to play a significant role in plant defense against insects and other pests (e.g., nematodes) by inhibiting the activity of digestive enzymes insect pests. While the protein has protease inhibitory activity, it does not appear to be responsible for any serious anti-nutritional activity in humans, probably because it tends to be heat labile [OECD, 2004].

The safety of the extensively modified CI-2 protein has not been studied in detail. Preliminary data are summarized below.

A.5. Structure-activity characterization.

The following information pertains to CI-2 protein.

Amino acid sequence identity with mammalian toxins.
No amino acid identity with known mammalian toxins was identified.

Pepsin digestion (under standard testing conditions).
The protein was partially digested at the end of 30 min, and fragments were visible up to 60 min.

Structural/functional comparability with comparator/source protein or non-protein substance.
Analysis of the physico-chemical properties (N-terminal amino acid sequencing, MALDI mass spectrometry, SDS-PAGE) of the engineered protein was conducted, as well as *in vitro* measurement of its thermodynamic and conformational stability (circular dichroism analysis, intrinsic fluorescence, and fluorescence quenching).

Sequences and molecular mass of the CI protein were as predicted. The modified protein also appears to have a native-like conformation and has thermodynamic stability comparable with many naturally occurring globular proteins, but less than that of the wild-type CI-2.

Functional comparability with the wild-type protein was evaluated using three mammalian digestive proteases and one bacterial protease with broad substrate specificity. Unlike the wild-type CI-2 which is an effective inhibitor of several proteases, the modified protein possessed detectable, but considerably reduced, inhibitory activity against chymotrypsin and subtilisin. Also unlike wild-type CI-2 which is completely resistant to trypsin digestion, the modified protein is only partially resistant to the protease.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
No unusual breakdown products of the protein in the modified plant are expected - no unusual protein fragments were identified by Western blot analysis.
The relative proteolytic stability of the modified CI-2 suggests that it can remain stable at high levels in the modified plant.

In mammalian metabolism from consumption of the gene product.
No unusual protein breakdown products are expected. However, given the protein's resistance to digestion, it may be necessary to examine the proteolytic profiles of the protein fragments.

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

Not applicable.

A.7.2. Between gene products introduced/modified and food constituents.

Given its resistance to digestion, the bioavailability of the protein and its potential interaction with the absorption of other food constituents should be addressed.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

Barley has a long history as a food source. While several proteases and amylase inhibitors, as well as lectins are present in barley, their physiological significance to humans is unknown [OECD, 2004]. However, barley in its processed form that is normally consumed is not known to be associated with human toxicity.

Applicable if the safety of donor organism is not known, or if the donor organism is a source of known toxicant or antinutrient.

B.1.1. Characterized gene modification.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Maize has a long history of consumption as food and does not contain significant toxicants or antinutritional factors (see also B.5.2).

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

As noted previously, the inhibitory activity of the engineered CI-2 protein in the modified plant against chymotrypsin and subtilisin was considerably reduced, compared to the wild-type protein, which is believed to have a defense function in the source plant.

B.3.2. Impact of gene product function in the modified plant.

The engineered CI-2 protein is not expected to affect regulatory, metabolic, or signalling pathways in the modified maize.

Applicable if gene product has an enzymatic function in the host plant.
B.3.3. Substrate specificity of gene product.

B.4. Potential for disrupting coding/regulatory DNA sequences in host plant.

B.4.1. In the inserted/modified DNA.

Southern hybridization and RT-PCR (reverse transcriptase-polymerase chain reaction) revealed integration of one copy of transgene at a single genetic locus with an incomplete inverted repeat.

Comparison of DNA insert and pre-insertion plant DNA (conducted using a combination of the Universal Genome Walker kit and PCR amplification) revealed rearrangements at the insertion site, including plant DNA deletion, duplication, and truncation. These rearrangements are common in transformation using recombinant plasmids that carry vector backbone sequences and have no impact on the expression of the target proteins. Bioinformatics analysis does not suggest the presence of unknown DNA sequences in the inserted sequences.

B.4.2. In genomic sequences bordering the site of insertion/modification.

Sequence analysis supports that the sequences flanking the insertion regions (about 500 bp) correspond to plant DNA.
Bioinformatics analysis does not suggest the presence of unknown DNA sequences in the border integration sequences.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Analysis included proximates, amino acid, and fatty acid profiles, as well as several minerals and vitamins, as suggested in the OECD consensus document for food use of maize. Levels of most nutrients analyzed in the modified and unmodified control are comparable, except for amino acids. Changes in amino acid content are expected, given the nature of the modification. These changes are not considered to be of nutritional significance, given the natural variation in amino acid content in cereal grains and other protein-containing foods.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

Trypsin and chymotrypsin inhibitors, as well as phytic acid and raffinose were analyzed, as suggested in the OECD consensus document for food use of maize. No differences were noted in their levels in the modified and unmodified control.

Analysis of DIMBOA was not recommended because of the high variability of its levels among maize varieties, and the fragmentary knowledge of its toxicology.

B.5.3. Other metabolites/parameters.

Analytes included furfural, ferulic acid, and p-coumaric acid, which are notable secondary metabolites in maize, as described in the OECD consensus document.

The protein is not expected to function in metabolic pathways in the modified plant. In concert with observable phenotype and function, analysis of key nutrients synthesized in primary metabolic pathways (B.5.1), known toxicants/antinutrients (B.5.2), and notable metabolites for the crop (B.5.3) arising from secondary metabolism, would be considered to be sufficient in scope to provide a reasonably sensitive indication of unintended effects of direct health relevance.

B.6. Health consequences of instability of the modified trait.

The modified trait is primarily for feed use - not intended to affect human health.

PART C - POTENTIAL FOR ALLERGENIC (IGE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN SUBSTANCES.

C.1. Allergenicity of donor organism (gene source).

Barely is a source of hordeins that are associated with gluten-sensitive enteropathy. It is also a source of a minimum of 4 allergenic proteins (BMAI-1, alpha-amylase, beta-amylase, gamma-3 hordein).

Protein gene products (factors.C.2-C.4).

C.2. Potential for protein gene product(s) to cross react with known allergens.

No matches of greater than eight consecutive amino acids were identified, nor were overall sequence identities of > 35% found.

C.3. Pepsin digestion (under standard testing conditions).

As noted in A.5, the protein was partially digested at the end of 30 min, and fragments were visible up to 60 min.

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factor (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

The native CI-2 protein is not classified as part of the protein families associated with allergenicity. No glycans were detected in glycoprotein assay.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

After heat treatment at 90° C for 30 minutes, the stability characteristic of the engineered protein is comparable to that of the wild-type protein in that they are both inactivated with respect to their inhibitory activity on proteases. The wild-type protein is known to be heat labile.

C.4.3. Abundance.

CI-2 protein was expressed at 10% of total seed protein.

C.5. Allergenicity of host plant.

Maize is not a common source of food allergen, but case reports of skin, gastrointestinal and respiratory symptoms have been reported. Allergenic protein(s) identified include: Zea m 1 (beta-expansin 1b) and Zea m 14 (lipid transfer protein; pathogenesis related protein PR14).

References.

OECD (2004) Consensus document on compositional considerations for new varieties of barley (*Hordeum vulgare L.*): key food and feed nutrients and antinutrients. Series on the Safety of Novel Foods and Feeds. No. 12. ENV/JM/MONO(2004)20.

Roesler and Rao (1999) Protein Engineering 12(11), 967-973. Conformation and stability of barley chymotrypsin inhibitor-2 (CI-2) mutants containing multiple lysine substitutions.

Roesler and Rao (2001) Journal of Agricultural and Food Chemistry 49, 3443-3451. Rapid gastric fluid digestion and biochemical characterization of engineered proteins enriched in essential amino acids.

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PRODUCT PROFILE

Modified trait	Insect resistance.
Intended food use of modified crop (host plant)	The use of sprouted seeds of alfalfa (*Medicago sativa*) as food has a very long history. Currently in North America, sprouted alfalfa seeds are often available in the fruit and vegetable sections of grocery stores, as well as in natural and health food stores. Seeds for sprouting are also available for sale. The frequency and quantity of consumption of sprouted alfalfa seeds and their contribution to nutrient can vary widely.
Gene (source) / Promoter (source)	A *cryIC* gene derived from *Bacillus thuringiensis* (Bt) was chemically synthesized by a template-directed ligation -PCR method. A cauliflower mosaic virus (CaMV) 35S promoter was included in the expression cassette. The synthetic gene was engineered to provide novel specificity and potency against recalcitrant target pests and preferential codon usage in dicotyledonous plants. Chemically phosphorylated adjacent oligonucleotides of the *cryIC* gene to be synthesized were assembled and ligated on a single-stranded, partially homologous template derived from the wild-type *cryIC* gene by a thermostable *Pfu* DNA ligase, using repeated cycles of melting, annealing, and ligation. The resulting synthetic DNA strands were selectively amplified by PCR with short specific flanking primers that were complementary only to the new synthetic DNA. Modifications of the synthetic gene did not alter the amino acid sequence of the minimal toxic fragment of the CryIC protein.
Transformation method	Agrobacterium-mediated transformation.
Metabolic reactions / pathways	Not applicable - CryIC protein, the direct gene product encoded by the synthetic gene, confers the desired trait of interest.
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	CryIC protein.
Secondary gene products	Not applicable

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop for food.

Average level of expression of CryIC protein: 0.05% of soluble protein in the modified crop, with a range between 0.01 - 0.1%.

A.2. Prior history of known or presumed safe consumption from foods.

Prior exposure to the wild-type CryIC protein is likely from adventitious presence of the source organism on some foods. Based on available information (A.5.3), the chemically synthesized CryIC protein appears to be functionally comparable to the wild-type protein.

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: No changes in the food uses of modified alfalfa are expected. Modified alfalfa will replace unmodified alfalfa in the same uses.

CryIC protein: No change of physiological significance with respect to protein intake is expected - given: 1) the generally low consumption of alfalfa as food, 2) the very low level of expression of the protein the modified crop, and 3) the low level of the modified protein expected to be present in the diet, estimated to be < 2 ppm of the diet (based on average vegetable intake of 0.1 g/kg b-w, which likely overestimates alfalfa intake for the general population).

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

The mode of action of the modified protein CryIC is comparable to other bacterial proteins of the same class, i.e., as delta-endotoxin of high specificity, attributable to the presence of specific binding sites that bind only to receptors on intestinal cells of target insect families/genera. This mode of action is not a concern for mammalian toxicity because the receptors for delta-endotoxins are not present in mammalian intestinal cells.

A.5. Structure-activity characterization.

Amino acid sequence identity with mammalian toxins.
No amino acid sequence identity with known mammalian protein toxins was identified.

Pepsin digestion (under standard testing conditions).
The protein was rapidly and completely digested in less than 7 min.

Structural/functional comparability with comparator/source protein or non-protein substance.
Specificity of toxicity to target pests was verified by insect bioassays.
As noted previously, modifications of the synthetic gene did not alter the amino acid sequence of the minimal toxic fragment of the CryIC protein. The protein had the expected molecular weight (based on electrophoretic mobility on SDS-PAGE) and comparable immunoreactivity (using Western blot analysis) when compared to the wild type protein.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
No unusual protein degradation products expected in the host plant - no unusual protein fragments were identified by Western blot analysis.

In mammalian metabolism from consumption of the gene product.
No concerns with ingested protein that is rapidly digested.

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. **Between gene products introduced/modified** (in the same or separate transformation events).

Not applicable.

A.7.2. Between gene products introduced/modified and food constituents.

Not expected based on known function and structure of the modified CryIC protein.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

Bt is a common bacterium with no known adverse effects on humans or animals. Bt microbial products are approved pesticides for use on food crops with a long history (about 40 years) of use. The synthetic gene was designed using the Bt-derived DNA sequence as a template.

Applicable if the safety of donor organism is not known, or if the donor organism is a source of known toxicant or antinutrient.

B.1.1. Characterized gene modification.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Alfalfa belongs to the Fabaceae family. Several toxicants, antinutrients, and secondary metabolites of potential concern have been identified in plants of this family. They include: 1) canavanine, which is a potentially toxic structural analogue of L-arginine that is stored by many legumes; 2) triterpenoid saponins, with soyasapogenols, zanhic acid glycosides and medicagenic acid being quantitatively the most important. Susceptibility to saponin toxicity (believed to be largely due to medicagenic and zanhic acid) varies with species. Saponins are highly toxic to fish and amphibians, but not to ruminants and swine; 3) condensed tannins (proanthocyanidins) can bind reversibly or irreversibly to proteins and can affect protein digestibility; 4) phytoestrogens, with coumesterols (coumestrol) being quantitatively more important than isoflavones. Coumesterols have been shown to cause estrogen-related disorders in animals. The alfalfa plant has not been associated with human toxicity. For ruminant animals that consume alfalfa forage, lignin and bloat are the principal anti-quality factors.

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

CryIC protein is not expected to be functional in the modified plant, except as an insecticidal protein against target pests by binding with intestinal cells of the target insects, forming pores, leading to cell lysis and eventual death of the insect.

B.3.2. Impact of gene product function in the modified plant.

CryIC protein does not have a regulatory role and is not expected to affect metabolic or signalling pathways of the host plant.

Applicable if gene product has an enzymatic function in the host plant.

B.3.3. Substrate specificity of gene product.

Not applicable - CryIC protein does not have an enzymatic function in the host plant.

B.4. Potential for disrupting coding/regulatory DNA sequences in host plant.

B.4.1. In the inserted/modified DNA.

Southern hybridization and RT-PCR (reverse transcriptase-polymerase chain reaction) revealed integration of one copy of transgene at a single genetic locus with an incomplete inverted repeat. See 4.2 below regarding the inserted sequence and the flanking regions.

B.4.2. In genomic sequences bordering the site of insertion/modification.

The entire sequence including the flanking regions (about 500bp) and the insert DNA was analyzed. In addition to the inserted genes, 2 ORFs (open reading frames) were identified. However, further bioinformatics analysis indicated these ORFs lacked the appropriate upstream transcriptional regulatory sequences, so are very unlikely to be transcribed.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Alfalfa as a food is generally consumed at low frequency and/or in low quantity (8-20 g serving) in the North American dietary context. Given that alfalfa has been promoted as highly nutritious, among a small segment of committed users, consumption may be 4-8 times that of the general population. Even at relatively high level of consumption, its contribution to nutrient intake remains minor.

The key nutrients analyzed were limited to the proximates and selected vitamins (beta-carotene, total folate, and vitamins C and K). Minor differences in levels in the seed between the modified and unmodified crops were within natural variation and do not have nutritional significance.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

No difference in the seed level of canavanine between modified and unmodified crop was identified.

B.5.3. Other metabolites/parameters.

The major metabolites described in B.2. were analyzed and compared between modified and unmodified crop. Limited quantitative data are available on secondary metabolites in this crop for reference purposes.

Some differences (20-50% higher in coumesterols in crops harvested in some locations) were found. It has been reported that levels of coumestrol in alfalfa forage range from 3-104 ppm, depending on genetic variation. Concentrations usually do not exceed 30-60 mg/kg dry matter, but can accumulate in plants subject to fungal attack. The difference between modified and unmodified crops falls within this reported range of variability.

The phenotype modified is conferred by the product of the inserted gene. It is generally assumed that the gene of the type introduced would have no interaction with existing plant metabolic pathways. Without evidence that would invalidate this assumption, the chemical analysis conducted for the key nutrients and known toxicants and significant secondary metabolites would be considered to be sufficient in scope.

B.6. Health consequences of instability of the modified trait.

The modified trait is not intended to affect human health.

PART C - POTENTIAL FOR ALLERGENIC (IgE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN SUBSTANCES.

C.1. Allergenicity of donor organism (gene source).

Bt is not a known source of allergen.

