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THE ASSEMBLY OF HEPATITIS B VIRUS CORE PARTICLES IN TRANSGENIC TOBACCO, CARROT AND RICE PLANTS

Zaman Alli

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ABSTRACT

The spread amongst humans of viral diseases such as acquired immunodeficiency syndrome (AIDS), hepatitis and severe acute respiratory syndrome (SARS) is alarming. A plant-based high fidelity production system is being developed with emphasis on producing antigens capable of being orally delivered to humans in plant packets. To test whether transgenic tobacco, carrot and rice plants can correctly process and assemble the hepatitis-B virus (HBV) core particle/antigen (HBcAg), they were transformed with a C-terminal truncated version of the HBcAg subunit coding sequence. Transgenic tobacco, carrot and rice plants processed the HBV subunits accurately indicating that these recombinant expression systems can be extended to produce other proteins at reduced costs.

In the wild-type expression construct (H1); the enhanced cauliflower mosaic virus double 35S (CaMV-d35S) promoter was fused to the alfalfa mosaic virus RNA 4 (AMV-RNA4) sequence to achieve greater translation of a C-terminal truncated HBV core particle subunit. A second expression construct (H2) was plant-codon optimized to match the *Arabidopsis thaliana* plant genome codon preferences. A third codon-optimized expression construct (H3) had a KDEL (lysyl-aspartyl-glutamyl-leucine) encoded sequence. While a fourth expression construct (H4) included an extensin signal sequence in place of the AMV-RNA4 sequence.

Western blotting analysis showed the presence of the HBcAg in transgenic tobacco, carrot and rice plants. The HBcAg levels increased from the H1 to the H4 transgenic tobacco lines. Plant codon-optimization of the HBcAg sequence and addition of the KDEL

encoded sequence led to higher levels of HBcAg. The most effective modification was observed when the extensin signal sequence replaced the AMV-RNA4 translation enhancer sequence resulting in the highest observed yields of HBcAg in both the leaves and seeds of the best H4 tobacco plant. In edible plants, higher levels of HBcAg were observed in carrot roots as opposed to carrot leaves and in rice seeds as opposed to rice leaves. Further analyses via electron microscopy indicated that the HBV subunits had assembled into virus-like particles of 25-30 nm diameter in all three plant systems. Therefore, these studies may aid in the global quest to develop cheap, safe and effective vaccines.

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AMV	Alfalfa Mosaic Virus
AP	Alkaline phosphatase
BA	6-benzyladenine
BCIP	5-bromo-4-chloro-3-indolylphosphate p-toluidine salt
Bis-Acrylamide	N,N'-methylene bisacrylamide
bp	Base pair
Bt	*Bacillus thuringiensis*
CaMV	Cauliflower Mosaic Virus
cDNA	Complementary DNA
CT	Cholera Toxin
DAP	Days after pollination
DEPC	Diethylpyrocarbonate
2,4-D	2,4-dichlorophenoxyacetic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
ER	Endoplasmic Reticulum
GUS	β-glucuronidase
HBV	Hepatitis B Virus
HBcAg	Hepatitis B Virus Core Antigen
HBsAg	Hepatitis B Virus Surface Antigen
HCMV	Human Cytomegalovirus
HIV	Human Immunodeficiency Virus
IDDM	Insulin Dependent Diabetes Mellitus
IG-BC	Immunodominant Glycoprotein B Complex
IgG/A	Immunoglobulin G/A
kb	Kilo base
kDa	Kilo Dalton
LB	Luria Bertani medium
LT-B	Heat labile enterotoxin β-subunit of *E. coli*
MW	Molecular weight
mRNA	Messenger RNA
MS	Murashige and Skoog medium
NAA	α-naphthalene acetic acid
NBT	Nitroblue tetrazolium chloride
ng	Nanogram
NOS-TER	Nopaline synthase terminator sequence of DNA from *A. tumefaciens*
nt	Nucleotide
PCR	Polymerase chain reaction
PG	Polygalacturonase

PVPP	Polyvinyl-polypyrrolidone anti-browning agent for plant phenolics
rpm	Revolutions per minute
rthGM-CSF	Recombinant human Granulocyte Macrophage Colony Stimulating Factor
SARS	Severe acute respiratory syndrome
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride and sodium citrate solution
TAE	Tris-acetic acid-EDTA buffer
TBS	Tris-buffered saline
T-DNA	Transfer DNA
TEMED	N,N,N',N'-tetramethylethylenediamine
TMV	Tobacco Mosaic Virus
TRIS	Tris (hydroxymethyl)aminomethane
TSP	Total soluble protein
USA	United States of America
WNV	West Nile Virus
YEP	Yeast extract, peptone and NaCl medium

Chapter 1

INTRODUCTION

Synopsis: food engineering, protein drugs and diseases

The Estonian biochemist Nikolai Lunin in 1881 reported that animals "cannot subsist on the known nutrients" and that other substances indispensable for optimal growth and development must be present in foods (Lunin, 1881). Not only did Lunin's Theory of "other substances" lead to the Nobel Prize winning work of F. G. Hopkins, but also F. G. Hopkins further developed the concept of "accessory food factors" (vitamins). Today one does not approach food as a delivery vehicle for just essential nutrients but as an appealing platform for optimizing human physical and mental performance. The goal here is based on the premise that food can be engineered to deliver a plethora of "other substances" both small and large. Starting with Lunin's work on pure food concentrates, two questions were asked: what "other substances" beside vitamins occur in the primal human food – grain kernels – as small molecules? Moreover, what "other substances" can be engineered into foods like leaves, grains and roots using molecular switches to ensure health-enhancing factors are biosynthesized in consumable plant tissues?

Initial work in Dr. Altosaar's laboratory on grains had uncovered two novel clones (Fabijanski *et al.*, 1988; Tanchak *et al.*, 1998) and encouraged the enrichment of basic food commodities with biotherapeutics (Robinson 1995; Ganz *et al.*, 1996; Sardana *et al.*, 1996, 1997, 2002; Panahi, 2002). Traditionally, therapeutic proteins used as protein drugs or vaccines were primarily derived from animal, viral or bacterial origin. Thus, insulin or interferon was extracted from dog pancreas or human blood while vaccines against bacteria or viruses were derived from killed or attenuated organisms. Over the past two decades,

advances in molecular biology have revolutionized the concept of recombinant protein expression. Hence, therapeutic proteins can be obtained from several expression systems including bacteria, yeast, animals, insect cells and plants. Please see US patent # 4,956,282 of Goodman *et al.* (1990) that claimed expression of a foreign protein in plant cells.