Protein gene products (factors C.2-C.4).

C.2. Potential for protein gene product(s) to cross react with known allergens.

No overall sequence identities of > 35% with known allergens were found. However, three exact matches were obtained with hexamer (Blo t11, Der p 11, Ole e 9) associated with paramyosin from mites and beta-1,3-glucanase from olives.

C.3. Pepsin digestion (under standard testing conditions).

As noted in A.5, the protein was rapidly and completely digested in less than 7 min.

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factors (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

The protein is not glycosylated when expressed in alfalfa. The absence of glycans in glycoprotein assay supports this conclusion.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

C.4.3. Abundance.

C.5. Allergenicity of host plant.

Alfalfa is not a common source of food allergen.

References.

Strizhov N et al. (1996) A synthetic *cryIC* gene, encoding a Bacillus thuringiensis δ-endotoxin, confers *Spodoptera* resistance in alfalfa and tobacco. Proceedings of National Academy of Sciences 93, 15012-15017.

PRODUCT PROFILE

Modified trait	Tolerance to herbicide (glufosinate).
Intended food use of modified crop (host plant)	Maize or field corn (*Zea mays L.*) is grown for the production of multiple ingredients such as starch, sweeteners, flaking grits, flour, and oils from wet or dry milling processing. Ingredients have wide applications in foods.
Gene (source) / Promoter (source)	Gene encoding the enzyme phosphinothricin acetyltransferase (PAT) derived from *Streptomyces hygroscopicus*, a common soil bacterium, is introduced under the control of constitutively expressed promoter CaMV 35S.
Transformation method	Biolistic transformation.
Metabolic reactions / pathways	Tolerance to herbicide (glufosinate) was developed by introducing an enzyme (PAT) that inactivates the active compound (phosphinothricin, or PPT) in the herbicide. The catabolism of glufosinate involves two competing metabolic pathways. (1) the deamination of glufosinate and subsequent conversion of PPO* to MPP or to MPHB. When PAT expression is low, the deamination pathway predominates, resulting in the residue metabolites MPHB and the main metabolite MPP, which is non-phytotoxic. An unstable intermediate PPO is formed via deamination, which is rapidly decarboxylated to the stable MPP. PPO can also be reduced to form MPHB, another final and stable product. (2) the N-acetylation of L-glufosinate by PAT. In L-PPT tolerant plants which express relatively high levels of PAT, this pathway predominates and the main residue metabolite of L-PPT catabolism is N-acetyl-phosphinothricin.
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	PAT enzyme.
Secondary gene products	Glufosinate and metabolites.

*Abbreviations: PAT=phosphinothricin N-acetyltransferase PPO=4-methylphosphinico-2-oxo-butanoic acid PPT= phosphinothricin MPP=3-methylphosphinico-proprionic acid MPHB=4-methyl-phosphinico-2-hydroxy-butanoic acid

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop.

PAT: Using a quantitative immunoblot, relatively low levels were found in the kernel, ranging from 3.1 to 6.0 μg/g dry weight (0.0174% total protein). It is not detectable in oil.

Glufosinate and metabolites: Below detection limits in both kernels (< 0.05 - 0.07 μg/g dry weight) and oil (< 0.05 ppm).

A.2. Prior history of known or presumed safe consumption from foods.

PAT: Adventitious presence is expected on some plant foods from the gene source which is a common soil bacterium.

Glufosinate and metabolites: These metabolites are present on foods with residual levels from a registered herbicide which has been in commercial use since 1999.

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: No change in the food use of modified corn is expected. Modified corn will replace unmodified corn in the same uses.

PAT: Change in dietary exposure to PAT enzyme may be extrapolated from expression levels, which were very low – no biologically significant changes relative to current exposure levels are expected for PAT.

Glufosinate and metabolites Potential for increase from the same or similar categories of foods would depend on PAT expression level and agricultural practices but should not exceed established upper limit.

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

PAT: Not a known toxicant. Enzymes are unlikely to be toxic.

Glufosinate and metabolites: Certain toxic endpoints have been reported in animals. : Tolerance[1] and ADI[2] levels have been established. There are no concerns with mutagenic and carcinogenic activity[3].

A.5. Structure-activity characterization.

Amino acid sequence identity with mammalian toxins.
PAT: No amino acid sequence identity with known mammalian protein toxins was identified.

Pepsin digestion (under standard testing conditions).
Rapid digestion in simulated gastric fluid - only trace amounts of PAT detected at 2 min.

Structural/functional comparability with comparator/source protein or non-protein substance.
The plant-derived PAT was judged to be comparable to the microbially-derived enzyme based on similar electrophoretic mobility, immunogenicity and enzymatic activity, as well as matching amino terminal amino acid sequence.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
PAT: No unusual breakdown products of protein in the modified plant are expected - no unusual protein fragments were identified by Western blot analysis.

[1] Tolerances for combined residues of glufosinate-ammonium and its metabolites (MPP and N-acetyl-L-glufosinate) expressed as 2-amino-4-(hydroxymethylphosphinyl) butanoic acid (glufosinate-ammonium free acid) equivalents have been established in the USA for transgenic corn, at 0.2 ppm. [OECD Series on Harmonization of Regulatory Oversight in Biotechnology No. 11 (1999) and No. 25(2002); U.S. EPA Glufosinate Ammonium; Pesticide Tolerance. Federal Register Vol. 68, No. 188, 55833-55849, 2003]. Maximum residual limit of 0.2 ppm for corn is also established in Canadian Food and Drug Regulations.

[2] An acceptable daily intake (ADI) value of 0.02 mg/kg body weight/day was accepted for glufosinate, and confirmed as group ADI for glufosinate-ammonium, MPP and N-acetyl-L-glufosinate [WHO Joint FAO/WHO Meeting on Pesticide Residues, Rome, 20-29 Sept, 1999]. Acceptable total exposure was established at 0.0063 mg/kg (acute), and 0.006 mg/kg/day (chronic) for U.S. population with 48% and 10% from food sources, respectively [U.S. EPA Glufosinate Ammonium; Pesticide Tolerance. Federal Register Vol. 68, No. 188, 55833-55849, 2003].

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Glufosinate and metabolites: Catabolism of PPT is described under "metabolic reactions/pathways" (p.1 of product profile). Limits have been established for degradation products of PPT in maize and other crops, to which application of PPT is permitted.
In mammalian metabolism from consumption of the gene product.
PAT: No concerns with a protein that is rapidly digested.

Glufosinate and metabolites: Effects of degradation products of glufosinate have been studied that led to the establishment of tolerance and ADI, as noted in a previous section.

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

Not applicable.

A.7.2. Between gene products introduced/modified and food constituents.

No interactions among food constituents and PAT enzyme and their metabolites are expected based on their levels in foods derived from the modified crop and their known functions and structures.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

Streptomyces hygroscopicus - not known to be pathogenic.

Applicable if the safety of donor organism is not known or if the donor organism is a source of known toxicant or antinutrient.
B.1.1. Characterized gene modification.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Corn is not a source of significant toxicants or antinutritional factors. Low levels of trypsin and chymotrypsin inhibitors are not considered nutritionally significant. Highly variable levels (0-1000 ppm) of DIMBOA (2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one) have been reported in green- and root-tissues of maize seedlings, with its glycoside form being the most prominent. The deglycosylated form, DIMBOA is toxic to insects; data on effects on humans and animals are limited. Other metabolites include:
a) Furfural: moderate acute toxicity and oral genotoxic carcinogen of low potency; partly removed by heating.
b) Ferulic acid and p-coumaric acid: natural pesticide against insects and fungi. As phenolic acids and weak anti-oxidants, their effects (positive and negative) on mutagenic substances have been reported.

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

The role of the PAT enzyme is well established, as described previously. It functioned as expected in the modified plant.

B.3.2. Impact of gene product function in the modified plant.

PAT metabolizes exogenously applied glufosinate in a pathway that is well-defined. It is not expected that this pathway would interact with the metabolism of the host plant.

Applicable if gene product has an enzymatic function in the modified plant.
B.3.3. Substrate specificity of gene product.

PAT is highly substrate-specific for L-PPT.

B.4. Potential for disrupting coding/regulatory DNA sequences in host plant.

B.4.1. In the inserted/modified DNA.

Southern blot analysis indicated that the transformation event contains two copies of the expression cassette at one insertion site (including a selectable marker to identify bacteria transformed with recombinant plasmid DNAs, which was not functional or expressed in the plant). PCR and sequencing analysis confirmed the estimations of the gene copy number by Southern blot analysis. Sequence analysis indicated that there were two potential open reading frames. Northern blot analysis indicated that the two ORFs were unlikely to be expressed, as no hybridization signals were observed when the total RNA was analyzed with a probe specific to the sequence.

B.4.2. In genomic sequences bordering the site of insertion/modification.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Compositional analysis of modified food included parameters suggested in the OECD Consensus Document to be analyzed for human food use of maize: proximate analysis, minerals, vitamins, amino acids, fatty acids. Levels in modified and unmodified control were comparable.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

Anti-nutrients analyzed included: phytic acid and raffinose. Levels in modified and unmodified control were comparable. Levels of trypsin and chymotrypsin inhibitors were also analyzed although their levels in maize are not nutritionally significant. Analysis of DIMBOA was not recommended as part of basic compositional analysis because of the high variability of its levels among maize varieties, and the fragmentary knowledge of its toxicology.

B.5.3. Other metabolites/parameters.

Secondary metabolites analyzed included: furfural, ferulic acid and p-coumaric acid. Levels in modified and unmodified control were comparable. No other metabolites were analyzed given the absence of changes in key nutrients, toxicants, and antinutrients.

B.6. Health consequences of instability of the modified trait.

Modified trait is not intended to affect human health.

PART C - POTENTIAL FOR ALLERGENIC (IgE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN SUBSTANCES.

C.1. Allergenicity of donor organism (gene source).

Streptomyces hygroscopicus - not known to be allergenic; transfer of allergenic protein from source organism unlikely.

Protein gene products (factors C.2-C.4).

C.2. Potential for protein gene product(s) to cross react with known allergens.

PAT: no amino acid sequence identity of > 35% with known allergens was found.

C.3. Pepsin digestion (under standard testing conditions).

As noted in A.5, PAT enzyme was rapidly digested in simulated gastric fluid - only trace amounts of PAT detected at 2 min.

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factor (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

Absence of glycosylation was confirmed by glycoprotein assays.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

C.4.3. Abundance.

C.5. Allergenicity of host plant.

Corn is not a common allergenic food, but case studies of skin, gastrointestinal and respiratory symptoms have been reported. Allergenic protein(s) identified: Zea m1 (beta-expansin 1b) and Zea m 14 (lipid transfer protein, homologue of pathogenesis related protein PR14).

PRODUCT PROFILE

Modified trait	Increased total levels of isoflavones, including genistein 2, for potential health benefits.
Intended food use of modified crop (host plant)	Soybeans (*Glycine max*) are primarily used for the production of oils (about 94% of soy food ingredients), protein fractions, and dietary fibre. Isolated soy protein is used in the production of infant formula and other food products as a source of amino acids. Soy-based foods are estimated to make up between 0.8% to 1.5% of a child's (age 1-6) and an infant's diet, respectively. For infants, about 80% of the soy-based food is expected to come from oil, and 20% from soy flour (flake) products.
Gene (source) / Promoter (source)	Two genes were introduced. 1) Chimeric gene (CRC) containing a fusion of nucleotide sequences of transcription factors C1 and the R (Lc allele) from maize (*Zea mays*) under the control of the seed-specific phaseolin promoter from the French bean (*Phaseolus vulgaris*). 2) A gene designed to promote cosuppression of flavavone 3-hydroxylase (F3H) developed from a homologous gene derived from carrot (*Daucus carota*).
Transformation method	Particle gun bombardment.
Metabolic reactions / pathways	C1 and R transcription factors activate phenylpropanoid pathway genes. Cosuppression of F3H blocks a branch pathway that competes with isoflavone synthase (IFS) for naringenin in the biosynthesis of flavonols and anthocyanidins.
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	Regulatory C1 and R proteins (transcription factors). Suppressed expression of F3H (enzyme).
Secondary gene products	Several metabolites of the phenylpropanoid pathway, including increased total levels of isoflavones.

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop for food.

The presence CRC and F3H transgenes in the transformed lines was identified by PCR.

Isoflavones: 4-fold higher levels of isoflavones (daidzein, genistein, and glycitein) than controls.

A.2. Prior history of known or presumed safe consumption from foods.

C1 and R proteins: As regulatory proteins in food crops, these proteins have prior dietary exposure.

Isoflavones: Isoflavones are largely restricted to the subfamily of Papilionoideae of the Fabaceae [Cassidy et al., 2000; Dixon, 2004]. Soybean is the predominant dietary source of isoflavones (daidzein and genistein and their glycosides, and smaller amounts of free and conjugated glycitein and coumestrol). Kidney bean, American groundnut, chickpea (biochanin A) and alfalfa sprouts are minor sources (formononetin glycosides and coumestrol) [Dixon, 2004].

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: The following estimates are based on the assumption that no major changes in consumption of modified soybean are expected.

C1 and R proteins: Change in dietary exposure is expected to be negligible considering the expected expression level of these proteins in the modified soybean relative to baseline exposure to dietary proteins in the diet.

Isoflavones: The reported increase in isoflavones levels in the modified soy is not expected to significantly increase isoflavones intake in Western populations. Even among subpopulations who consume higher than average amounts of soya products (e.g., individuals with intolerance to dairy products or who follow a vegetarian diet), the increase in isoflavones would be expected to be within the range of isoflavones intake of populations with high soya consumption.

Levels of isoflavones reported in the literature vary between 0.5 to over 10 mg/kg, depending on the variety and growing conditions [Dixon, 2004; Fletcher, 2003; Romanti et al., 2003]. High variability in the intakes isoflavones is also apparent among populations. Estimates of consumption of soya in the Asian countries vary between 10-35 g/d and 30-50 g/d [Manach et al., 2004; Cornwell et al., 2004]. A mean intake of 25-40 mg isoflavones/d, with a maximum intake of 100 mg/d, has been reported [Manach et al., 2004]. Americans and Europeans, who eat little soya (primarily as analogs of meat and dairy products), consume only a few milligrams of isoflavones per day [Manach et al., 2004]. The incorporation of growing quantities of soya ingredients into processed food products could result in an increase in isoflavones intake.

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

C1 and R proteins: No safety concerns are expected based on their history of consumption from foods and low levels in the diet.

Isoflavones: A major effect of isoflavones of interest is their ability to bind to estrogen receptors (ER), which function as ligand-inducible transcription factors for genes involved in cell growth, proliferation, and differentiation. Soy-derived genistein and daidzein and their metabolites have different binding affinities for the two types of ER (ERα and ERβ). Compounds that bind to ER can result in either agonistic or antagonistic activity and have the potential to disrupt estrogenic signalling. Estrogenic and/or antiestrogenic activities of isoflavones may reduce but also stimulate estrogen-dependent tumour growth depending on the target cell, dose and timing of exposure [Mueller et al., 2004]. Isoflavones may also induce adverse developmental effects.

A.5. Structure-activity characterization.

Amino acid sequence identity with mammalian toxins.
C1 and R proteins: No amino acid sequence identity with mammalian toxins is expected with endogenous proteins of crop plants.

Pepsin digestion (under standard testing conditions).
C1 and R proteins: The proteins were rapidly and completely digested in less than 1 min.

Structural/functional comparability with comparator/source protein or non-protein substance.
C1 and R proteins: The proteins have the expected molecular weight (based on electrophoretic mobility). Relative to the wild-type protein, the protein expressed in the modified plant had comparable immunogenicity (Western blots with antibodies specific to the test protein) and amino acid sequences (N-terminal sequencing).

Isoflavones: Isoflavone aglycones and their glucose-conjugates were measured for each individual isoflavones (daidzin, genistin, and glycitin) by HPLC. Their structural identities were verified by comparing with known standards.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
C1 and R proteins: No unusual breakdown products of the proteins in the modified plant are expected.

Isoflavones: Isoflavones are endogenous substances in soybean. No unusual metabolites are expected in the modified plant at the moderately increased level.

In mammalian metabolism from consumption of the gene product.
C1 and R proteins: No concerns with ingested protein that is rapidly digested.

Isoflavones: The bioavailability of dietary isoflavones can affect their metabolic effects following ingestion. Isoflavones present in the food in the form of glycosides cannot be absorbed in their native form. They must be hydrolyzed by intestinal enzymes or by colonic microflora before they can be absorbed [Manach et al., 2004]. As a general rule, the metabolites are believed to be rapidly eliminated from the plasma. Knowledge on the important metabolites of isoflavones that are biologically active is limited [Cassidy et al., 2000; Manach et al., 2004]. There is considerable individual variability in metabolic response to a known dose of isoflavones-rich food, as well as major inter-species differences in some metabolic processes [Cassidy et al., 2000].

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

No synergistic effects with potential for mammalian toxicity are expected based on the function of the proteins. The genes introduced have separate effects on the metabolites of the phenylpropanoid pathway.

A.7.2. Between gene products introduced/modified and food constituents.

No interactions with food constituents are expected at the expressed level that would affect nutrient absorption. Isoflavones are inherent constituents in soybean, although the effects of food matrix on the bioavailability of dietary isoflavones have not been examined in detail.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

Maize and carrots have a long history of consumption as food and do not contain significant toxicants or antinutritional factors.

Applicable if the safety of donor organism is not known, or if the donor organism is a source of known toxicant or antinutrient.
B.1.1. Characterized gene modification.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Soybean belongs to an extremely large legume family, Fabaceae, which encompasses numerous genera that are toxic. Substances of relevance in soybean include:
Antinutrients: include trypsin inhibitors, lectins – both are subject to degradation upon heating; stachyose and raffinose, and phytic acid. Non-ruminants are particularly susceptible to the adverse effects of trypsin inhibitors in raw soybeans.

Other antinutrients, with the exception of phytic acid, do not appear to be of practical significance in animal nutrition where soybeans and soybean meals area major protein source [Cheeke, 1998].

Bioactive substances: phytoestrogens (isoflavones).

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

C1 and R proteins: Over-expression of *C1* and R (*Lc*) genes has been studied in several species. These studies support the use of transcription factors to induce the accumulation of metabolites in target tissues, including those tissues in which most of the relevant enzymatic activities are normally insufficient.

F3H (suppressed): The suppression of the expression of the F3H protein had the expected effect of reducing the levels of flavonols and anthocyanidins.

Isoflavones: are part of a large group of phenolic compounds in plants that are believed to have antimicrobial function in disease resistance. Isoflavones are constitutively expressed and can also be induced in response to stress. The isoflavonoids are widely distributed in the plant kingdom, being mostly limited to the subfamily of Papilionoideae of Fabaceae [Cassidy et al., 2000; Dixon, 2004].

B.3.2. Impact of gene product function in the modified plant.

Expressed C1 and R proteins and suppressed expression of F3H: The induction of pathway genes is only a small part of the metabolic and cellular changes that result from overexpressing transcriptional activators. Increased vesicular trafficking, as well as the expression of several hundreds of genes can also be affected; and only a subset of these genes encode cognate pathway enzymes. As transcription factors, C1 and R activate multiple genes in the phenylpropanoid pathway, and possibly affecting the expression of other genes as well, as described above. The complex mechanisms of transcriptional regulation can also be seen in the synergistic interaction between other activators in the related families of transcription factors [Broun, 2004].

Applicable if gene product has an enzymatic function in the host plant.
B.3.3. Substrate specificity of gene product.

C1 and R proteins: Not applicable.

F3H (suppressed): Substrate specificity was indicated in *in vitro* enzyme assays.

B.4. Potential for disrupting coding/regulatory DNA sequences in host plant.

B.4.1. In the inserted/modified DNA.

Transgenic lines exhibiting integration of one copy of transgene at a single genetic locus were selected. Southern hybridization and RT-PCR (reverse transcriptase-polymerase chain reaction) revealed incomplete repeat sequences.

Comparison of DNA insert before and after introduction into plant DNA (conducted using PCR amplification) revealed rearrangements at the insertion site, including DNA deletion and duplication. These rearrangements are common in transformation using recombinant plasmids that carry vector backbone sequences. Several partial insert gene fragments were judged non-functional using Northern blot analysis. No hybridization signals were observed when RNA was probed using gene-specific sequence. Bioinformatics analysis and PCR assays do not suggest the presence of open reading frames in the inserted sequences.

B.4.2. In genomic sequences bordering the site of insertion/modification.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Compositional analysis of the modified food included parameters suggested in the OECD Consensus Document for food use of soybean: proximate analysis, individual amino acids, fatty acids, and selected vitamins.

Significant changes were found with several parameters compared with isogenic parental line, but were within natural variation reported in the literature.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

Analytes included phytic acid, trypsin inhibitor, lectins, stachyose and raffinose, in addition to isoflavones (noted in A.3).

Significant changes were found with several parameters compared with isogenic parental line, but were within natural variation reported in the literature.

B.5.3. Other metabolites/parameters.

Other metabolites of the phenylpropanoid pathway were analyzed, principally intermediates in the synthesis of daidzein, glycitein, and genistein, and synthesis of flavonols that are downstream of the F3H enzyme.

An average of 20-fold increase (from 3 ppm to 68 ppm) in the flavonol kaempferol (range 30 -150 ppm) was detected. This was probably due to incomplete suppression of F3H.

There is limited evidence that kaempferol is active in short term genetic tests: it was mutagenic in bacteria and insects and in mammalian cells *in vitro*. It also induced micronuclei in mice. The data are inadequate to make an evaluation in one carcinogenicity study in rats by administration in the diet. No data are available to evaluate teratogenicity in experimental animals and in humans. No data are available to assess chromosomal and carcinogenic effects of this compound in humans, despite wide human exposure to this compound. Its natural occurrence as aglycone is widely distributed in fruit and other edible plants.

The increased level of kaempferol (30 - 150 ppm) is within the range of occurrence in the edible parts of plant origin (30 -300 ppm in flower of cauliflower, leaf of chives and cabbage; 500 ppm in pea) [Duke, 1992].

B.6. Health consequences of instability of the modified trait.

Modified trait is health-related. Seedbanks are being developed to ensure lot-to-lot consistency in the expression of the target gene products.

PART C - POTENTIAL FOR ALLERGENIC (IGE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN SUBSTANCES.

C.1. Allergenicity of donor organism (gene source).

Source of C1 and R: Maize is not a common allergenic food, but case reports of skin, gastrointestinal and respiratory symptoms have been reported. Allergenic protein(s) identified include: Zea m 1 (beta-expansin 1b) and Zea m 14 (lipid transfer protein; pathogenesis related protein PR14).

Source of F3H: Carrot is not a common source of allergen, but it has several allergenic proteins (Dau c 1 and Dau c4) that are homologues of Bet v 1 (pathogenesis related protein PR10) and profilin, respectively.

Product ID: L (Soybean_Isoflavones)

Protein gene products (factors C.2-C.4).

C.2. Potential for protein gene product(s) to cross react with known allergens.

C1 and R: These proteins are not known to be associated with allergenicity. In addition, no overall sequence identity of 35% or higher was found when compared with known allergens.

C.3. Pepsin digestion (under standard testing conditions).

The protein was rapidly and completely digested in less than 1 min.

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factor (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

C.4.3. Abundance.

C.5. Allergenicity of host plant.

Soybean is a common allergenic food, containing several antigenic proteins. Several gastrointestinal reactions have been attributed to the globulin fraction of soybean proteins that comprises about 85% of total protein.

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PRODUCT PROFILE

Modified trait	Insect resistance.
Intended food use of modified crop (host plant)	Potatoes are increasingly consumed in a processed form (e.g., French fries and potato chips) while the direct consumption of potatoes (e.g., boiled potatoes) has declined dramatically. Potatoes should be cooked before eating because of the indigestibility of non-gelatinised starch and the presence of anti-nutritional proteins.
Gene (source) / Promoter (source)	Gene encoding a lectin protein, Galanthus nivalis agglutinin (GNA), from snowdrop (*Galanthus nivalis*) was introduced under the control of constitutively expressed cauliflower mosaic virus 35S promoter.
Transformation method	Agrobacterium-mediated transformation.
Metabolic reactions / pathways	The GNA protein is an immediate product encoded by the gene introduced in the crop and confers the desired phenotype.
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	GNA lectin protein.
Secondary gene products	Not applicable.

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop.

Up to 2.0% of total soluble protein.

A.2. Prior history of known or presumed safe consumption from foods.

No prior dietary exposure.

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: No change in the food use of the modified potato is expected. Modified potato will replace unmodified potato in the same uses.

GNA protein: An expression level of up to 2.0% total soluble protein in the modified potato is estimated to result in an intake of approximately 0.8 g/day of the GNA lectin for the general population, based on current level of potato consumption (90th percentile for consumers only of potato, assuming consumption of 100% modified potato).

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

GNA lectin is a monocot mannose-specific binding protein. It has been hypothesized that carbohydrate-binding proteins can be accumulated in large quantities in seeds and/or vegetative storage organs of plant species as part of plant defense mechanisms [Peumans and Van Damme, 1996]. GNA shares similar molecular structure and sugar-binding specificity with lectins from four other plant families (Alliaceae, Araceae, Liliaceae, and Orchidaceae).

Although typical lectins from different plant sources exhibit a considerable degree of structural similarity, lectin GNA from snowdrop has lower toxicity to mammals compared to lectins from kidney bean (PHA) and wheat germ (WGA) [Peumans and Van Damme, 1996]. The antinutritional effects of ingested lectins are likely due to their ability to combine with specific receptor sites of intestinal cells and to cause a non-specific interference with nutrient absorption and utilization. The severity of these adverse effects will depend on the gut region to which the lectin will bind. Lectins have also been associated with binding to intestinal and red blood cells. Other adverse effects of plant lectins include: morphological and physiological modifications in the small intestine, inhibition of intestinal and brush border enzymes, and systematic effects, affecting the immune system, hormonal homeostasis, and enlargement of the liver and hypertrophy. It is plausible to suppose that the adverse effects of dietary lectins observed in experimental animals will be comparable in humans [Vasconcelos and Oliveira, 2004]. Most of the information on the toxicity of lectins in humans has been derived from acute toxicity in incidences of accidental poisoning from PHA.

The ability of dietary lectins to interact with the immune system in facilitating the autoimmune process in genetically susceptible individuals was reviewed by Cordain et al. [2000]. Dietary lectins may interact with enterocytes and lymphocytes to facilitate the translocation of gut-derived bacterial and dietary antigens to peripheral tissues and cause persistent peripheral antigenic stimulation. This may cause antibodies or T-lymphocytes to cross-react with both foreign and endogenous peptides.

A.5. Structure-activity characterization.

Amino acid sequence identity with mammalian toxins.
No amino acid sequence identity with known protein toxin was identified.

Pepsin digestion (under standard testing conditions).
Lectin is highly resistant to digestion.

Structural/functional comparability with comparator/source protein or non-protein substance.
Comparability in molecular weight between potato-derived and native GNA was indicated by similar electrophoretic mobility on SDS-PAGE. Comparable immunogenicity was suggested by Western immunoblot analysis. N-terminal amino acid sequencing and mass spectrometry of proteolytic fragments did not reveal the presence of N-terminal signal peptide and N-linked glycosylation sequences. Specificity of toxicity to target pests was verified by insect assays, as expected based on known activity of the native protein.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
No unusual breakdown products of protein in host plant are expected.

In mammalian metabolism from consumption of the gene product.
In rats, some lectins increase their binding sites to gut tissue by mediating changes in the glycosylation of the gut, with associated disruption of the brush borders and disorganization of the main absorptive cells. Lectins bound to epithelial cells may be taken up systematically by endocytosis, released by exocytosis into the intracellular space from where they are subsequently transported throughout the body [Cordain et al., 2000].

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

Not applicable.

A.7.2. Between gene products introduced/modified and food constituents.

There is the potential for disruption of nutrient absorption, as described above (A.6 - mammalian metabolism).

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

There is limited information on toxic metabolites in snowdrop (*Galanthus nivalis*). Taxonomically, *Galanthus nivalis* is part of the Amaryllidaceae plant family. Several genera in this family, including the *Galanthus* genus, have caused poisonings in humans and animals when consumed during times of food shortages. The bulbs of these species contain galanthamine, a competitive cholinesterase inhibitor, as well as other related alkaloids [Lopez et al., 2002].

Applicable if the safety of donor organism is not known or if the donor organism is a source of known toxicant or antinutrient.

B.1.1. Characterized gene modification.

The complete sequence of the DNA insert used in the genetic modification was characterized. There is no evidence of other unidentified DNA sequence being transferred to the host plant.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Potato belongs to the Solanaceae plant family that encompasses a large number of toxic plant species. Potato is a source of known toxicants for which limits have been established. Major metabolites of concern include:

Toxicants: potatoes contain several types of alkaloids, with glycoalkaloids being the most important, 95% of which consist of α-chaconine and α-solanine, present in higher concentrations at the periphery, in green and sprouted tubers; not destroyed during cooking and frying. The widely accepted tolerance limit of total glycoalkaloids in tuber is 200 mg/kg, fresh weight, was based on acute and/or subacute effects. New standards based on chronic effects may be warranted [Korpan et al., 2004]. Susceptibility of humans to glycoalkaloids appears to be high and variable: 1-5 mg/kg body weight oral doses are marginally to severely toxic; 3-6 mg/kg body weight can be lethal. Margin of exposure for potato glycoalkaloids may be between 2 and 6, assuming a low observed effect level in humans of 2 mg/kg bw, an average level of glycoalkaloids in potato and an average daily consumption of potato in western countries [Nordic Working Group, 1991].

Anti-nutrients: several protease inhibitors and lectins are present, inhibiting the activity of trypsin, chymotrypsin and other proteases, or binding to specific carbohydrate structures on cell surfaces (e.g., intestinal or blood cells) - an activity that is largely inactivated by boiling and other thermal processes.

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

GNA in the modified potato exhibited the expected phenotype. It reduces damage by target insect pests.

B.3.2. Impact of gene product function in the modified plant.

GNA is not expected to have metabolic, regulatory, or signalling function in the host plant.

Applicable if gene product has an enzymatic function in the modified plant.
B.3.3. Substrate specificity of gene product.

Not applicable.

B.4. Potential for disrupting coding/regulatory DNA sequences in the modified plant.

B.4.1. In the inserted/modified DNA.

Insertion of the T-DNA occurred at 4-5 loci. At least 1 locus contained copies of the T-DNA organized in inverted orientations with incomplete copies of the promoters. For 2 copies of the T-DNA, transfer of the T-DNA resulted in incomplete resolution of the right border. Plasmid sequences beyond the left and right borders were inserted. Complex integrations are common in transformation using recombinant plasmids that carry vector backbone sequences.

However, the coding regions of all the genetic elements (including selectable markers) remained intact. Bioinformatics analysis does not suggest the presence of unknown DNA sequences in the inserted sequences.

B.4.2. In genomic sequences bordering the site of insertion/modification.

B.5. Chemical analysis of food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Analysis included proximates (dry matter), sugars, including reducing sugars, protein and vitamin C, as suggested in the OECD consensus document for food use of potato. Small differences in macro- and micronutrients between transgenic and control potatoes were within the normal range of variation reported for commercial varieties.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

Total glycoalkaloids were below the guidelines of 20 mg/100 g fresh weight for potatoes.