In 1981, Gottlieb *et al.* identified pneumonia and Kaposi's sarcoma as symptoms of a new disease that was later identified as the acquired immunodeficiency syndrome (AIDS) (Zakowski *et al.,* 1982). However, one year elapsed before the AIDS virus was identified. During the lag phase involved in identifying the human immunodeficiency virus (HIV) and its mode of action, death from AIDS was certain. However, advanced techniques are available today for identification of disease causing organisms. For example, with the outbreak of severe acute respiratory syndrome (SARS) in Canada, only three months elapsed before the genome was identified (Marra *et al.*, 2003; Rota *et al.,* 2003) and two glycoproteins were characterized (Krokhin *et al.,* 2003). Thus, humanity has advanced tremendously in science. Therefore, vaccines help to prevent diseases and indirectly calm the anxiety generated from disease outbreaks.

Conventional recombinant protein expression systems

Some available recombinant expression systems include bacterial, yeast, insect cells, mammalian cells, urine, transgenic animals and crops. Recombinant proteins can be easily expressed in bacteria using commercially available plasmid vectors. The *E. coli* bacterium is used routinely for amplification of various DNA fragments and protein expression. Several plasmid vectors are available commercially for bacterial cloning, nearly all are based on the critical functionality embedded in the pBR322 (Bolivar *et al.,* 1977; reviewed by Balbas *et al.,* 1988). Recombinant protein expression studies were initially conducted in bacteria with somatostatin being the first (Itakura *et al.,* 1977), rat

proinsulin the second (Villa-Komaroff *et al.,* 1978) and later, human insulin became the third (Goeddel *et al.,* 1979). Additional proteins were also expressed in bacteria including Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) (Burgess *et al.,* 1987), insulin-like growth factor-I (IGF-I) (Joly *et al.,* 1998), human proinsulin (Winter *et al.,* 2001) and antibodies (Jurado *et al.,* 2002). Most of these proteins were found to be biologically active when tested or administered *in vivo*.

However, bacterial recombinant systems have the possibility of producing improperly folded proteins that aggregate in the form of inactive inclusion bodies. In *E. coli,* the signal peptide Lam B directs secretion to the outer membrane (Josefsson *et al.,* 1982) and this may decrease inclusion body formations. In the absence of an *E. coli* signal peptide, additional costs to re-fold the recombinant proteins were incurred (Burgess *et al.,* 1987; Watt *et al.,* 1997). Although some *E. coli* derived proteins can be made active after re-folding, a therapeutic protein such as human erythropoietin requires glycosylation for its *in vivo* activities and bacteria will not serve as a suitable host (Tsuda *et al.,* 1990). Therefore, proteins requiring these modifications for their *in vivo* activities could not be produced in *E. coli*. Thus, other cell types have been investigated as recombinant protein hosts including yeast cells (Marty *et al.,* 2001), human Namalwa cells (Okamoto *et al.,* 1990) and insect cells such as *Bombyx mori* and *Spodoptera frugiperda* (Kokuho *et al.,* 1999).

Eukaryotic cell systems offered some distinct advantages over bacterial systems in terms of the accomplishment of complex processing (e.g. splicing of heteronuclear RNA) including glycosylation and correct folding of proteins. The advantage of obtaining structurally authentic proteins from yeast system led to a new phenomenon of aberrant O-linked glycosylation that was normally not observed for the native proteins (Ernst *et al.,*

1987, 1992). Thus, masking of antigenic sites of yeast derived vaccines may prevent immune recognition and decrease protection against the challenge pathogen.

For mammalian cell culture expression systems, ample use has been made of Chinese hamster ovary (CHO) cells or COS-1 cells from monkey kidney to produce: GM-CSF (Maliszewski *et al.,* 1988), erythropoietin (Coscarella *et al.,* 1997) and thrombopoietin (Kaszubska *et al.,* 2000). These proteins harvested from mammalian cell culture systems were also found to be as biologically active as the native proteins (Luo *et al.,* 1995). Since the gene regulatory mechanisms of both plants and animals are similar, mammalian systems were a major achievement over bacterial systems (Kuhlemeier, 1992). However, mammalian systems still had several disadvantages. Mammalian cells lack cell walls and when grown in culture, the decreased rate of stirring made it difficult to scale-up these systems. Mammalian cells multiply slower than *E. coli* cells making it a slow system with additional costs for maintenance of constant temperature, pH, levels of metabolic wastes and oxygen levels (Ganz *et al.,* 1995). In addition to the high maintenance costs, expenditure on fetal bovine serum coupled with the need to obtain purified end-product led to increased costs.

Other investigators preferred to express human recombinant proteins in the milk of transgenic animals since mammary glands can perform correct post-translational modifications such as complex glycosylation (Lubon *et al.*, 1996, reviewed by Karatzas, 2003). The idea that milk from transgenic animals could be a production medium for recombinant proteins was first demonstrated by Simons *et al.* (1987). The milk of the transgenic mice accumulated the sheep β-lactoglobulin protein. Others have since expressed a variety of medically important proteins in transgenic milk including: protein C (Velander *et al.,* 1992), IGF-1 (Zinovieva *et al.,* 1998), thrombopoietin (Sohn *et al.,* 1999)

and lactoferrin (van Berkel *et al.*, 2002). But, recombinant proteins secreted in milk were not always cleaved or glycosylated correctly (Drew *et al.*, 1995). Sometimes, milk-specific promoters become "leaky" resulting in aberrant expression in undesirable tissues. This can cause physiological problems including immunogenicity and obesity in animals (Meade and Ziomek, 1998). Further, generation of transgenic animals in general is extremely expensive. The cost of producing one transgenic sheep could be as high as US $60,000 and as high as US $500,000 for one transgenic cow (Janne *et al.*, 1998). Further, transgenic animal expression systems might still be infected by human pathogens, as are mammalian cell cultures.

Other investigators have invested in baculoviral expression systems where insect cells or insect larvae were infected with host specific viruses (Shi *et al.*, 1996; Inumaru *et al.*, 1998; review by Jarvis, 2003). Some important proteins expressed in insect cells include rubella virus envelope proteins (Mottershead *et al.*, 1997), human calpain I (Meyer *et al.*, 1996) and porcine interleukin-12 (Kokuho *et al.*, 1999). Most of the proteins obtained from baculoviral-infected cells were also found to be biologically active (Meyer *et al.*, 1996; Muneta *et al.*, 2000). However, insect cell systems are complicated and still have similar disadvantages as other animal cell culture systems.

The overriding concern that conventional expression systems are prone to pathogenic contaminations leads one to investigate further the suitability of edible plant parts as "protein pills". In addition, the discovery of prions due to new variant Creutzfeld-Jacob Disease (CJD) and the spread of bovine spongiform encephalopathy (BSE) have exemplified the need for safe therapeutics (Duffy *et al.*, 1974; Creange *et al.*, 1995; reviewed by Bradley, 2001). The transmission of CJD was linked to transplantation of human tissues and organs from person to person (Croes *et al.*, 2001, 2002). Thus,

endeavours to secure an ideal but safe protein expression system are still being actively pursued. Recombinant systems capable of rapid scale-up, high yields of safe biologically active proteins will be best suited for this task. Thus, plant recombinant systems may provide a unique vehicle necessary for the production and assembly of safe and biologically active therapeutic proteins geared towards universal acceptance and distribution.