B.5.3. Other metabolites/parameters.

The GNA protein is not expected to interact with the host plant's metabolic pathways. This, in conjunction with the absence of phenotypic alterations and the lack of significant changes in the above factors, no other metabolites were analyzed.

B.6. Health consequences of instability of the modified trait.

The modified trait is not intended to affect human health.

PART C - POTENTIAL FOR ALLERGENIC (IGE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN SUBSTANCES.

C.1. Allergenicity of donor organism (gene source).

There are limited data on Galanthus nivalis as an allergenic source. Amaryllis in the same Amaryllidaceae family has been associated with occupational asthma [Jansen et al., 1996]. Sixteen common plant lectins can trigger histamine release from basophils. Antibodies to common dietary legume lectins are present in the sera of healthy humans.

Protein gene products (factors C.2-C.4).

C.2. Potential for protein gene product(s) to cross react with known allergens.

No amino acid sequence identity of > 35% with known allergens was found.

C.3. Pepsin digestion (under standard testing conditions).

As noted in A.5, GNA is resistant to digestion.

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factor (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

As note in A.5, no N-terminal signal peptide and N-linked glycosylation sequences were detected in the potato-derived GNA. The absence of post-translational glycosylation was also confirmed by glycoprotein assays.

C.4.2. Heat stability (NOT applicable to lipid transfer protein).

GNA is heat stable.

C.4.3. Abundance.

GNA is expressed at up to 2.0% of total soluble protein.

C.5. Allergenicity of host plant.

Potato is not a common source of allergens. However, potato contains multiple heat-labile proteins which can induce immediate hypersensitivity reactions when raw potatoes are consumed. Patatin, the main storage protein in potatoes, has been associated with allergic reactions in sensitive children - allergenicity of patatin to be confirmed. In addition, IgE binding to several proteins belonging to the family of soybean trypsin inhibitors was observed. Allergenic proteins include: cathepsin D inhibitor, cysteine protease inhibitor, and aspartic protease inhibitor.

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PRODUCT PROFILE

Modified trait	Enhanced protein quality.
Intended food use of modified crop (host plant)	Little whole kernel or processed maize (field corn) is consumed by humans worldwide when compared to maize-based food ingredients. Maize-based ingredients have wide applications in foods and include starch, sweeteners, flaking grits, flour, and oils from wet or dry milling processing.
Gene (source) / Promoter (source)	11S globulin cDNA from amaranth (*Amaranth hypochondriacus*) was under the control of rice glutelin-1.
Transformation method	Biolistic transformation.
Metabolic reactions / pathways	Not applicable - the primary gene product is not expected to function in biosynthetic pathways of the modified crop.
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	Amarantin - a seed storage protein.
Secondary gene products	Not applicable.

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop for food.

Amarantin was expressed at 0.5-1.2% of total protein.

A.2. Prior history of known or presumed safe consumption from foods.

Exposure to amaranth or amarantin is expected to be low in North America and limited to a small segment of the population. Historically, amaranth was a basic food in the diets of Meso-American civilizations.

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: No major changes in the food use of modified maize are expected.

Amarantin: Modified maize will provide a new dietary source of exposure to amarantin, of below 1 g /day for consumers only at 90th percentile intake of maize (using an over-estimate of 100% modified maize). This increased level of protein consumption from the modified maize containing amarantin is expected to be within the range of normal variation in protein level in the diet.

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

Given the historical and current dietary intake of amarantin protein, at least in some populations, no safety concerns are expected at the anticipated level of dietary exposure to amarantin and to the associated changes in the levels of amino acids.

A.5. Structure-activity characterization.

Amino acid sequence identity with mammalian toxins.
No amino acid sequence identity with known mammalian toxins was identified or expected.

Pepsin digestion (under standard testing conditions).
The protein was almost completely digested within 20 min in pepsin digestion using simulated gastric fluids, followed by complete degradation within 15 min in simulated intestinal fluid.

Structural/functional comparability with comparator/source protein or non-protein substance.
The maize-derived amarantin has expected molecular weight of 54 kDa (electrophoretic mobility) and amino acid sequences (N-terminal sequencing) relative to the wild-type amarantin.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
No unusual breakdown products of protein in the modified plant are expected - no unusual protein fragments were identified by Western blot analysis.

In mammalian metabolism from consumption of the gene product.
The protein was moderately digested in pepsin digestibility test - no unusual digestion products expected.

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

Not applicable.

A.7.2. Between gene products introduced/modified and food constituents.

Not expected based on digestibility profile of dietary proteins.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

Amaranth has a historical and current food use in some populations. It is not known to be a source of significant toxicants or antinutrients. Unidentified, heat labile growth inhibiting factors have been reported, possibly consisting of lectins, trypsin inhibitors and phytates. Other antinutrients of potential concern include oxalates, nitrates, saponins, and tannins [Cheeke, 1998].

Applicable if the safety of donor organism is not known, or if the donor organism is a source of known toxicant or antinutrient.

B.1.1. Characterized gene modification.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Maize has a long history of consumption as food and does not contain significant toxicants or antinutritional factors (see also B.5.2).

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

The amaranth-derived protein is expected to provide a function in the modified maize similar to that in the source organism - as a storage protein.

B.3.2. Impact of gene product function in the modified plant.

The introduction of the amarantin will affect metabolic control of amino acid biosynthetic pathways and the accumulation of seed storage protein. However, the protein is not known to have a direct regulatory or signalling function in the native crop.

Applicable if gene product has an enzymatic function in the host plant.
B.3.3. Substrate specificity of gene product.

Not applicable - amarantin is not expected to function in biosynthetic pathways of the modified plant.

B.4. Potential for disrupting coding/regulatory DNA sequences in host plant.

B.4.1. In the inserted/modified DNA.

Southern hybridization and RT-PCR (reverse transcriptase-polymerase chain reaction) revealed integration of one copy of transgene at a single genetic locus with an expected molecular size.

Bioinformatics analysis and PCR assays do not suggest the presence of unknown DNA sequences in the inserted sequences.

B.4.2. In genomic sequences bordering the site of insertion/modification.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents, where applicable).

Analysis included proximates, amino acid, and fatty acid profiles, as well as several minerals and vitamins, as suggested in the OECD consensus document for food use of maize.

Total protein content was increased up to 30%, with between 20-40% change in individual amino acids, including lysine, tryptophan, and isoleucine. The associated changes in multiple amino acids with the introduction of the amarantin protein are not unexpected, given the nature of the intended modification. The amino acid scoring pattern supports the view that the protein quality of the modified maize was improved compared to the unmodified maize.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents, where applicable).

Phytic acid and raffinose were analyzed, as suggested in the OECD consensus document for food use of maize. No differences were noted in their levels in the modified and unmodified control.

Analysis of DIMBOA was not recommended because of the high variability of its levels among maize varieties, and the fragmentary knowledge of its toxicology.

B.5.3. Other metabolites/parameters.

Analytes included furfural, ferulic acid, and p-coumaric acid, which are notable secondary metabolites in maize, as described in the OECD consensus document.

Changes in metabolites mostly likely to be affected by the modification (i.e., proteins and amino acids) are described in B.5.1. Given that the changes in amino acid levels (B.5.1) are not unexpected and that there are no changes in the other metabolites analyzed (B.5.2 and B.5.3) or in the phenotype, no additional metabolites were analyzed.

B.6. Health consequences of instability of the modified trait.

The modified trait is intended to enhance the nutritional quality of the food derived from the modified crop. Depending on the dietary context, the modified food may or may not have practical health relevance.

PART C - POTENTIAL FOR ALLERGENIC (IGE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN SUBSTANCES.

C.1. Allergenicity of donor organism (gene source).

Amaranth is not a common source of allergens. However, a storage protein (11S globulin) in amaranth is a homologue of common allergens: Ber e 2, Cor a 9, and Ses i 6 present in Brazil nut, hazel, and sesame.

Protein gene products (factors C.2-C.4).

C.2. Potential for protein gene product(s) to cross react with known allergens.

Overall sequence identities of > 35% with known allergens were found.
Several exact matches were obtained with octamers, heptamers and hexamers, 2, 4, and 7, respectively. Most of the matches obtained with at least six contiguous amino acids identified were from 11S legume-related proteins and one from *Arachis hypogea*. Longer matches of greater than 40% identity with allergens in buckwheat, Brazil nuts, peanuts and soybeans have also been reported [Goodman et al., 2005]. The large amino acid similarity could be expected due to large sequence similarity among globulins.

C.3. Pepsin digestion (under standard testing conditions).

As noted in A. 5.2, the protein was almost completely digested within 20 min in pepsin digestion using simulated gastric fluids, followed by complete degradation within 15 min in simulated intestinal fluid.

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factors (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

Storage protein 11S globulin is a homologue of common allergens: Ber e 2, Cor a 9, and Ses i 6 present in Brazil nut, hazel, and sesame.

C.2. Heat stability (NOT applicable to lipid transfer proteins).

C.4.3. Abundance.

Amarantin was expressed at 0.5-1.2% of total protein.

C.5. Allergenicity of host plant.

Maize is not a common allergenic food, but case reports of skin, gastrointestinal and respiratory symptoms have been reported. Allergenic protein(s) identified include: Zea m 1 (beta-expansin 1b) and Zea m 14 (lipid transfer protein; pathogenesis related protein PR14).

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PRODUCT PROFILE

Modified trait	Delayed fruit ripening.
Intended food use of modified crop (host plant)	Tomato (*Lycopersicon esculentum*) is cultivated primarily for human consumption, either fresh or processed.
Gene (source) / Promoter (source)	The gene encoding an enzyme (ACCd*) derived from *Pseudomonas chlororaphis* was introduced in tomato under the control of constitutively expressed 35S promoter from figwort mosaic virus.
Transformation method	Agrobacterium-mediated transformation.
Metabolic reactions / pathways	Delayed ripening is achieved by reducing the biosynthesis of ethylene (a plant hormone important in fruit ripening), which was mediated by increasing the conversion of an essential precursor (ACC) to other metabolites, *ammonia and alpha-ketobutyrate. Ammonia* is redistributed through amino acid pathways, of which *alpha-ketobutyrate* is an intermediate metabolite. The biosynthesis of ethylene involves the conversion of methionine in a series of reactions including SAM and ACC.
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	ACCd enzyme.
Secondary gene products	*Ammonia, alpha-ketobutyrate and ethylene*: NOT assessed. No biologically significant change in dietary exposure to these intermediate metabolites are expected from altered metabolism in the modified crop that exhibits normal phenotype. No known safety concerns are expected with these existing components of edible plants and plant-derived foods at naturally occurring levels.

*Abbreviations: ACCd=1-aminocyclopropane-1-carboxylic acid deaminase SAM=S-adenosylmethionine ACC=1-aminocyclopropane-1-carboxylic acid

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop.

Level of Accd: <50 μg/g , fresh weight, or <0.4% total fruit protein, or <0.004% fruit fresh weight.

A.2. Prior history of known or presumed safe consumption from foods.

Dietary exposure to the enzyme Accd is likely from some plant foods through the adventitious presence of the gene source, which is a common soil bacterium.

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: No change in the food use of modified tomato is expected. Modified food will replace unmodified tomato in some uses.

Accd enzyme: No biologically significant increase in consumption is expected relative to current intakes based on expression level in the red ripe tomato fruit of the modified crop. Protein intake from tomatoes is <0.7% adult human recommended dietary allowances.

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

Low probability of an enzyme being toxic.

A.5. Structure-activity characterization.

Amino acid sequence identity with mammalian toxins.
No amino acid sequence identity with known toxin was found.

Pepsin digestion (under standard testing conditions).
Readily degraded under simulated mammalian digestive conditions, with no protein detected at 15 seconds, as determined by Western blot and confirmed by loss of Accd functional enzyme activity in the simulated digestive fluids.

Structural/functional comparability with comparator/source protein or non-protein substance.
Comparability to the native protein was judged by similarity in molecular weight (based on electrophoretic mobility), immunogenicity (Western blots with antibodies specific to the test protein), amino acid sequences (N-terminal sequencing), and enzymatic activities (deamination of ACC to *ammonia and alpha-ketobutyrate*).

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
No unusual breakdown products of protein in the modified plant are expected - no unusual protein bands were identified by Western blot analysis.

In mammalian metabolism from consumption of the gene product.
No unusual metabolic breakdown products of rapidly digested protein are expected.

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

Not applicable.

A.7.2. Between gene products introduced/modified and food constituents.

No interactions between Accd and food constituents that would affect nutrient bioavailability are expected based on its known function.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

Pseudomonas chlororaphis: A non-pathogenic soil bacterium.

Applicable if the safety of donor organism is not known or if the donor organism is a source of known toxicant or antinutrient.

B.1.1. Characterized gene modification.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Tomato belongs to the Solanaceae plant family that encompasses a large number of toxic plant species. Tomato is a source of steroidal glycoalkaloids, including alpha-tomatine, which is the primary toxic agent in tomatoes. Alpha-tomatine is present at higher concentration in green tomatoes (870 μg/g) compared to 450 and 360 μg/g in yellow and red tomatoes, respectively. Consumption of green tomato plants by pigs resulted in illness or death. Small amounts can also be found in the ripe fruit of certain tomato varieties close to the wild form (e.g., "tomadoses" in the Antilles). The alkaloid can be easily bred out. Alpha-tomatine and tomatidine (aglycone form of tomatine) have not been tested in oral toxicity or cancer bioassays other than intravenous injections in mice in which tomatidine was slightly more toxic than the parent glycoside [Beier, 1990].

Other glycoalkaloids include solanine and chaconine. The alkaloids cause membrane disruption in the digestive system and have anticholinesterase activity on the central nervous system. They have also been shown to be potentially teratogenic. Recent studies have shown that many food plants in the Solanaceae also contain significant levels of calystegines, polyhydroxytropane alkaloids that are potent inhibitors of glucosidases [Asano et al., 1997]. These alkaloids may also be involved in digestive disturbances through inhibition of gut enzymes. Antinutrients such as lectins and oxalate can also be present.

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

Accd is a novel enzyme in the ethylene biosynthesis pathway in tomato.

B.3.2. Impact of gene product function in the modified plant.

Tomato fruit ripening involves a series of coordinated biochemical reactions that result in fruit compositional changes and in fruit flavour development, including chlorophyll degradation and lycopene synthesis. Unintended effects with potential health-related concerns include: increased accumulation of metals and mycotoxin due to enhanced susceptibility of the modified plant to pathogens [Grichko et al., 2000; Nie et al. 2002; Klee, 2002].

Ethylene signaling pathway affects many metabolic processes in plants. The genes encoding the enzymes of the pathway are part of a multigene family.

Applicable if gene product has an enzymatic function in the host plant.

B.3.3. Substrate specificity of gene product.

B.4. Potential for disrupting coding/regulatory DNA sequences in host plant.

B.4.1. In the inserted/modified DNA.

Accd gene is present as a single, complete copy at a single locus and the size of DNA fragment is of the predicted size. Structural integrity of the inserted DNA was maintained during transfer (based on determination of molecular weight). Two additional tandem, incomplete copies of the *accd* gene at a different locus were not functional based on Northern blot and PCR assays.

B.4.2. In genomic sequences bordering the site of insertion/modification.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Compositional analysis of modified food included: total solids, protein, ash, carbohydrates, vitamins A and C, and folic acid. No statistically significant differences for any of the parameters between the modified and control tomatoes.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

In mature tomatoes, tomatine levels were low with no significant differences between modified and control lines. Comparable decrease in tomatine levels from immature to mature tomatoes was observed in both the modified and control crops. The initial levels in the immature fruit was slightly higher in the modified fruit (51.5 μg/g fresh fruit) compared with that in the control fruit (37.9 μg/g fresh fruit), but the levels were within those reported in the literature (49-900 μg/g fresh fruit).