Plant Biotechnology

These limitations discussed above severely hindered the expansion of conventional bioreactors forcing scientists in search of "green bioreactors." Plants were investigated in attempts to reduce some of the limitations of early expression systems. The first set of transgenic plants ever recorded by modern society was in 1983 (Fraley *et al.,* 1983; Roychoudhury and Lam, 1983). The petunia or tobacco plant was transformed with an antibiotic resistance gene. Eukaryotic systems allowed for expression of cloned genes that can be glycosylated. Applied biotechnology has been used to produce plant-based antibodies, vaccine antigens, entomocidal proteins, human and industrial enzymes and cytokines (Ma *et al.,* 1994; Fiedler *et al.,* 1997; Conrad and Fiedler, 1998; Cramer *et al.,* 1999; Chargelegue *et al.,* 2001; Huang *et al.,* 2002). For effective recombinant protein expression, strong promoters from plant or viral origin have been used. For stabilized protein accumulation in specific compartments, a host or sometimes a foreign signal sequence is encoded in the expression construct (Holmberg *et al.*, 2001; Sojikul *et al.*, 2003). In some cases, a plant viral leader sequence is used for increased translation of the mRNA (Gallie *et al.,* 1987; Jobling and Gehrke, 1987; Sleat *et al.,* 1987; Gallie and Walbot, 1992; Dowson *et al.,* 1993; Gallie, 2002). Therefore, these sequence considerations in plant expression constructs are reviewed below.

Non-translated mRNA sequences

The region of the mRNA occupied by a leader sequence is from the 5`CAP site and extends towards the protein translation initiation site containing the AUG codon. Untranslated leader (UTL) sequences can be obtained from a variety of genes including the alfalfa mosaic virus subgenomic RNA 4 gene, brome mosaic virus gene and the omega factor gene from the tobacco mosaic virus (TMV). These sequences have high AT nucleotide content (up to 72%) and act to enhance translation of steady state mRNAs in tobacco, carrot and rice cells (Gallie *et al.,* 1989). During protein translation of the mRNAs, it is believed that UTL sequences efficiently attract the 40S ribosomal subunit of eukaryotes due to their unstructured arrangement (Heidecker and Messing, 1986; Ryabova *et al.*, 1993). Datla *et al.*, (1993) showed increased reporter enzyme activities when the AMV-RNA4 UTL sequence was used in different expression constructs to drive the synthesis of the β-glucuronidase (GUS) reporter enzyme. Each expression construct used either the natural (CaMV-35S) or enhanced (CaMV-d35S) promoter fused to the AMV-RNA4 UTL sequence. The GUS specific activities increased up to 20-fold when the AMV-RNA4 untranslated leader sequence was included in the CaMV-35S construct. However, a four-fold increase was observed when the AMV-RNA4 UTL sequence was included in the CaMV-d35S construct. For comparison, the CaMV-d35S promoter and the AMV-RNA4 UTL sequence led to the highest observed GUS specific activities in both transient and stable transgenic lines. Caution should be exercised when using these UTL sequences since they may demonstrate monocot or dicot preferences. For example, the UTL sequence of the TMV was shown to enhance gene expression up to 30 times in tobacco protoplasts while this enhancement in maize protoplasts was only up to 2.6 times (Gallie *et al.*, 1989; Gallie and Young, 1994). Given that every report that used a viral

UTL sequence resulted in increased expression of recombinant proteins, it would be a logical choice to include such a sequence within plant expression constructs.

Sometimes, addition of an intron to an expression construct leads to increased protein expression (Callis *et al.,* 1987a; Mascarenhas *et al.,* 1990; Luehrsen and Walbot, 1991; Bhattacharyya *et al.*, 2003; Rascon-Cruz *et al.*, 2004). The most studied intron is the alcohol dehydrogenase-1 (ADH-1) intron. For example, when the ADH-1 intron was present in reporter gene expression constructs, up to four-fold increase in reporter gene activities were observed (Luehrsen and Walbot, 1991). Recently, Rascon-Cruz *et al.* (2004) used the ADH-1 intron with the glutelin-1 promoter from rice or the CaMV-d35S promoter. Upon analyses, the recombinant protein was correctly processed and the levels in maize endosperm cells appeared to increase when the ADH-1 intron was present. However, the current view on introns remains controversial due to the presence of other sequences (promoter, signal sequence) that may have a consorted effect on expression level. However, the strength of the associated promoter may play a major role in dictating the plant expression level than would the intron (Mascarenhas *et al.,* 1990). Therefore, introns in plant expression constructs lead to more complexities in designing expression constructs and may not provide much increase in gene expression.

To achieve more stable translatable mRNAs, addition of polyadenylated tails is needed (Callis *et al.,* 1987b; Gallie *et al.*, 1995; Tanguay and Gallie, 1996). Several non-coding sequences for termination of transcription and polyadenylation are currently used and include the nopaline synthetase terminator sequence from *Agrobacterium tumefaciens* (NOS-TER) and the vegetative storage terminator from soybean (Chikwamba *et al.*, 2002). These two sequences apparently are very effective in increasing the mRNA half-life, once any destabilizing 3` sequences have been removed from the original coding

sequences. Gallie *et al.* (1995) showed that the GUS reporter mRNA was stabilized when polyadenylated. Further, the critical length for maximum stability of polyadenylated mRNA seems to be 19 bases (Tanguay and Gallie, 1996). When the firefly luciferase reporter mRNA contained 19 bases in its polyadenylated tail, a 97-fold increase in mRNA stability resulted but as the polyadenylated tail length increased, the mRNA stability decreased from 97-fold (Tanguay and Gallie, 1996). Therefore, to achieve high expression levels of recombinant proteins, these sequence considerations must be taken into account in designing plant expression constructs.

Synthetic genes

Many bacterial and non-plant genes/cDNAs contain deleterious sequences that affect expression in plant recombinant systems. They include high AT nucleotide content, improper mRNA splice sites, improper polyadenylation signals and improper amino acid codon-composition. Expression of many bacterial, viral and animal genes/cDNAs in plants were not detected, improperly processed or resulted in very poor yields. Therefore, synthetic genes are assembled that modify the deleterious sequences such that mRNA splice sites and polyadenylation sites are removed from the coding sequences, AT nucleotide content are reduced and/or plant preferred codons are incorporated. For example, increase in expression was observed for the following synthetic coding sequences when compared to the natural sequences expressed in plants: *E. coli* heat-labile enterotoxin (Mason *et al.,* 1998; Chikwamba *et al.*, 2002), *Bacillus thuringiensis* crystal insecticidal proteins (Cheng *et al.,* 1998), a thermostable β-glucanase enzyme (Horvath *et al.,* 2000), the spruce budworm antifreeze protein (Holmberg *et al.*, 2001) and HBsAg (Sojikul *et al.,* 2003). These observations show that synthetic genes/cDNAs can also result in the production of active recombinant proteins with higher yields than the expression of the

native genes/cDNAs and hence synthetic genes/cDNAs should be included in plant expression constructs.