B.5.3. Other metabolites/parameters.

Acidity of the tomato fruit inhibits the growth of thermophilic organisms during processing and is an important quality trait for food safety in the thermal processing of tomatoes by. No differences in pH were found between modified and control tomatoes.

B.6. Health consequences of instability of the modified trait.

Modified trait is not intended to affect human health.

PART C - POTENTIAL FOR ALLERGENIC (IGE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN SUBSTANCES.

C.1. Allergenicity of donor organism (gene source).

Pseudomonas chlororaphis: Bacteria are generally not known to be allergenic.

Protein gene products (factors C.2-C.4).

C.2. Potential for protein gene product(s) to cross react with known allergens.

No identity of > 35% was found when the deduced amino acid sequence of the introduced protein was compared to the sequences of known allergens.

C.3. Pepsin digestion (under standard testing conditions).

As noted in A.5, the modified Accd enzyme was readily degraded under simulated mammalian digestive conditions, as determined by Western blot and confirmed by loss of Accd functional enzyme activity.

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factor (C.3) is insufficient or equivocal, or if the criteria for the factor is not met.***

C.4.1. Structural/functional characteristics.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

C.4.3. Abundance.

C.5. Allergenicity of host plant.

Tomato is not a major source of allergens, but several glycoproteins are known to be allergenic, including: profilin, beta-fructofuranosidase, and clones 1 and 2, anther specific LAT52 protein, thaumatin-like protein NP24. The latter two proteins are non-IUIS* allergens.

* International Union of Immunological Societies.

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PRODUCT PROFILE

Modified trait	Resistance to insects.
Intended food use of modified crop (host plant)	Potatoes (*Solanum tuberosum*) are increasingly consumed in a processed form (e.g., French fries and potato chips) while the direct consumption of potatoes (e.g., boiled potatoes) has declined dramatically. Potatoes should be cooked before eating because of the indigestibility of non-gelatinised starch and the presence of anti-nutritional proteins.
Gene (source) / Promoter (source)	**Cry**: Gene encoding an insecticidal protein (Cry1Ac) from *Bacillus thuringiensis subsp. kurstaki.* **GNA**: Gene encoding a lectin protein, Galanthus nivalis agglutinin (GNA), from snowdrop (*Galanthus nivalis*). Both genes were under the control of constitutively expressed cauliflower mosaic virus 35S promoter.
Transformation method	Agrobacterium-mediated transformation. A recombinant expression system was used to produce Cry/GNA fusion protein, generated by fusing the C-terminus of Cry to the N-terminus of GNA. No change in amino acid sequence of the component proteins.
Metabolic reactions / pathways	Both the Cry and GNA proteins are immediate products encoded by the gene introduced in the crop and confer the desired phenotype.
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	The component proteins of the Cry/GNA fusion protein: 1) Cry and 2) GNA proteins.
Secondary gene products	Not applicable.

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop.

Cry: < 0.05 ppm, or 0.0001% total protein.

GNA: Up to 2.0% of total soluble protein.

A.2. Prior history of known or presumed safe consumption from foods.

Cry: Cry1Ac is among several *Bt* proteins that have a long history (approximately 40 years) of safe application in *Bt* microbial products as pesticide approved for use on food crops. In these formats, *Bt* insecticidal crystal proteins (ICPs) are present as protoxins, which require solubilisation in the midgut of the target insect and the conversion of the protoxin to the biologically active toxin by proteolytic enzymes. In *Bt* crops, the plant tissues produce specific ICPs in a soluble form.

GNA: No prior dietary exposure.

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: No change in the food use of modified potato is expected. Modified food will replace unmodified tomato in some uses.

Cry: Given the expression level and estimated potato consumption of consumers only at the 90th percentile intakes (assuming consumption of 100% modified potatoes), human daily intakes of the Cry protein are estimated to be less than 0.1 μg/kg bw/day, compared to NOELs (acute toxicity) published for Cry1Ac protein (> 4200 mg/kg/day). Wide margin of exposure is also expected based on toxicity of several families of Cry protein reported in the literature. No tolerance limit is required for this class of pesticidal proteins.

GNA: An expression level of up to 2.0% total soluble protein in the modified potato is estimated to result in an intake of approximately 0.8 g/day of the GNA lectin for the general population, based on current level of potato consumption (90th percentile for consumers only of potato, assuming consumption of 100% modified potato).

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

Cry: The insecticidal efficacy of *Bt* ICPs, which are delta-endotoxins, depends on specific membrane receptor binding by the C-terminal domain of the active toxin and pore formation by the N-terminal domain with subsequent lysis of the epithelial cells. Receptor binding by the ICP is the major determinant of host specificity by the different *Bt* ICPs[IPCS, 1999].

Bt crystal proteins bind only to receptors on intestinal cells of a narrow range of lepidopteran and coleopteran insects. No receptors for delta-endotoxins of *B. thuringiensis* in mammalian intestinal cells have been identified. Cry1Ac protein is insecticidal only to lepidopteran insects. The specific mechanism of toxicity is not applicable to humans.

GNA: GNA lectin is a monocot mannose-specific binding protein. It has been hypothesized that carbohydrate-binding proteins can be accumulated in large quantities in seeds and/or vegetative storage organs of plant species as part of plant defense mechanisms [Peumans and Van Damme, 1996]. GNA shares similar molecular structure and sugar-binding specificity with lectins from four other plant families (Alliaceae, Araceae, Liliaceae, and Orchidaceae).

Although typical lectins from different plant sources exhibit a considerable degree of structural similarity, lectin GNA from snowdrop has lower toxicity to mammals compared to lectins from kidney bean (PHA) and wheat germ (WGA) [Peumans and Van Damme, 1996]. The antinutritional effects of ingested lectins are likely due to their ability to combine with specific receptor sites of intestinal cells and to cause a non-specific interference with nutrient absorption and utilization. The severity of these adverse effects will depend on the gut region to which the lectin will bind. Lectins have also been associated with binding to intestinal and red blood cells. Other adverse effects of plant lectins include: morphological and physiological modifications in the small intestine, inhibition of intestinal and brush border enzymes, and systematic effects, affecting the immune system, hormonal homeostasis, and enlargement of the liver and hypertrophy. It is plausible to suppose that the adverse effects of dietary lectins observed in experimental animals will be comparable in humans [Vasconcelos and Oliveira, 2004]. Most of the information on the toxicity of lectins in humans has been derived from acute toxicity in incidences of accidental poisoning from PHA.

The ability of dietary lectins to interact with the immune system in facilitating the autoimmune process in genetically susceptible individuals was reviewed by Cordain et al. [2000]. Dietary lectins may interact with enterocytes and lymphocytes to facilitate the translocation of gut-derived bacterial and dietary antigens to peripheral tissues and cause persistent peripheral antigenic stimulation. This may cause antibodies or T-lymphocytes to cross-react with both foreign and endogenous peptides.

A.5. Structure-activity characterization.

Amino acid sequence identity with mammalian toxins.
No amino acid sequence identity with known protein toxin was identified for Cry and GNA.

Pepsin digestion (under standard testing conditions).
Cry: Rapid digestion - no traces of Cry1Ac protein detected at 2 min.

GNA: The protein is highly resistant to digestion.

Structural/functional comparability with comparator/source protein or non-protein substance.
Cry: Equivalence to source protein (*Bt*-derived Cry1Ac) is based on molecular weight (electrophoretic mobility), immunogenicity (Western blots with antibodies specific to the test protein), and functional activity (bioassay on target organism).

GNA: Comparability in molecular weight between potato-derived and native GNA was indicated by similar electrophoretic mobility on SDS-PAGE. Comparable immunogenicity was suggested by Western immunoblot analysis. N-terminal amino acid sequencing and mass spectrometry of proteolytic fragments did not reveal the presence of N-terminal signal peptide and N-linked glycosylation sequences. Specificity of toxicity to target pests was verified by insect assays, as expected based on known activity of the native protein.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
No unusual breakdown products of Cry or GNA protein in the modified plant are expected.

In mammalian metabolism from consumption of the gene product.
Cry: No concerns with ingested protein that is rapidly digested.

GNA: In rats, some lectins increase their binding sites to gut tissue by mediating changes in the glycosylation of the gut, with associated disruption of the brush borders and disorganization of the main absorptive cells. Lectins bound to epithelial cells may be taken up systematically by endocytosis, released by exocytosis into the intracellular space from where they are subsequently transported throughout the body [Cordain et al., 2000].

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

A possibility exists that GNA can facilitate the penetration by cry protein of the brush-border membranes of the gut lumen by increased permeability of the membranes or by lectin-mediated co-endocytosis [Peumans and Van Damme, 1996].

A.7.2. Between gene products introduced/modified and food constituents.

Cry: No interactions between cry protein and food constituents that would affect nutrient bioavailability are expected based on its known function.

GNA: There is the potential for disruption of nutrient absorption, as described above (A.6).

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

Cry: *Bacillus thuringiensis subsp. kurstaki* is a common bacteria with no known adverse effects on humans or animals.

GNA: There is limited information on toxic metabolites in snowdrop (*Galanthus nivalis*). Taxonomically, *Galanthus nivalis* is part of the Amaryllidaceae plant family. Several genera in this family, including the *Galanthus* genus, have caused poisonings in humans and animals when consumed during times of food shortages. The bulbs of these species contain galanthamine, a competitive cholinesterase inhibitor as well as other related alkaloids [Lopez et al., 2002].

Applicable if the safety of donor organism is not known, or if the donor organism is a source of known toxicant or antinutrient.

B.1.1. Characterized gene modification.

Cry: Not applicable.

GNA: The complete sequence of the DNA insert used in the genetic modification was characterized. No other unknown DNA sequence was transferred to the host plant.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Potato belongs to the Solanaceae plant family that encompasses a large number of toxic plant species. Potato is a source of known toxicants for which limits have been established. Major metabolites of concern include:

Toxicants: Several types of alkaloids, with glycoalkaloids being the most important, 95% of which consist of α-chaconine and α-solanine, which are present in higher concentrations at the periphery, in green and sprouted tubers. These glycoalkaloids are not destroyed during cooking and frying. The widely accepted tolerance limit of total glycoalkaloids in tuber is 200 mg/kg, fresh weight, was based on acute and/or subacute effects. New standards based on chronic effects may be warranted [Korpan et al., 2004]. Susceptibility of humans to glycoalkaloids appears to be high and variable: 1-5 mg/kg body weight oral doses are marginally to severely toxic; 3-6 mg/kg body weight can be lethal. Margin of exposure for potato glycoalkaloids may be between 2 and 6, assuming a low observed effect level in humans of 2 mg/kg bw, an average level of glycoalkaloids in potato and an average daily consumption of potato in western countries [Nordic Working Group, 1991].

Anti-nutrients: Several protease inhibitors and lectins are present, inhibiting the activity of trypsin, chymotrypsin and other proteases, or binding to specific carbohydrate structures on cell surfaces (e.g., intestinal or blood cells) - an activity that is largely inactivated by boiling and other thermal processes.

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

Both Cry and GNA proteins exhibited the expected phenotype in reducing damage by target insect pests. These proteins are not expected to have other functions in the modified plant, except as insecticidal proteins.

B.3.2. Impact of gene product function in the modified plant.

Both Cry and GNA proteins are not expected to have metabolic, regulatory, or signalling function in the modified plant.

Applicable if gene product has an enzymatic function in the modified plant.
B.3.3. Substrate specificity of gene product.

Not applicable to both Cry and GNA proteins.

B.4. Potential for disrupting coding/regulatory DNA sequences in the modified plant.

B.4.1. In the inserted/modified DNA.

Transformation event was characterized using Southern blot, polymerase chain reaction, and nucleotide sequence analyses. Molecular analyses indicated that the transformation event contained 2 copies of the expression cassette. Several copies of other genetic elements were identified at 1 insertion site (including a selectable marker to identify bacteria transformed with recombinant plasmid DNAs) but they were not expressed or functional. This was supported by Northern blot and Western blot analyses.

B.4.2. In genomic sequences bordering the site of insertion/modification.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Analysis included proximates (dry matter), sugars, including reducing sugars, protein and vitamin C, as suggested in the OECD consensus document for food use of potato. Differences in macro- and micronutrients between transgenic and control potatoes were within the normal range of variation reported for commercial varieties.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

Total glycoalkaloids were below the guidelines of 20 mg/100 g fresh weight for potatoes.

B.5.3. Other metabolites/parameters.

No other metabolites were analyzed. Analysis of nutrients and known toxicants and antinutrients is considered sufficient in detecting unintended changes with the potential for adverse health effects, given the factors considered above, including the lack of significant changes in key nutrients, toxicants, and antinutrients. The GNA protein is not expected to interact with the host plant's metabolic pathways. Based on these observations and the absence of phenotypic alterations, no other metabolites were analyzed.

B.6. Health consequences of instability of the modified trait.

The modified trait is not intended to affect human health.

PART C - POTENTIAL FOR ALLERGENIC (IGE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN SUBSTANCES.

C.1. Allergenicity of donor organism (gene source).

Cry: *Bacillus thuringiensis subsp kurstaki* - bacteria not known to be allergenic. **GNA**: There are limited data on *Galanthus nivalis* as an allergenic source. Amaryllis in the same Amaryllidaceae family has been associated with occupational asthma [Jansen et al., 1996]. Sixteen common plant lectins can trigger histamine release from basophils. Antibodies to common dietary legume lectins are present in the sera of healthy humans.

Protein gene products (factors C.2-C.4).

C.2. Potential for protein gene product(s) to cross react with known allergens.

No amino acid sequence identity with known allergens was found for both Cry and GNA proteins.

C.3. Pepsin digestion (under standard testing conditions).

Cry: Rapid digestion - no traces of Cry1Ac in < 1 min.

GNA: As noted in A.5, GNA is resistant to digestion.

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factor (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

Cry: Absence of post-translational modification (glycosylation) was confirmed by glycoprotein assays.

GNA: As note in A.5, no N-terminal signal peptide and N-linked glycosylation sequences were detected in the potato-derived GNA. The absence of post-translational glycosylation was also confirmed by glycoprotein assays.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

Cry: Not heat stable.

GNA: GNA is heat stable.

C.4.3. Abundance.

Cry: < 0.0001% total protein.

GNA: Up to 2.0% total soluble protein.

C.5. Allergenicity of host plant.

Potato is not a common source of allergens. However, potato contains multiple heat-labile proteins which can induce immediate hypersensitivity reactions when raw potatoes are consumed. Patatin, the main storage protein in potatoes, has been associated with allergic reactions in sensitive children - allergenicity of patatin to be confirmed. In addition, IgE binding to several proteins belonging to the family of soybean trypsin inhibitors was observed. Allergenic proteins include: cathepsin D inhibitor, cysteine protease inhibitor, and aspartic protease inhibitor.

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PRODUCT PROFILE

Modified trait	Broad spectrum pathogen resistance through systemic acquired resistance and induced systemic resistance[3].
Intended food use of modified crop (host plant)	Tomato (*Lycopersicon esculentum*) is cultivated primarily for human consumption, either fresh or processed.
Gene (source) / Promoter (source)	Non-expresser of pathogenesis-related genes 1 (*NPR1*) from *Arabidopsis thaliana* was introduced under the control of cauliflower mosaic virus (CaMV) 35S promoter.
Transformation method	Agrobacterium-mediated transformation.
Metabolic reactions / pathways	Nuclear NPR1 is a regulatory protein that activates the expression of pathogenesis-related (*PR*) genes through interaction with a subgroup of the TGA family of transcription factors in systemic acquired resistance. In pathogen infection, a cascade of events are triggered by the accumulation of salicylic acid induced by the infection. In addition, NPR1 also has a cytosolic function in the cross-talk between jasmonic acid- and ethylene-dependent defense pathways in induced systemic response and in insect-inflicted wound response [Dong, 2004; Pieterse and van Loon, 2004].
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	NPR1 protein and pathogenesis-related (PR) proteins (see A.1).
Secondary gene products	N/A.