Targeting sequences for sorting of proteins from plant cells

Apart from the use of strong promoters, removal of deleterious sequences from plant expression constructs, subcellular targeting of recombinant proteins appears as a major determinant in protein stability and hence this affects the expression level, significantly. After transport of the matured messenger RNA to the plant cell cytosol, proteins destined for the secretory pathway are first synthesized on the rough endoplasmic reticulum (ER) and are co-translationally translocated into the ER lumen. This process results in the cleavage of the N-terminal signal peptides (Yamagata and Tanaka, 1986). Plant cells have unique features imprinted into their endomembrane system that are not observed in other cell types. For example, large precursor proteins are localized and assembled in the ER lumen or vacuole (Vitale and Chrispeels, 1992). Therefore, plant ER is important in the synthesis of therapeutic proteins (Vitale and Denecke, 1999). Following post-translational modifications in the ER, proteins are then transported via secretory vesicles to the Golgi apparatus for further modifications. Final processing in the Golgi apparatus releases the matured proteins for deposition into different subcellular compartments of the endomembrane system (Okita and Rogers, 1996). Plant and animal cells need very specific peptide sequences to allow correct targeting of secretory proteins. This information may be a specific three-dimensional arrangement of amino acid residues such as a signal patch or a signal peptide. While working on the immunoglobulin light chains, Blobel and Dobberstein (1975) proposed that the signal peptide of a protein was located at its amino-terminal. This 1975 "signal hypothesis" led to the 1999 Nobel Prize of Gunter Blobel. The role of different signal peptides in the secretory pathway has been delineated and has been used to express heterologous proteins in different host systems

(Holmberg *et al.*, 2001). The glutelin signal peptide targets matured proteins for deposition into type-II protein bodies of seeds and specifically in the seed storage vacuoles of rice (Okita, 1996; Okita *et al.*, 1989). In the absence of the glutelin signal peptide but presence of the glutelin promoter, recombinant proteins appear to be confined to the starchy endosperm cells (Rascon-Cruz *et al.*, 2004). The compartmentalization of proteins in rice protein storage bodies was reviewed by Okita (1996). Inclusion of an extensin signal sequence will result in a recombinant protein being targeted to the cell wall (Chen and Varner, 1985). For secretion outside the cell, the proteins will be released across the plasma membrane (Okita and Rogers, 1996).

According to this "signal hypothesis", higher levels of expression are possible when signal peptides are used to direct recombinant proteins into specific subcellular compartments. When three enzymes involved in the polyhydroxybutyrate synthetic pathway were targeted to the chloroplast of *A. thaliana* using a pea transit peptide, the dry leaves accumulated up to 14% of this polymer (Nawrath *et al.*, 1994). However, only up to 100 μg/g of this polymer was observed in the leaves of transgenic plants when these enzymes were expressed in the cytoplasm (Poirier *et al.*, 1995). Thus it is believed, that the presence of plant signal sequences in plant expression constructs are the major contributing factor to augmented yields. Likewise, when the KDEL tetrapeptide was included at the carboxyl terminal as an ER retention signal, higher levels of single-chain antibody fragments (scFvs) in transgenic potato (Schouten *et al.*, 1997) and heat-labile enterotoxin β-subunit of *E. coli* in maize seeds (Chikwamba *et al.*, 2002) were observed. Coincidentally, the expression construct of Chikwamba *et al.* (2002) also contained an *E. coli* bacterial signal peptide from the heat-labile enterotoxin β-subunit. However, the work of Panahi *et al.* (2004) did not show any differences in expression levels when a bacterial

leader/signal peptide, Lam B, was used in transgenic plants. Although the Lam B signal peptide was correctly cleaved to generate biologically active IGF-1, inclusion of the Lam B signal peptide in plant expression constructs allowed it to act in a parasitic manner to drain the pool of amino acids and processing enzymes in the transgenic plant. Thus the bacterial signal peptide used by Chikwamba *et al.* (2002) may not have contributed to the increased expression of the antigen and the expression constructs were not designed to allow for this determination. Therefore, a good plant expression construct should include a plant signal peptide and a KDEL encoded sequence but not a non-functional "parasitic" targeting sequence.

Not all non-plant signal peptides are considered "parasitic" in plants. When a murine signal peptide was used to target a scFv antibody to the apoplast of transgenic tobacco cells, higher yields resulted than when the same signal peptide was not included in the expression construct (Fischer *et al.*, 1999). Further, the leaves and seeds of transgenic tobacco as well as potato tubers showed increased accumulation of recombinant scFv antibodies when the KDEL targeting peptide was used for retention of these proteins in the lumen of the ER (Conrad and Fiedler, 1998). However, the human serum albumin (HSA) prepro-sequence in transgenic tobacco and potato plants was inefficiently processed resulting in poor maturation of the HSA (Sijmons *et al.*, 1990). When the tobacco pathogen related protein leader peptide was used to target the HSA to the extracellular apoplastic space, correct cleavage of the plant signal peptide allowed for the detection of matured HSA having same molecular weight (MW) as the matured HSA. The functionality of the tobacco pathogen related protein leader peptide was also recently demonstrated when a synthetic spruce budworm antifreeze protein were observed to accumulate in tobacco apoplasts (Holmberg *et al.*, 2001). Given that plant and sometimes

animal signal peptides lead to better processing and accumulation of recombinant proteins in transgenic plants, they should be considered an integral part of all expression constructs. To generate transgenic plants, there must be at least one easy and efficient method. Therefore, *A. tumefaciens* mediated DNA transfer to plant cells is now detailed.

Agrobacterium tumefaciens mediated DNA transfer to plant cells

A protein expression vector needs a viable mechanism to obtain transient or stable expression in the plant hosts. Several methods exist for the mobilization and transfer of the DNA sequence of interest into diverse hosts and include microinjection (Tsulaia *et al.,* 2003), electroporation (Chowrira *et al.,* 1995), viral delivery (Crystal *et al.,* 1994; Foley *et al.,* 1997), particle gun bombardment (Christou, 1997; Taylor and Fauquet, 2002) and *A. tumefaciens* mediated transformation. Walden *et al.* (1995), Newell (2000) and Gelvin (2003) have reviewed gene transfer mechanisms into plants. Many of the gene transfer methods to plants are today routinely conducted using *A. tumefaciens.* Improvements in the *A. tumefaciens* technique allowed for both monocot and dicot transformations (Wu *et al.,* 2002a, b; reviewed by Lindsey, 1996).