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop for food.

NPR1 protein: The transgenic protein with the expected molecular size was detected in the transgenic line using Western blot. Average expression level in the edible part of the crop was less than 0.01% protein.

PR proteins: Compared to the wild-type plants in which *PR* genes are constitutively expressed at very low levels, the expression of a few *PR* genes (*PR1b1*, *PR1p6*, *GLUa*, *GLUb*, *CHI3*, and *CHI9*) was enhanced in the transgenic line. Using Western blot, the proteins encoded by these *PR* genes were identified. They include: basic pathogenesis-related proteins 1, acidic and basic glucanases, and chitinases. Constitutive levels of PR proteins in the transgenic line remained very low (< 0.1% total protein) compared to induced levels (1% total protein for each PR protein), which were comparable to those reported for wild-type plants [Heil and Bostock, 2002].

[3] Modes of action of induced pathogen resistance in plants include (1) systemic acquired resistance (SAR), (2) induced systemic resistance (ISR), (3) wound-induced defense, and (4) R-gene-mediated resistance (also termed gene-for-gene resistance). SAR is triggered by necrotizing pathogens, whereas ISR is activated upon colonization of roots by selected strains of non-pathogenic rhizobacteria. Wound-induced defense is typically elicited upon tissue damage such as that caused by feeding insects, and R-gene-mediated resistance is a response that detects specific pathogen races through the recognition of pathogen-encoded Avirulence (Avr) proteins. R-gene mediated resistance is an immediate response in local tissue to an attack by a pathogen or herbivore, whereas SAR and ISR provide temporary post-attack immunity to other plant tissues.

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A.2. Prior history of known or presumed safe consumption from foods.

NPR1 protein: Homologues of NPR1 have been identified in several food crops, including rice, sunflower, and oilseed rape. Given their important role in pathogen resistance in plants, NPR1-like proteins are expected to be common in other food crops.

PR proteins: endogenous to the tomato plant.

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: No major changes in food use of the modified crop are expected. Modified tomato will replace unmodified tomato in the same uses.

NPR1 protein: NPR1 in the modified tomato presents an additional source of the protein to the diet at very low level, estimated to be < 1 μg/kg-bw or < 20 ppb diet.

PR protein: PR proteins are also expected to be present at low levels in the transgenic line. Their enhanced expression is not expected to result in physiologically significant changes in dietary exposure relative to natural variations in their constitutive or induced expression levels. *No further assessment of potential toxic and antinutritional effects will be necessary.*

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

NPR1 protein: Taking into consideration that the structure and function of a protein like NPR1 would have been conserved across a wide variety of species, including those that have a dietary history, NPR1 protein derived from *Arabidopsis thaliana* is not expected to pose safety concerns. It has been proposed that NPR1 possesses extensive sequence conservation with the mammalian protein I_kB_α and that it may represent the plant homologue of I_kB_α [Després et al., 2000].

A.5. Structure-activity characterization.

Amino acid sequence comparison with mammalian toxins.
NPR1 protein: No similarity in amino acid sequence identity with known protein toxin was identified.

Pepsin digestion (under standard testing conditions).
NPR1 protein: The protein was rapidly and completely digested in less than 1 min.

Structural/functional comparability with comparator/source protein or non-protein substance.
NPR1 protein: The tomato-derived NPR1 protein has the expected molecular weight compared to the protein derived from *Arabidopsis thaliana.* NPR1 in the modified tomato also exhibited the expected functional characteristics. When challenged with specific pathogens, the modified lines exhibited increased level of resistance and lower disease symptom severity compared to the wild-type plants.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
NPR1 protein: No unusual degradation products of protein are expected.

In mammalian metabolism from consumption of the gene product.
NPR1 protein: The protein was rapidly digested in pepsin digestibility test - no unusual protein degradation products are expected.

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

Not applicable - Only one protein (NPR1) was introduced. The expression of multiple endogenous PR proteins is part of normal induced plant defense response and is not expected to pose new toxic concerns for humans.

A.7.2. Between gene products introduced/modified and food constituents.

Both NPR1 and PR proteins: Not expected, based on their levels in the diet and rapid and complete digestion.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

NPR1 protein: The protein is from a non-edible plant, *A. thaliana,* which belongs taxonomically to the Family of *Brassicaceae* (alt. *Cruciferae*) and subfamily of *Brassicoideae.* Several species in the family contain either cyanogenic or goitrogenic substances.

Applicable if the safety of donor organism is not known, or if the donor organism is a source of known toxicant or antinutrient.

B.1.1. Characterized gene modification.

The sequence of the DNA insert used in the genetic modification was characterized. There is no evidence of other unidentified sequences being transferred to the host plant.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Tomato belongs to the Solanaceae plant family that encompasses a large number of toxic plant species. Tomato is a source of steroidal glycoalkaloids, including alpha-tomatine, which is the primary toxic agent in tomatoes. Alpha-tomatine is present at higher concentration in green tomatoes (870 μg/g) compared to 450 and 360 μg/g in yellow and red tomatoes, respectively. Consumption of green tomato plants by pigs resulted in illness or death. Small amounts can also be found in the ripe fruit of certain tomato varieties close to the wild form (e.g., "tomadoses" in the Antilles). The alkaloid can be easily bred out. Alpha-tomatine and tomatidine (aglycone form of tomatine) have not been tested in oral toxicity or cancer bioassays other than intravenous injections in mice in which tomatidine was slightly more toxic than the parent glycoside [Beier, 1990].

Other glycoalkaloids include solanine and chaconine. The alkaloids cause membrane disruption in the digestive system and have anticholinesterase activity on the central nervous system. They have also been shown to be potentially teratogenic. Recent studies have shown that many food plants in the Solanaceae also contain significant levels of calystegines, polyhydroxytropane alkaloids that are potent inhibitors of glucosidases [Asano et al., 1997]. These alkaloids may also be involved in digestive disturbances through inhibition of gut enzymes. Antinutrients such as lectins and oxalate can also be present.

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

Function of NPR1 protein - see description under "Metabolic reactions/pathways" on p.1.

B.3.2. Impact of gene product function in the modified plant.

NPR1 protein is a regulatory protein that interacts with transcription factors and functions in the cross-talk between jasmonic acid- and ethylene-dependent defense pathways.

Applicable if gene product has an enzymatic function in the host plant.
B.3.3. Substrate specificity of gene product.

Not applicable - NPR1 protein does not have an enzymatic function in the modified plant.

B.4. Potential for disrupting coding/regulatory DNA sequences in host plant.

B.4.1. In the inserted/modified DNA.

Southern hybridization and RT-PCR (reverse transcriptase-polymerase chain reaction) revealed integration of one copy of transgene at a single genetic locus with an incomplete inverted repeat.

Comparison of DNA insert and pre-insertion plant DNA revealed rearrangements at the insertion site, including plant DNA deletion, duplication, and truncation. These rearrangements are common in transformation using recombinant plasmids that carry vector backbone sequences and have no impact on the expression of the target proteins.

Bioinformatics analysis does not suggest the presence of unknown DNA sequences in the inserted sequences.

B.4.2. In genomic sequences bordering the site of insertion/modification.

Sequence analysis supports that the sequences flanking the insertion regions (about 500 bp) correspond to plant DNA.

Bioinformatics analysis does not suggest the presence of unknown DNA sequences in the border integration sequences.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Compositional analysis of modified food included: total solids, protein, ash, carbohydrates, vitamins A and C, and folic acid. No statistically significant differences for any of the parameters between the modified and control tomatoes.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

In mature tomatoes, tomatine levels were low with no significant differences between modified and control lines. Comparable decrease in tomatine levels from immature to mature tomatoes was observed in both the modified and control crops. The initial levels in the immature modified fruit (51.5 μg/g fresh fruit) was slightly higher compared with that in the control fruit at a similar stage of maturity (37.9 μg/g fresh fruit), but the levels were within those reported in the literature (49-900 μg/g fresh fruit).

B.5.3. Other metabolites/parameters.

Acidity of the tomato fruit inhibits the growth of thermophilic organisms during processing and is an important quality trait for food safety in the thermal processing of tomatoes. No differences in pH were found between modified and control tomatoes.

Changes in the induced levels of PR proteins were assessed using challenge tests with specific pathogens. The assessment included glucanases and chitinases that are homologues of known allergens in the pathogenesis-related protein families. Changes in the induced levels were highly variable in both the modified and control plants.

B.6. Health consequences of instability of the modified trait.

Modified trait is not intended to affect human health.

PART C - POTENTIAL FOR ALLERGENIC (IGE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN PRIMARY AND SECONDARY GENE PRODUCTS.

C.1. Allergenicity of donor organism (gene source).

A thaliana is a source of 2 putative (non IUIS*) allergenic proteins: Ara t 8 (profilin) and Ara t expansin (homologue to group I allergens of grass pollen).

Protein gene products (factors C.2-C.4).

C.2. Potential for protein gene product(s) to cross react with known allergens.

NPR1 protein: For the 80 amino acids sliding window alignments, 35-36% amino acid sequence identity was found with 2 known allergens in 1 out of 3 allergen databases [Allermatch , FARRP, SDAP]. The 2 allergens identified were: Equ c 3 (Ag3 - albumin from domestic horse) and Sol i 4 (an insect allergen). No exact match for 6-8 amino acids, or homologous allergenic proteins were identified using full FASTA alignment in all 3 databases.

C.3. Pepsin digestion (under standard testing conditions).

NPR1 protein: The protein was rapidly and completely digested in less than 1 min.

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factor (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

C.4.3. Abundance.

See A.1.

C.5. Allergenicity of host plant.

Tomato is not a major source of allergens, but several glycoproteins are known to be allergenic, including: profilin, beta-fructofuranosidase, and clones 1 and 2, anther specific LAT52 protein, thaumatin-like protein NP24. The latter two proteins are non-IUIS* allergens.

* International Union of Immunological Societies.

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Allergen databases (accessed November18, 2005):

Allermatch: http://allermatch.org.

FARRP (The Food Allergy Research and Resource Program): http://www.allergenonline.com.

SDAP (Structural Database of Allergenic Proteins): http://fermi.utmb.edu/SDAP/sdap_who.html.

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PRODUCT PROFILE

Modified trait	Increased iron level.
Intended food use of modified crop (host plant)	Rice (*Oryza sativa*), polished or brown, is used for food in various forms. In limited amounts, rice is also used to manufacture starch.
Gene (source) / Promoter (source)	Ferritin gene from soybean (*Glycine max L.*) was expressed under the control of a rice seed-storage protein glutelin promoter GluB-1.
Transformation method	Agrobacterium-mediated transformation.
Metabolic reactions / pathways	In plants, ferritin synthesis is tightly regulated both spatially and temporally, in part through the differential expression of the various members of a small gene family. Ferritin synthesis is also responsive to developmental and environmental cues. Plant ferritin gene regulation is iron-dependent and appears to consist of a complex interplay of transcriptional and posttranscriptional mechanisms and cellular processes involving plant hormones, oxidative steps and Ser/Thr phosphatase [Briat et al., 1999].
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	Ferritin protein and associated iron.
Secondary gene products	

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop for food.

Ferritin protein: is estimated to be expressed at a level up to 0.3% of the total seed protein.

Iron: There was a 3-fold increase in the mature whole seeds of the modified crop, varying between 13.3 to 38.1 μg/g dry weight, compared to 8.6 to 14.3 μg/g dry weight in the unmodified crop.

A.2. Prior history of known or presumed safe consumption from foods.

Both the ferritin protein and iron are food constituents naturally occurring in the diet.

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: The following analysis assumes that iron fortification of rice and other foods is discretionary from a regulatory perspective.

No major changes in food use of the modified crop are expected in population groups for whom rice is a staple food that is regularly consumed. For individuals who are not regular consumers of rice, the modified crop may replace part of the consumption of other grain-based foods because of its iron content. As a result, consumption of modified rice may increase for these individuals. As an ingredient, modified rice can also replace unmodified rice in the same uses.

Ferritin protein: Modified rice will provide an additional source of ferritin protein of less than 1 g in the diet for rice consumers only at 90th percentile rice intake in North America (using an overestimate of 100% modified rice). This increased level of protein consumption from modified rice containing the ferritin protein is within the range of protein intakes.

Iron intake: Increased iron intake can result from an increase in the frequency and/or quantity of consumption of modified rice. For some consumers, the iron content in a meal-size portion of modified rice (2.0-5.7 mg iron/150 g dry weight) would be sufficient to provide 15-50% of the daily adult iron requirement (approximately 13-15 mg Fe).

In North America, the highest median reported intake of iron from food and supplements (approximately 19 mg/day) was by men between 31 to 50 years of age. The highest intake from food and supplements at the 90th percentile reported for any life stage and gender group, excluding pregnancy and lactation, was approximately 34 mg/day for men 51 years of age and older.

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

Ferritin protein: There are no toxicological concerns with a protein that is a food constituent.

Iron: Iron is a redox-active transition metal. In the body iron is shielded from unwanted participation in redox reactions by transport and storage mechanisms that hold the iron in the ferric state. If these mechanisms are overwhelmed, the free iron will immediately be chelated by cellular compounds and readily participate in redox reactions catalyzing the formation of highly toxic free radicals or the initiation of lipid peroxidation.

Acute adverse effects of iron are mostly associated with intake of iron supplements. Chronic iron accumulation in the liver is a risk factor for hepatocellular carcinoma in patients with hemochromatosis. Dietary iron intake may also be a risk factor for cardiovascular disease although current evidence does not provide convincing support for a causal relationship. Tolerable upper intake levels (40 and 45 mg/day for children, and adults, respectively) have been developed based on gastrointestinal effects following supplemental intakes of iron salts [IOM, 2000]. Relative to current levels of total iron intakes from foods and supplements, the risk of adverse effects from dietary sources appears to be low. Certain susceptible groups may not be protected by the upper intake levels. They include individuals with hereditary hemochromatosis, chronic alcoholism, alcoholic cirrhosis, and other liver diseases, iron-loading abnormalities, particularly thalassemias, congenital atransferrinemia, and aceruloplasminemia [IOM, 2000]. Effective screening is needed for early detection of genetic abnormalities among susceptible groups.

A.5. Structure-activity characterization.

Amino acid sequence comparison with mammalian toxins.
Ferritin protein: No similarity in amino acid sequence identity with known mammalian toxins was found.

Pepsin digestion (under standard testing conditions).
Ferritin protein: The recombinant ferritin protein is resistant to proteolysis. This characteristic is similar to that of the native protein from soybean and other dietary ferritins that survive gastric digestion [Theil, 2004]. This property presumably facilitates iron absorption in the gut.

Structural/functional comparability with comparator/source protein or non-protein substance.
Ferritin protein: Soybean ferritin was synthesized as a 28 kDa protein in transgenic rice seeds, corresponding to the expected size of that observed in the native soybean cotyledon. Additional evidence supporting the structural equivalence between the soybean- and rice-derived proteins was provided by tryptic peptide mass fingerprinting with MALDI-TOF mass spectrometry and sequencing of two internal peptides. The levels of iron binding and

accumulation between the two proteins were not comparable. This was not unexpected as iron uptake and transport in plants are affected by many factors [Qu et al., 2005].