In nature, the roots of *A. tumefaciens*-infected plants acquire a phenotypic characteristic of tumours but this does not result in the production of transgenic plants (Zambryski *et al.,* 1980). The *A. tumefaciens* tumour-inducing (Ti) plasmid is known to influence positively the development of tumours. However, modifications of the Ti plasmids have led to derivatives that have no tumourogenicity (Turk *et al.,* 1993; reviewed by Newell, 2000; Gelvin, 2003). The regions outside of the transfer DNA (T-DNA) borders of the Ti plasmid allow for the T-DNA processing and genomic integration into plant cells. Sometimes, this may insert into an inactive region of the genome which may result in gene silencing. The virulence genes (*vir*) on the Ti plasmid encode several

polypeptides. There are six *vir* genes (*vir* A, B, C, D, E and G) for which some function has been correlated as being essential or are involved in enhancing the transformation efficiency (reviewed by Tzfira and Citovsky, 2002; Gelvin, 2003).

The *vir* A gene product senses acetosyringone (AS) released by damaged or stressed plant tissues while the *vir* G gene product regulates transcriptional activation of the *vir* genes (Stachel and Zambryski, 1986; Turk *et al.*, 1994). The *vir* C and D gene products encode endonucleases for nicking the 25 bp direct repeats present at the T-DNA border regions (Stachel *et al.*, 1987). The single strand binding proteins that are encoded by the *vir* E gene binds single stranded T-DNA. The *vir* E gene product appears to function in pore formation in either the bacterium or plant cell (Dumas *et al.*, 2001). At the bacterial-plant cell interface, the *vir* B gene products (25 kDa, 33 kDa and 80 kDa) may be associated with transferring the T-DNA (Engstrom *et al.*, 1987). The design of the modified T-DNA allows it to flank the expression constructs that encode the desired gene products (Datla *et al.*, 1992). Co-cultivation of plant tissues with *A. tumefaciens* is the first step for the release and transfer of the main single stranded component (T-DNA) for random integration into plant genomes (Horsch *et al.*, 1985).

Upon infection by *A. tumefaciens*, the host plant then synthesizes opines (nopaline and octopine) and the antibiotic degrading enzyme that is encoded between the T-DNA borders. In plant transformation, cells that have been modified by the new genes hopefully can still grow into a clump of unorganised cells known as a callus. Plant calli can then be made to reorganise and differentiate into plants, as is the case of tobacco and rice. Plantlets may eventually grow into mature transgenic plants which are more amenable to molecular and biochemical analyses than are small calli. Not all transformations are successful since the integration of transgenes within the plant genome is not controlled and when a

transgene enters a transcriptionally inactive region of the genome, little or no expression of the transgene occurs. This is one of the major setbacks of generating transgenic plants. Further, many transgenes may have adverse effects on the host plant that could result in abnormal morphology such as stunted growth or infertility. These conditions may occur when a transgene is inserted into a vital plant gene or when a recombinant protein has an adverse effect on the plant. Thus, all expression systems have merits and demerits and they must be considered carefully for recombinant expression.

Effective plant-derived antigens

Several antigens from different infectious agents have been successfully produced in plants including HIV-1 p24 protein (Zhang *et al.,* 2002), human cytomegalovirus (HCMV) immunodominant glycoprotein B (gB) complex (Tackaberry *et al.,* 1999; 2003), HBV nucleocapsid protein (Tsuda *et al.*, 1998), alfalfa mosaic virus coat protein/HIV-1 V3 loop fusion (Yusibov *et al.,* 1997), *E. coli* heat-labile enterotoxin β-subunit (Haq *et al.,* 1995), hepatitis B surface antigen (Thanavala *et al.,* 1995) and rabies glycoprotein (McGarvey *et al.,* 1995). The application of the human hepatitis B virus core antigen from transgenic tobacco plants has been studied as a positive control for serological diagnosis in a clinical setting (Tsuda *et al.,* 1998). The plant expression construct was placed under the control of the CaMV 35S promoter for transcription and eventual synthesis of the entire coding sequence of the HBV core protein. Transgenic tobacco plants assembled the HBcAgs into virus like particles (VLPs) with diameters of 25 to 30 nm and this was consistent with the size observed for expression in other recombinant systems. The plant-derived HBcAgs retained their antigenicity as were *E. coli* derived HBcAgs. The maximum observed HBcAgs expression in fresh tobacco leaf tissue was 24 μg/g (0.002%). Thus, the wild-type HBcAgs coding sequence and the CaMV 35S promoter resulted in low expression of the HBcAgs in tobacco plants. Indeed, low expression not greater than

approximately 0.5% appears to be the norm in plant expression studies where the coding sequence is not from plant source and/or a plant signal sequence is not present. For example, the HBsAg observed in transgenic potato tuber was 8.35 μg/g. Although this expression construct had a viral etch leader sequence encoded between the CaMV 35S promoter and the wild-type HBsAg coding sequence, this value was even lower than that observed for the full-length HBcAg expressed in transgenic tobacco plant where no viral leader sequence was present. Further, the natural endoplasmic retention sequence found in the HBsAg coding sequence was not removed. However, the two different plant systems (tobacco and potato) as well as the tissue types may have influenced the expression levels. Further experiments were conducted in tobacco to achieve higher HBsAg levels by using the CaMV d35S, a viral etch leader sequence, the soybean vegetative storage protein (VSP) alpha S signal peptide and a synthetic plant-optimized HBsAg coding sequence (Sojikul *et al.,* 2003). Various combinations of the different components were used to generate six different expression vectors with or without a KDEL encoded sequence. The HBsAg maximum expression increased to 226 μg/g in the rapid test tobacco plant system when the soybean vegetative storage protein signal peptide was present. Therefore, several augmentation sequences are needed to achieve high levels of recombinant proteins in plant systems.

In feeding trials, several bacterial and viral antigens produced in plants were equivalent as immunogens compared to their counterparts derived from other commercial sources in laboratory animals (Arakawa *et al.,* 1998a, b; Modelska *et al.,* 1998; Kong *et al.,* 2001; Lauterslager *et al.,* 2001). For example, Modelska *et al.* (1998) attempted oral immunization when plant tissues were used to deliver a vaccine antigen from the rabies virus resulting in production of IgA antibodies in mice. The effectiveness of a vaccine is

measured when a vaccinated animal is challenged by the specific pathogen and the animal does not develop the infection as is known for the commercially available HBV vaccine. In terms of a plant derived antigen that offered protection against the challenge organism, this was shown for oral boosting of mice with a tobacco-derived C-terminal intimin protein from *E. coli* O157:H7 (Judge *et al.*, 2004). Immunization resulted in mucosal protection from bloody diarrhoea. Thus, plant-derived therapeutics were as effective as those derived from other sources and this makes plant expression systems attractive. Targeted protein expression in edible tissues of many plant species, might in some instances not require further processing or purification. Thus, *in situ* fabrication of antigens in foods makes oral delivery possible without purification. Curtiss and Cardineau, 1997, patented the concept of oral delivery of plant-derived antigens.