Iron: Studies of iron diffusion and iron uptake using food extracts in an i*n vitro* Caco-2 cell digestion model suggested differences in iron bioavailability between the native and recombinant soybean ferritin proteins. This could be attributable to different phytate content in the food extracts. Further analysis using phytase-digested extracts and purified ferritin supports the view that iron bioavailability from the two proteins is comparable.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
Ferritin protein: The absence of proteolytic products or unusual bands in Western blot analysis indicated that the soybean ferritin was stable in the rice seed.

In mammalian metabolism from consumption of the gene product.
Ferritin protein and iron: There are no concerns with the digestion and metabolism of ferritin protein and associated iron that is derived from a food source and that is structurally similar to the native protein.

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

Not applicable.

A.7.2. Between gene products introduced/modified and food constituents.

Ferritin protein and iron: No adverse interactions between endogenous food constituents in rice and the heterologous ferritin protein and increased iron levels are expected, based on the known biological function and levels in the modified rice. Iron availability is affected by other dietary factors. However, the effect of dietary iron on the absorption of other mineral nutrients is generally not a concern.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

Soybean belongs to an extremely large legume family, Fabaceae, which encompasses numerous genera that are toxic. Substances of relevance in soybean include:
Antinutrients: trypsin inhibitors, lectins – both subject to degradation upon heating; stachyose and raffinose, and phytic acid. Non-ruminants are particularly susceptible to the adverse effects of trypsin inhibitors in raw soybeans. Other antinutrients, with the exception of phytic acid, do not appear to be of practical significance in animal nutrition where soybeans and soybean meals area major protein source [Cheeke, 1998].
Bioactive substances: phytoestrogens (isoflavones).

Applicable if the safety of donor organism is not known, or if the donor organism is a source of known toxicant or antinutrient.
B.1.1. Characterized gene modification.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Rice is a well-established food crop with no known toxicants in the form customarily consumed. The bran fraction contains antinutrients (phytic acid, trypsin inhibitor, lectins).

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

Ferritins are part of the iron storage mechanism with a capacity to store up to 4500 iron atoms in their cavity in a soluble and bioavailable form. Ferritins provide an iron storage function at early stages of plant development for the synthesis of iron-containing proteins involved in photosynthesis. During germination, ferritins are degraded and iron is used for the growth of the seedling. They are also key proteins in long-term iron storage. It is also known that free-radical-mediated alteration of proteins is a signal for their degradation.

Our knowledge of the role that ferritins play in plant physiology is still very limited. Studies overexpressing ferritins in transgenic plants are beginning to increase our understanding of their role in plants and any metabolic consequences that may be relevant to the safety assessment of foods derived from plants overexpressing ferritins.

Iron is required in essential processes in plants, such as respiration and photosynthesis. It is involved in electron transfer reactions in these processes. Iron is also an essential cofactor of enzymes required for fundamental reactions such as DNA synthesis, or plant-specific pathways like nitrogen fixation or phytohormone synthesis. Due to their immobility, plants are highly dependent on their environment and need tight regulation of iron uptake, transport and storage to ensure optimal development.

B.3.2. Impact of gene product function in the modified plant.

Although ferritin protein is important in the storage and release of iron for essential plant processes, knowledge about its role in the metabolism of secondary metabolites is limited. Any disruptions in plant metabolism due to overexpression of ferritin protein in the modified crop affecting essential processes are likely to result in observable changes in phenotype that would signal the need for additional scrutiny for unintended effects.

Applicable if gene product has an enzymatic function in the host plant.
B.3.3. Substrate specificity of gene product.

B.4. Potential for disrupting coding/regulatory DNA sequences in host plant.

B.4.1. In the inserted/modified DNA.

Southern blot analysis identified at one locus, 2 intact copies of the expression cassette. A truncated gene was identified on another locus, but was determined to be non functional by bioinformatics, Northern, and Western analyses. Bioinformatics analysis did not identify additional unknown sequences.

B.4.2. In genomic sequences bordering the site of insertion/modification.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Compositional analysis of the modified food included parameters suggested in the OECD Consensus Document for food use of rice: proximate analysis, amino acids (including valine, leucine or isoleucine), and fatty acids. No differences between the modified lines and conventional commercial lines were detected.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

Amylase and phytic acid were analyzed, in addition to trypsin inhibitor and lectin. For all the varieties tested, the levels of trypsin inhibitor and lectins were below the threshold of detection. No differences between the modified lines and conventional commercial lines were detected.

B.5.3. Other metabolites/parameters.

Given the lack of genomic changes that may indicate expression of unknown genes and the absence of phenotypic changes, analysis of key nutrients and known toxicants and antinutrients in modified rice kernels is considered sufficient. No other metabolites were analyzed.

B.6. Health consequences of instability of the modified trait.

The modified rice is intended to affect a nutritional attribute of rice for human consumption. Methods are being developed to facilitate rapid field testing of iron level in the crop. Master seedbank and working seedbank are also being developed to ensure consistent lot-to-lot growth of the plant and expression of the target gene products.

PART C - POTENTIAL FOR ALLERGENIC (IGE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN PRIMARY AND SECONDARY GENE PRODUCTS.

C.1. Allergenicity of donor organism (gene source).

Soybean is a commonly allergenic food, containing several antigenic proteins. Several gastrointestinal reactions have been attributed to the 7S and 11S globulins in soybean proteins that comprise about 85% of total proteins.

Protein gene products (factors C.2-C.4).

C.2. Potential for protein gene product(s) to cross react with known allergens.

No overall amino acid sequence identity of > 35 % with known allergens in an 80 amino acids sliding window or exact matches of 6-8 contiguous amino acids were found.

C.3. Pepsin digestion (under standard testing conditions).

As noted in A.5, both the recombinant and native ferritin proteins are resistant to proteolysis.

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factor (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

No post-translational glycan formation was identified by glycoprotein assay.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

Heat stable.

C.4.3. Abundance.

Up to 0.3% of total seed protein.

C.5. Allergenicity of host plant.

Rice is a less common allergenic source in a population for whom rice is not a major component of the diet. Allergenic reaction (atopic dermatitis) is associated with a 16 kDa protein, which is a member of the alpha-amylase/trypsin inhibitor family of proteins. Other allergenic proteins in the 14-15 kDa range, present in the albumin plus globulin protein fraction of the rice endosperm, have also been reported.

References.

Briat J-F et al. (1999) Regulation of plant ferritin synthesis: how and why. Cellular and Molecular Life Sciences 56, 155-166.

Goto F et al. (1999) Iron fortification of rice seed by the soybean ferritin gene. Nature Biotechnology 17, 282-286.

IOM (Institute of Medicine) (2000) Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, boron, chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. Washington, DC: National Academies Press.

Qu LQ et al. (2005) Iron accumulation does not parallel the high expression level of ferritin in transgenic rice seeds. Planta 222(2), 225-233.

Theil EC (2004) Iron, ferritin, and nutrition. Annual Review of Nutrition 24, 327-343.

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PRODUCT PROFILE

Modified trait	Resistance to insects.
Intended food use of modified crop (host plant)	Potatoes (*Solanum tuberosum*) are increasingly consumed in a processed form (e.g., French fries and potato chips) while the direct consumption of potatoes (e.g., boiled potatoes) has declined dramatically. Potatoes should be cooked before eating because of the indigestibility of non-gelatinised starch and the presence of anti-nutritional proteins.
Gene (source) / Promoter (source)	**Cry**: Gene encoding an insecticidal protein (Cry3A) from *Bacillus thuringiensis subsp. tenebrionis* was introduced under the control of constitutively expressed cauliflower mosaic virus 35S promoter. The Cry3A gene was modified by site-directed mutagenesis to contain plant-preferred codons in order to maximize protein expression in plant cells. These modifications did not alter the predicted amino acid sequence of the Cry3A protein.
Transformation method	Agrobacterium-mediated transformation.
Metabolic reactions / pathways	Cry protein is an immediate product encoded by the gene introduced in the crop and confers the desired phenotype.
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	Cry protein.
Secondary gene products	

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop for food.

Cry: 0.004% total protein (0.8 µg/g fresh wt).

A.2. Prior history of known or presumed safe consumption from foods.

Cry: Cry3A is among several *Bt* proteins that have a long history (approximately 40 years) of safe application in *Bt* microbial products as pesticide approved for use on food crops. In these formats, *Bt* insecticidal crystal proteins (ICPs) are present as protoxins, which require solubilisation in the midgut of the target insect and the conversion of the protoxin to the biologically active toxin by proteolytic enzymes. In *Bt* crops, the plant tissues produce specific ICPs in a soluble form.

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: No change in the food use of modified potato is expected. Modified food may replace some existing commercial potato cultivars in potato product applications.

Cry: Given the expression level and estimated potato consumption of consumers only at 90th percentile intake (assuming consumption of 100% modified potato), human daily intakes are estimated to be less than 0.01 mg/kg bw/day, compared to NOELs (acute toxicity) published for Cry3A protein (> 5000 mg/kg/day). Wide margin of exposure is also expected based on toxicity of several families of Cry protein reported in the literature. No tolerance limit is required for this class of pesticidal proteins.

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

Cry: The insecticidal efficacy of *Bt* ICPs, which are delta-endotoxins, depends on specific membrane receptor binding by the C-terminal domain of the active toxin and pore formation by the N-terminal domain with subsequent lysis of the epithelial cells. Receptor binding by the ICP is the major determinant of host specificity by the different *Bt* ICPs [IPCS, 1999]. Following binding, cation-specific pores are formed that disrupt midgut ion flow thereby causing gut paralysis and eventual death from bacterial sepsis.

Bt crystal proteins bind only to receptors on intestinal cells of a narrow range of lepidopteran and coleopteran insects. No receptors for delta-endotoxins of *B. thuringiensis* in mammalian intestinal cells have been identified. Cry3A protein is insecticidal only to coleopteran insects. The specific mechanism of toxicity is not applicable to humans.

A.5. Structure-activity characterization.

Amino acid sequence identity with mammalian toxins.
No amino acid sequence identity with known protein toxin was identified.

Pepsin digestion (under standard testing conditions).
Cry: Rapid digestion - no traces of Cry3A protein detected at 2 min.

Structural/functional comparability with comparator/source protein or non-protein substance.
Cry: Comparison with source protein (*Bt*-derived Cry3A) was based on molecular weight, immunogenicity, amino-terminal sequence, and functional activity.

Equivalence in apparent molecular weight and immunogenicity was demonstrated using Western blot analysis (based on an immunogenic protein band of the expected molecular size detected with antibodies specific to the test protein). In *B. thuringiensis*, the expression of the *cry3A* gene results in the production of two proteins, a full length protein (73 kDa, 644 amino acids) and a smaller molecular weight species (68 kDa, 597 amino acids). This latter protein lacks 48 amino acids from the N-terminus but retains insecticidal activity. Western blot analysis of protein extracts from transgenic potato tissue demonstrated the presence of a 55 kDa species in addition to the Cry3A proteins of the expected molecular size. Further studies confirmed that the 68 kDa protein product underwent processing or degradation in the plant cell into a smaller protein of 55 kDa, which was also observed in some commercial *Bt* preparations.

Amino-terminal sequencing identified a shorter core sequence in the full length species of the potato-derived Cry3A protein, when compared to the native Cry3A protein. However, an exact match was obtained with the predicted sequence of the remaining trypsinised Cry3A core fragment. It has not been determined whether the removal of the first several amino acids in the potato-derived protein occurred *in vivo* or whether it occurred during the purification of the protein for analysis. While potential glycosylation sites were present on the potato-derived Cry3A protein, the protein has not been specifically targeted to the endoplasmic reticulum where glycosylation occurs. No glycosylation would be expected. The absence of glycosylation was confirmed by a glycoprotein detection assay.

Bioassay with target and non-target insects confirmed expected functionality of the potato-derived Cry3A protein.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.
No unusual breakdown products of Cry protein in the modified plant were detected, except possibly *in vivo* removal of several amino acids in the core fragment of the full length 73 kDa species, and degradation of the 55 kDa species, as noted in A.5.

In mammalian metabolism from consumption of the gene product.
Cry: No concerns with ingested protein that is rapidly digested.

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

Not applicable.

A.7.2. Between gene products introduced/modified and food constituents.

Cry: No interactions between cry protein and food constituents that would affect nutrient bioavailability are expected based on its known function.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

Cry: *Bacillus thuringiensis subsp. tenebrionis* is a common bacteria with no known adverse effects on humans or animals.

Applicable if the safety of donor organism is not known, or if the donor organism is a source of known toxicant or antinutrient.

B.1.1. Characterized gene modification.

B.1.2. Poorly characterized gene modification.

B.2. Safety of host plant.

Potato belongs to the Solanaceae plant family that encompasses a large number of toxic plant species. Potato is a source of known toxicants for which limits have been established. Major metabolites of concern include:

Toxicants: Several types of alkaloids, with glycoalkaloids being the most important, 95% of which consist of α-chaconine and α-solanine, which are present in higher concentrations at the periphery, in green and sprouted tubers. These glycoalkaloids are not destroyed during cooking and frying. The widely accepted tolerance limit of total glycoalkaloids in tuber is 200 mg/kg, fresh weight, was based on acute and/or subacute effects. New standards based on chronic effects may be warranted [Korpan et al., 2004]. Susceptibility of humans to glycoalkaloids appears to be high and variable: 1-5 mg/kg body weight oral doses are marginally to severely toxic; 3-6 mg/kg body weight can be lethal. Margin of exposure for potato glycoalkaloids may be between 2 and 6, assuming a low observed effect level in humans of 2 mg/kg bw, an average level of glycoalkaloids in potato and an average daily consumption of potato in western countries [Nordic Working Group, 1991].

Anti-nutrients: Several protease inhibitors and lectins are present, inhibiting the activity of trypsin, chymotrypsin and other proteases, or binding to specific carbohydrate structures on cell surfaces (e.g., intestinal or blood cells) - an activity that is largely inactivated by boiling and other thermal processes.

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

Cry exhibited the expected phenotype in reducing damage by target insect pests. The protein is not expected to have other functions in the modified plant, except as insecticidal proteins.

B.3.2. Impact of gene product function in the modified plant.

Cry is not expected to have metabolic, regulatory, or signalling function in the modified plant.

Applicable if gene product has an enzymatic function in the host plant.
B.3.3. Substrate specificity of gene product.

B.4. Potential for disrupting coding/regulatory DNA sequences in host plant.

B.4.1. In the inserted/modified DNA.

Two copies of the *Cry3A* gene were identified by Southern blot, PCR and sequencing analyses at one insertion site. Nucleotide sequence analysis of the inserted DNA region indicated two potential open reading frames (partial fragments of selectable marker gene to identify bacteria transformed with recombinant plasmid DNAs). Northern blot analysis was conducted by probing the total RNA isolated from potato tissues with a sequence specific to the marker gene. The lack of hybridization signals confirmed that the two ORFs were unlikely to be expressed.

B.4.2. In genomic sequences bordering the site of insertion/modification.

No potentially translatable ORFs extending from the insert into the flanking genomic region were identified.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Key nutrients suggested in the OECD consensus document for the food use of potato were analyzed in the modified potato and compared to the unmodified control, as well as to literature values and to the composition of commercial varieties. No significant differences were observed for the majority of the nutrients analyzed. The only exceptions were values for tyrosine and calcium, which were low for both the modified crop and the unmodified control when compared to literature values. This is most likely due to environmental factors and is not considered to be treatment related.

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

Total glycoalkaloids (GAs) were significantly higher in the modified potato compared to control, but were below the guidelines of 20 mg/100 g fresh weight for potatoes.

B.5.3. Other metabolites/parameters.

The scope of analytes assessed (B.5.1 and B.5.2) was considered adequate, given that the introduced Cry3A protein is not expected to interact with the metabolic pathways of the host plant. While significant differences were found in some metabolites (tyrosine, calcium, and total GAs), the differences are judged not to have health relevance. These observations, in conjunction with the absence of phenotypic alterations, would support the view that unintended changes with the potential for adverse health effects are of low concern for food derived from the modified crop.