Not all plant-derived antigens were shown to ellicit an oral immune response due mainly to the low amounts of immunizing dose and/or the absence of mucosal adjuvants. For example, the HCMV gB expressed in transgenic plants accumulated up to 0.31 μg/mg (0.03% TSP) (Tackaberry *et al.*, 1999). This value was very low due to poor perception that did not include plant-codon optimization. Oral immunization of mice failed perhaps because of the low doses used as compared to other studies in which higher doses (~20X higher) of transgenic plant tissues were used (Alli *et al.*, 2002a, b). For example, the maximum vaccination doses that were found effective in stimulating immune responses for the Norwalk virus capsid protein derived from potato tubers and the respiratory syncytial virus-F protein derived from tomatoes were 80 μg (Mason *et al.* 1996) and 130 μg (Sandhu *et al.* 2000) respectively. However, a novel mechanism to increase recombinant protein expression in plants appears to involve growth of plants in constant environments such as an underground mine. When the same HCMV gB transgenic plants were grown in an

underground mine, the expression increased from 0.03% to 1.07% (Tackaberry *et al.*, 2003). These experiments may allow others to focus their future endeavours towards achieving higher antigen doses in plant tissues intended for oral, *in situ*, administration.

In many oral immunization studies, use was made of highly effective oral adjuvants including the cholera toxin (CT) β-subunit or the heat labile enterotoxin β-subunit (LT-B) of *E. coli*. These adjuvants are not known to cause the disease, as do the holotoxins (Haq *et al.* 1995; Tacket and Mason, 1999; Lauterslager *et al.* 2001). Other researchers have investigated DNA and live attenuated viral vectors for vaccination studies (Lebedev *et al.*, 2003; Goncharova *et al.*, 2002). Table 1 shows a summary of some biologically active proteins that were successfully produced in transgenic plants. In plant recombinant systems, if a vaccine is produced in an edible plant, it is possible to avoid the routine purification steps and deliver the vaccine "*in situ.*" Therefore, higher levels of antigens expressed in plant tissues are desired. Thus, this thesis studies the augmented expression of the highly immunogenic HBcAg for future multivalent oral vaccinations. This thesis studies the hepatitis B virus core particle and will not be complete without a discussion of the hepatitis B virus.

Table 1. Biologically active human proteins produced in transgenic plant systems

Protein Source	Peptide Encoded	Plant System	Maximum Expression	Biological Activity
Enterotoxigenic *E. coli*	Heat-labile toxin B-subunit	Tobacco Potato	<0.01 % TSP [a] 0.19 % TSP	[1]Orally immunogenic
Human	GM-CSF	Tobacco Rice	0.03 % TSP 1.3 % TSP	[2]Stimulated TF-1 cell proliferation
Hepatitis B virus	Surface envelope	Tobacco Potato	<0.01 % TSP <0.01 % FW [b]	[3]Orally immunogenic
Human	IGF-1	Tobacco Rice	0.02 % TSP 0.04 % TSP	[4]Stimulated neuroblastoma cells to differentiate
Hepatitis B virus	Core Particle	Tobacco	<0.01 % FW	[5]Serologically detected
Norwalk virus	Capsid protein	Tobacco Potato	0.23 % TSP 0.37 % TSP	[6]Orally immunogenic

The table represents a summary of recombinant products that were harvested from different plant systems. The source organisms of the proteins, the plant expression system, the yield and the biological activity observed are stated for each protein. The yields ranged from 0.01% of total soluble proteins when the Hepatitis B virus surface antigen was expressed in tobacco plants to 1.3% of total soluble proteins when the hGM-CSF was expressed in rice endosperm cells. References for superscript numbers are: 1 Haq *et al.*, 1995, Tacket *et al.*, 1998; 2 Sardana *et al.*, 2002; 2004; 3 Mason *et al.*, 1992; Richter *et al.*, 2000; 4 Panahi, 2002, Panahi *et al.*, 2003; 5 Tsuda *et al.*, 1998; 6 Mason *et al.*, 1996, Tacket *et al.*, 2000. [a] TSP, total soluble protein. [b]FW, fresh weight.

Current state of knowledge: hepatitis infections

Recent estimates of viral hepatitis infection rates indicate that over 90% of the world population has been infected with one of the now identified five human hepatitis viruses (Morton, 2001). Among these, hepatitis A has infected approximately 5 billion people and hepatitis E, 2 billion alone. While these two viruses cause self-limited acute infection resolved without further health consequences, hepatitis B and C viruses are the major etiological agents responsible for the development of the chronic carrier state that can lead to the induction of cirrhosis and hepatocellular carcinoma (Koch *et al.,* 1984; Tokino, *et al.,* 1987; Tu *et al.,* 2001; Blumberg, 2002; Strauss and Strauss, 2002). Currently 350 million people are chronic carriers of the hepatitis B virus and no effective cure exists for this infection.

The human hepatitis B virus (HBV) belongs to the genus *Orthohepadnavirus* in the family of Hepadnaviridae. Jaundice associated with liver disease has been known for the past 25 centuries and was depicted by Hippocrates. The HBV is related to other hepatitis viruses including the Duck Hepatitis B Virus, Ground Squirrel Hepatitis Virus and Woodchuck Hepatitis Virus. Viral hepatitis infections were eventually correlated with blood transfusions and blood-derived products used in the preparation of vaccines (Hill, 1965; Singleton *et al.,* 1971). Vaccines linked to the HBV disease include smallpox derived from human lymph and yellow fever vaccines that contained human serum. Later, other bodily fluids were found to contain the HBV including genitalia fluids and those from the nasal and oral cavities (Bancroft *et al.,* 1977; Scott *et al.,* 1980; Davison *et al.,* 1987). The highly infective nature of the HBV was demonstrated when transmission was observed from human saliva, containing complete virions, to gibbons (Bancroft *et al.,* 1977).

McCallum, 1947, first classified the hepatitis B viral disease as homologous serum hepatitis. Blumberg *et al.,* in 1967, were able to identify a novel antigen in an Australian aborigine and this antigen (named Au) was later correlated to the hepatitis B infection. Dane *et al.* (1970) identified the Au antigen by electron microscopy and showed an explicit link between Dane particles, serum Au antigen and hepatitis B viral infections. The Dane particle was identified as a 42 nm diameter particle and is also known as the hepatitis B virus surface antigen (HBsAg) (Figure 1). The hepatitis B virus core antigen (HBcAg) consists of a 28-32 nm diameter core particle, the viral DNA and the polymerase (Schodel *et al.,* 1996). There are two types of "empty" secreted HBcAgs found in HBV infected individuals. One is a 22 nm diameter secreted filament while the other is a 22nm diameter secreted sphere. In 1972, the defective hepatitis B "e" antigen (HBeAg) was identified (Magnius and Espmark, 1972). The HBeAg is a fusion protein that consists of 159 amino acids of which the last 149 are derived from the HBcAg. It took another year for the HBV DNA polymerase to be identified within the core of the virus (Kaplan *et al.,* 1973). Within a year, the association of the HBV DNA polymerase activity was linked to the presence of the viral hepatitis B disease (Krugman *et al.,* 1974). The HBV is the smallest known DNA virus discovered to date having a 3.2 kb genome (Strauss and Strauss, 2002). The organization of the hepatitis B viral genome and the derived encoded protein products are shown in Figure 2.

After infection with the HBV, the immune system attempts to clear the infection (Lazinski, 1998). A review of effective "mucosal immune responses" has been published by Ogra *et al.* (2001). This immune response results in increased cellular proliferation of liver cells. The mechanism by which HBV infects liver cells is unknown and the HBV has no tropism for other tissues. This makes it more difficult to design drugs that block cellular

Figure 1. The hepatitis B virus particle types currently used for global immunizations. The figure shows the three known types of HBV surface antigens that are used for effective human vaccinations against the specific pathogen. The complete hepatitis B virion or Dane particle (42 nm in diameter) consists of the genome, polymerase, core antigen and the surface coat proteins. The three immunologically important surface peptides are derived from gene S such that the shortest consists of the S (small) domain, the Pre S2 consists of the M (medium) domain and the Pre S1 consists of the L (large) domain. The current HBV vaccine is derived from yeast and does not contain the HBV genome, polymerase or HBcAg as depicted with the Dane particle. In some countries, the secreted 22 nm diameter filaments derived from the blood of chronic HBV carriers, are used for vaccinations. The secreted 22 nm diameter sphere consists of the three immunologically important surface peptides and can also be used for HBV vaccinations. This picture was adapted from source http://www.iprimus.ca /~harlequin/HBV/hbvparts.htm.

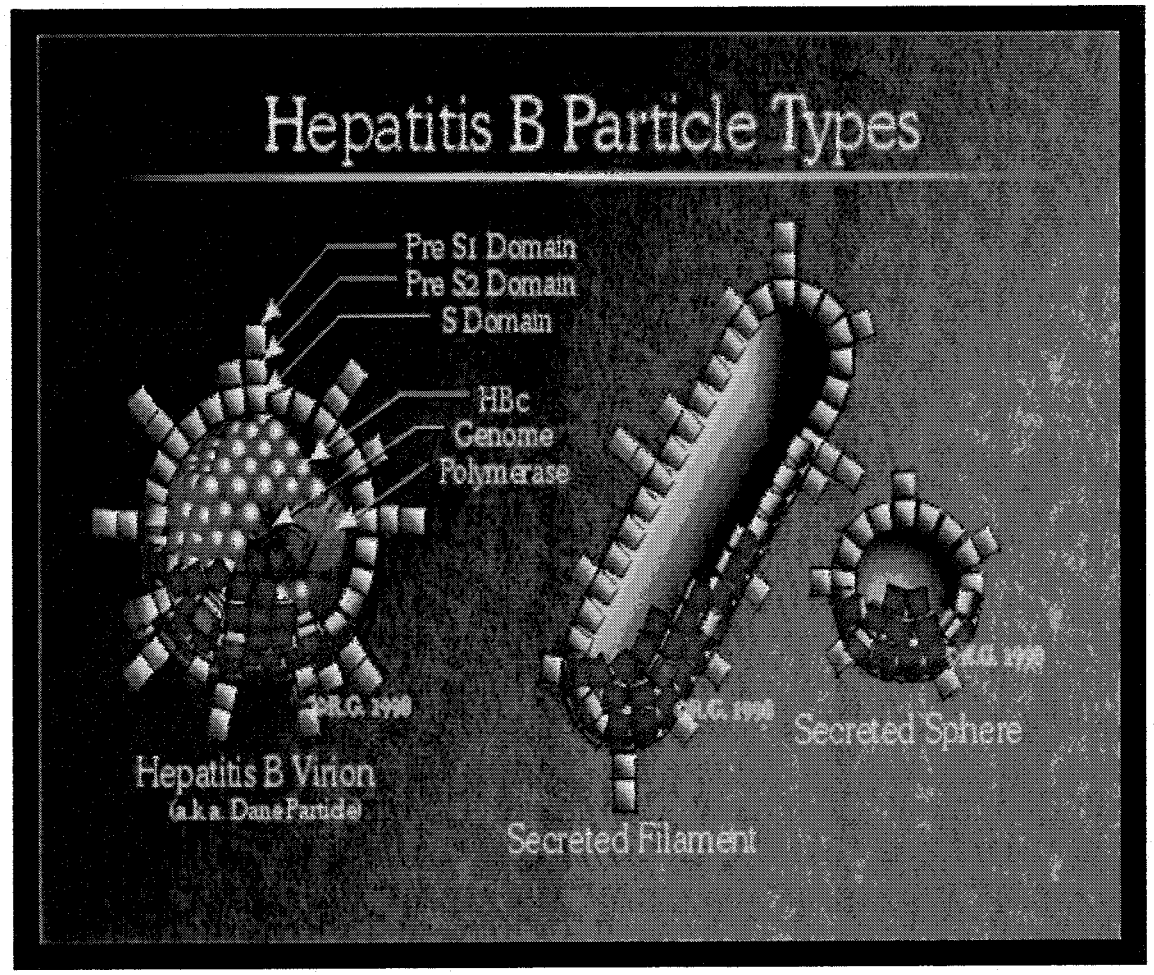
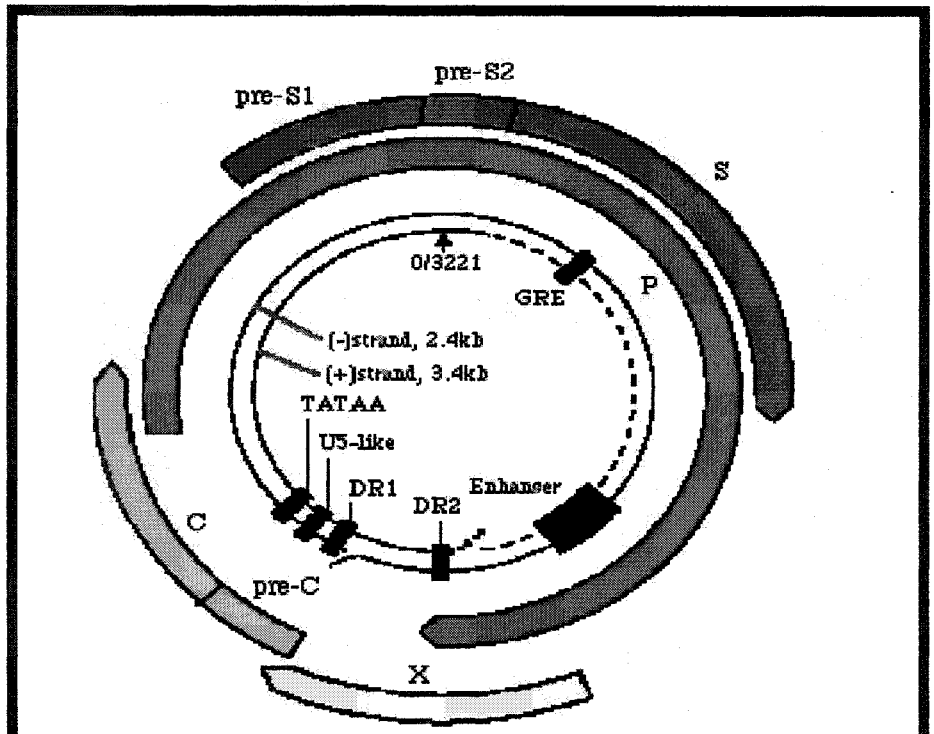