B.6. Health consequences of instability of the modified trait.

The modified trait is not intended to affect human health.

PART C - POTENTIAL FOR ALLERGENIC (IgE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN PRIMARY AND SECONDARY GENE PRODUCTS.

C.1. Allergenicity of donor organism (gene source).

Cry: *Bacillus thuringiensis subsp tenebrionis* - bacteria not known to be allergenic.

Protein gene products (factors C.2-C.4).

C.2. Potential for protein gene product(s) to cross react with known allergens.

No amino acid sequence identity of ≥ 35% with known allergens was found.

C.3. Pepsin digestion (under standard testing conditions).

Cry: Rapid digestion - no traces of Cry3A at 2 min.

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factor (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

Cry: Absence of post-translational modification (glycosylation) was confirmed by glycoprotein assays.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

C.4.3. Abundance.

Cry: 0.004% total protein.

C.5. Allergenicity of host plant.

Potato is not a common source of allergens. However, potato contains multiple heat-labile proteins which can induce immediate hypersensitivity reactions when raw potatoes are consumed. Patatin, the main storage protein in potatoes, has been associated with allergic reactions in sensitive children - allergenicity of patatin to be confirmed. In addition, IgE binding to several proteins belonging to the family of soybean trypsin inhibitors was observed. Allergenic proteins include: cathepsin D inhibitor, cysteine protease inhibitor, and aspartic protease inhibitor.

Reference

Korpan YI et al. (2004). Potato glycoalkaloids : true safety or false sense of security? Trends in Biotechnology 22(3), 147-151.

Nordic Working Group (1991) Food and New Biotechnology. Novelty, Safety and Control Aspects of Foods Made by New Biotechnology. Copenhagen: Nordic Council of Ministers, Nordic Working Group on Food Toxicology and Risk Assessment, p.18.

PRODUCT PROFILE

Modified trait	Multiple traits (bacterial resistance against tuber soft rot, viral resistance to potato virus X, and tolerance to frost).
Intended food use of modified crop (host plant)	Potatoes (*Solanum tuberosum*) are increasingly consumed in a processed form (e.g., French fries and potato chips) while the direct consumption of potatoes (e.g., boiled potatoes) has declined dramatically. Potatoes should be cooked before eating because of the indigestibility of non-gelatinised starch and the presence of anti-nutritional proteins.
Gene (source) / Promoter (source)	*Solanum commersonii*, like other wild species of Solanum, provides a useful source of allelic diversity for the cultivated potato. However, the genes of interest have not been physically identified and isolated but were selected for among hybrid cells and regenerated plants. Tolerance to biotic and abiotic stresses in *S. commersonii* is likely to be mediated by multiple genes.
Transformation method	Somatic hybridization through protoplast fusion between a cultivated potato variety and *S. commersonii* and backcrossing of the somatic hybrid three times to the parental line.
Metabolic reactions / pathways	Protoplast fusion is a practical way to transfer multiple polygenic traits from a species that presents crossing barriers. Quantitative trait loci (QTL) for resistance to tuber soft rot have been identified on all 12 potato chromosomes in Solanum somatic hybrids, with putative QTL for tuber resistance on 10 chromosomes [Zimnoch-Guzowska et al., 2000]. Similarly, multiple proteins have been associated with cold tolerance in *S. commersonii* [Massarelli et al, 2002; Seppänen et al., 2000; Vega et al., 2004]. While homozygosity for a dominant gene for resistance to potato virus X in the wild species has been suggested, the gene has not been characterized [Parrella and Cardi, 1999].
INTENDED GENE PRODUCTS TO BE ASSESSED	
Primary gene products	Not identified.
Secondary gene products	

PART A - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - INTENDED CHANGES FROM PRIMARY AND SECONDARY GENE PRODUCTS INTRODUCED/MODIFIED.

Dietary Exposure to Gene Product(s).

A.1. Expression of gene product(s) in the edible part(s) of the modified crop for food.

A.2. Prior history of known or presumed safe consumption from foods.

See A.3 below.

A.3. Expected change in total dietary exposure (based on theoretical estimates).

Food derived from the modified crop: No change in the food use of modified potato is expected. Modified food may replace some existing commercial potato cultivars in potato product applications.

No new dietary exposures are likely to arise, nor are changes outside the ranges of levels generally seen in commercialized crop varieties expected, if the genetic material leading to the production of a substance in the modified crop is closely related to the recipient food plant [EPA, 2001]. Closely related species can be determined on the basis of sexual compatibility that produces a viable zygote. This presupposes that the donor plant is within the same family of plants that have contributed traits to the recipient food plant through sexual recombination, and is therefore considered to share a common pool of genetic material. It is also assumed that less uncertainty exists about how the gene of the donor species will function in the recipient plant and how its products may affect the plant's phenotype.

Two species are considered sexually compatible if they are capable of forming a viable zygote through the fusion of two gametes, including the use of bridging and/or wide crosses between plants, or vegetative reproduction [EPA, 2001]. While protoplast fusion is excluded from the definition for sexual compatibility, in other recombination events, viable sexual hybrids between *S. tuberosum* and *S. commersonii* have been produced with and without the use of bridging [Carputo et al., 1997; Masuelli et al., 1992]. Therefore, a similar justification can be made that no new or substantially modified level of exposure to the intended gene products in the somatic hybrid would be expected.

Nature of Gene Products.

A.4. Prior knowledge of safety / mode of action (including considerations of food preparation/processing methods, e.g. the modified food or ingredient may be prepared/processed differently from the food it replaces, or impurities may be associated with processing).

While the precise identities of the gene products are not known in the somatic hybrid, on the basis of the rationale given in A.3, there is reasonable certainty that, no new substances or substantially modified level of exposure to the intended gene products of concern to human health, would be expected in the food derived from the modified potato. Therefore, intended effects will not be further assessed. Factors in Part B (unintended changes) and factor C.5 (unintended changes with respect to the allergenicity of the host plant) will be assessed.

A.5. Structure-activity characterization.

Amino acid sequence comparison with mammalian toxins.

Pepsin digestion.

Structural/functional comparability with comparator/source protein or non-protein substance.

A.6. Digestion and degradation products (based on known function and metabolism of the introduced/modified gene products).

In edible parts of modified crop.

In mammalian metabolism from consumption of the gene product.

A.7. Synergism and interactions (based on known functions of the introduced/modified gene products).

A.7.1. Between gene products introduced/modified (in the same or separate transformation events).

A.7.2. Between gene products introduced/modified and food constituents.

PART B - POTENTIAL FOR TOXIC AND ANTINUTRITIONAL EFFECTS - UNINTENDED CHANGES IN THE MODIFIED FOOD FROM GENETIC MODIFICATION OF THE HOST PLANT.

B.1. Safety of donor organism (gene source).

S. commersonii (cmm) is a tuber-bearing wild species that shows a high level of resistance to a number of potato diseases. It is part of the Solanaceae family that encompasses a large number of toxic plant species. Principal toxicants in the Solanaceae family include: tropane alkaloids with anticholinergic activity in non-food genera (e.g., Brugmansia, Daucus, Duboisia, Hyoscyamus), steroidal glycoalkaloids (GAs) and polyhydroxy alkaloids or calystegines in genera that provide several major food crops such as potatoes, tomatoes, eggplants and garden peppers. Gastroenteritis, teratogenicity, and neurotoxicity have been associated with steroidal alkaloids and calystegines. Cultivated potato tubers contain only the GAs α-solanine and α-chaconine, while other types of GAs are present in *S. commersonii*, including commersonine, demissine, tomatine, dehydrocommersonine, and delta-5-demissine. *S. commersonii* has high content of total GAs (over 400 mg/kg of tuber fresh weigh) [Pianzzola et al., 2005; Vazquez et al., 1997]. There are little data on the toxicity of alkaloids in wild *Solanum* species [Cheeke, 1998].

Applicable if the safety of donor organism is not known, or if the donor organism is a source of known toxicant or antinutrient.

B.1.1. Characterized gene modification.

B.1.2. Poorly characterized gene modification.

Commersonii-specific RFLP (restricted fragment length polymorphism) analysis provided evidence for recombination on 5 out of 12 chromosomes on the somatic hybrid. *Commersonii*-specific RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism) analyses were used to estimate the wild genome content in the backcross generations, determined to be 31% and 26%, respectively, in the hybrids of the third backcross generation (BC_3). RFLP markers specific to the short and long arms of specific potato chromosomes were used to identify the presence of complete and partial chromosomes derived from *S. commersonii*. Further, a sequential GISH and FISH (genomic and fluorescence in situ hybridization) approach coupled with chromosome-specific cytogenetic DNA markers revealed the number and identity of the alien chromosomes and their potato homologues in the breeding lines. The BC_3 hybrid has 47 chromosomes, including four copies of chromosome 8, three from potato and one from the wild species.

B.2. Safety of host plant.

Potato belongs to the Solanaceae plant family that encompasses a large number of toxic plant species. Potato is a source of known toxicants for which limits have been established. Major metabolites of concern include:

Toxicants: Several types of alkaloids, with glycoalkaloids being the most important, 95% of which consist of α-chaconine and α-solanine, which are present in higher concentrations at the periphery, in green and sprouted tubers. These glycoalkaloids are not destroyed during cooking and frying. The widely accepted tolerance limit of total glycoalkaloids in tuber is 200 mg/kg, fresh weight, was based on acute and/or subacute effects. New standards based on chronic effects may be warranted [Korpan et al., 2004]. Susceptibility of humans to glycoalkaloids appears to be high and variable: 1-5 mg/kg body weight oral doses are marginally to severely toxic; 3-6 mg/kg body weight can be lethal. Margin of exposure for potato glycoalkaloids may be between 2 and 6, assuming a low observed effect level in humans of 2 mg/kg bw, an average level of glycoalkaloids in potato and an average daily consumption of potato in western countries [Nordic Working Group, 1991].

Anti-nutrients: Several protease inhibitors and lectins are present, inhibiting the activity of trypsin, chymotrypsin and other proteases, or binding to specific carbohydrate structures on cell surfaces (e.g., intestinal or blood cells) - an activity that is largely inactivated by boiling and other thermal processes.

B.3. Potential for novel/altered patterns of metabolites (based on prior knowledge and biochemical studies in the modified plant).

B.3.1. Knowledge of gene product function.

B.3.2. Impact of gene product function in the modified plant.

Applicable if gene product has an enzymatic function in the host plant.
B.3.3. Substrate specificity of gene product.

B.4. Potential for disrupting coding/regulatory DNA sequences in host plant.

B.4.1. In the inserted/modified DNA.

B.4.2. In genomic sequences bordering the site of insertion/modification.

B.5. Chemical analysis of the food derived from the modified plant.

B.5.1. Key nutrients (as suggested for food use in applicable OECD consensus documents).

Key nutrients suggested in the OECD consensus document for the food use of potato were analyzed in several lines of the BC_3 hybrids, and compared to the unmodified controls (cultivated and wild parents), as well as to literature values and the composition of commercial varieties. Compared to the parental lines, nutrient and proximate analyses revealed considerable variation in the interspecific hybrid, particularly in soluble sugars and soluble proteins (up to 2-fold range). The protein pattern was intermediate between that of the wild species and the cultivated control. This was particularly evident in a group of high molecular weight proteins (~100 kDa on SDS-PAGE).

B.5.2. Known toxicants/antinutrients (as suggested for food use in applicable OECD consensus documents).

Total glycoalkaloids (GAs) were significantly reduced in BC_3 compared to the wild species but higher compared to the cultivated parent and were present in wider variation. Total GAs in the majority of the BC_3 hybrid lines remained below the guidelines of 20 mg/100 g fresh weight for potatoes, although the levels in a few lines exceeded this limit and were removed from further development. Among the individual glycoalkaloids, α-chaconine levels were significantly higher in several hybrid lines with considerable variation (80-260 ppm) than in the cultivated control (120-130 ppm). In addition, other GAs characteristic of the wild species were present in the hybrid, but were at lower levels than in the wild parent. Little is known about the genetic control and inheritance of GAs in Solanum species [Carputo et al., 2003].

The presence of other GAs (i.e. GAs not expected in the parental lines) cannot be ruled out as other chromatographic peaks in the same region as the known GAs were present. It was reported in the literature that a new GA not present in either parent was identified in *tuberosum-brevidens* hybrids [Laurila et al, 1996; Väänänen et al., 2005]. The formation of a new GA was hypothesized to result from hydrogenation of an endogenous GA in the cultivated parent by an enzyme of the wild species, or from possible recombination events between different types of sugar aglycones.

B.5.3. Other metabolites/parameters.

Additional analyses were conducted on: specific hemagglutination activity and several morphological and food quality parameters.

No changes in heat stability and binding property of lectins to red cells in the BC_3 hybrid were detected when compared to the cultivated variety.

Chlorogenic acid was high in the wild species and remained highly variable in the hybrid lines. Chlorogenic acid can cause postcooking blackening upon reacting with ferric ions. Chlorogenic acid is apparently hydrolyzed in the stomach to caffeic and quinic acids. Caffeic acid is also a naturally occurring chemical carcinogen found in higher plants [NRC, 1996: 66].

Food quality - As expected, soluble solids and dry matter content in the hybrid lines were significantly different from those in the cultivated variety and had a profile similar to that in the wild species, which is a useful attribute in potato processing. Starch structure was not significantly altered compared to the cultivated variety, measured in terms of crystallinity of starch granules and the average size of starch crystallites. Antioxidative ability was measured separately on the hydrophilic and lipophilic parts and were low in both but more variable in the hydrophilic part. No differences were noted compared to the cultivated parent.

Wide variability in many parameters analyzed in potato- *Commersonii* somatic and sexual hybrids have also been reported in the literature. High within-group variability in somatic hybrid is thought to be important because the success of plant breeding depends on the variability available. While significant changes were detected in some quality related parameters (e.g., soluble solids and dry matter), they were not health relevant. Other metabolites such as GAs and chlorogenic acid were also at levels different from the cultivated variety but were at levels intermediate between the two parental species. As expected, total GAs were below the recommended limit in the majority of the hybrid lines. Among the parameters considered, glycoalkaloids content was the most sensitive to variation. Its measurement, in conjunction with a wide spectrum of biochemical, morphological, and functional parameters analyzed, should provide a reasonable basis for assessing the potential for deleterious unintended changes in the backcross somatic hybrid.

B.6. Health consequences of instability of the modified trait.

The modified trait is not intended to affect human health.

PART C - POTENTIAL FOR ALLERGENIC (IGE-MEDIATED) EFFECTS - APPLICABLE TO PROTEIN PRIMARY AND SECONDARY GENE PRODUCTS.

C.1. Allergenicity of donor organism (gene source).

Protein gene products (factors C.2-C.4).

C.2. Potential for protein gene product(s) to cross react with known allergens.

C.3. Pepsin digestion (under standard testing conditions).

C.4. Secondary factors (C.4.1-C.4.3). ***Applicable if information on factor (C.3) is insufficient or equivocal, or if the criterion for the factor is not met.***

C.4.1. Structural/functional characteristics.

C.4.2. Heat stability (NOT applicable to lipid transfer proteins).

C.4.3. Abundance.

C.5. Allergenicity of host plant.

Potato is not a common source of allergens. However, potato contains multiple heat-labile proteins which can induce immediate hypersensitivity reactions when raw potatoes are consumed. Patatin, the main storage protein in potatoes, has been associated with allergic reactions in sensitive children - allergenicity of patatin to be confirmed. In addition, IgE binding to several proteins belonging to the family of soybean trypsin inhibitors was observed. Allergenic proteins include: cathepsin D inhibitor, cysteine protease inhibitor, and aspartic protease inhibitor.

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