Hepatitis B Particle Types
Pre S1 Domain
Pre S2 Domain
S Domain
HBc
Genome
Polymerase
Hepatitis B Virion
(a.k.a. Dane Particle)
Secreted Filament
Secreted Sphere

Figure 1

Figure 2. The organization of the hepatitis B virus genome and the encoded protein products derived from the partial double stranded DNA. The four known gene products are depicted in blue, green, yellow and red. Gene P encodes the polymerase, gene C encodes the core protein subunit, gene X encodes a viral protein that is involved in transactivation of viral transcription and gene S encodes three different polypeptides that form the viral surface coat - derived from alternative translation start sites. This picture was adapted from Alan Cann, http://www-micro.msb.le.ac.uk/335/hepatitis1.gif.



Source: www-micro.msb.le.ac.uk/335/hepatitis1.gif.

Figure 2

entry and hence, the reliance on HBV vaccines. Initially, immunoglobulin (Ig) class M antibodies (Abs) appear in the blood targeting HBV surface antigen (HBsAg) as well as HBV core particles. Later, highly specific IgG class antibodies are released. This results in 90% clearance via destruction of infected hepatocytes while 10% of patients advance to chronic infection. There are no effective treatments for chronically infected HBV patients and this pool serves to maintain the HBV in the population. An approximate one million HBV infected individuals die annually (Strauss and Strauss, 2002).

The HBV genome can exist as an episomal plasmid known as a covalently closed circular DNA (cccDNA) or it can integrate within the host genome. The cccDNA represents the active viral genome that is used for replication and transcription. The role designated for each encoded protein from the HBV genome is not fully understood. Thus, the reliance on effective HBV vaccines is deemed necessary. However, several proteins have been identified that compose the structural building blocks of the virus. They include three outer surface proteins (preS1, preS2 and S) that vary in length due to alternate translation initiation codons, the HBV core protein, a polymerase and protein X. Protein X is believed to turn-on viral and host genes. The hepatitis D virus (HDV), discovered in 1977, is a subviral agent requiring the HBV envelope for its assembly and so cannot replicate in the absence of HBV. Vaccinations against the HBV have lead to a rapid decline in HDV infections.

Hepatitis B vaccine

Hepatitis B vaccine, developed in 1982 by SmithKline Beecham Biologicals, is an example of a recombinant DNA technology product that has been highly successful. It is marketed under the brand name Engerix-B®. The HBV vaccine is produced in yeast cells expressing only a non-infectious subunit of the whole virus particle (HBsAg). The vaccine

is highly effective and safe due to broad protection offered against all subtypes of the hepatitis B virus. Others have investigated live recombinant hepatitis B virus vaccine (Paoletti *et al.,* 1984), edible hepatitis B virus vaccines derived from lettuce, lupin, and potato (Kapusta *et al.,* 1999, 2001; Richter *et al.,* 2000) and multivalent hepatitis B vaccines (Musacchio *et al.,* 2001). Unlike the HIV-1 viral gp120 surface antigen that constantly mutates, the low mutation rates of the hepatitis B virus have been useful in this respect. Hepatitis B vaccination requires several injections to achieve full protection, however; it is not beneficial to the 350 million chronically ill people already infected with the HBV and antiviral drugs are not as effective in treating the disease (Ben-Ari *et al.,* 2001; Das *et al.,* 2001; Ayres *et al.,* 2003).

Chronic hepatitis B carriers serve as the HBV reservoir and are responsible for both horizontal and vertical transmissions worldwide (Hutchinson *et al.,* 1965; Schweitzer *et al.,* 1975; Stevens *et al.,* 1975; Xu *et al.,* 2001). Several reports have shown that the HBV may be reactivated after transplantation (reviewed by de Villa *et al.,* 2003). Liver donors who were HBsAg negative and HBcAg positive were able to transmit the HBV to recipients who were uninfected prior to receiving organ donations (Dickson *et al.,* 1997; Uemoto *et al.,* 1998; Roque-Afonso *et al.,* 2002). However, there was no HBV transmission reported to HBV negative recipients when hearts of donors were HBsAg negative, HBcAg positive and HBV immunoglobulin M (IgM) negative (Wachs *et al.,* 1995). Although hepatitis B immunoglobulin may prevent transmission of the virus, treatment is expensive and complicated (Tong *et al.,* 1985; Roque-Afonso *et al.,* 2002). Thus vaccines are needed to prevent the spread of the HBV.

The hepatitis B vaccine remains expensive and out of reach for the poor nations of the world. Adjuvant and other compounds found in many vaccines may result in adverse

effects and permanent tissue damage. They include aluminium hydroxide, formaldehyde, thimerosal and phenol. In the commercial Engerix-B® hepatitis B vaccine, there is 0.50 mg aluminium and <1.0 mcg mercury in each adult dose (source: Prescribing Information for Engerix-B®, SmithKline Beecham Biologicals, 2001). Fears about vaccines were disseminated by the New Jersey based "Cure Autism Now" group as well as Dr. Viera Scheibner (1993). Claims that vaccines were not effective or beneficial to humans due to contaminations and/or the presence of toxic adjuvants have contributed to public fears. Further, the discovery by the Food and Drug Administration (FDA) of high levels of mercury accumulation in some vaccinated infants and the recommendation that this preservative be eliminated from childhood vaccines also lead to "fears" (Ball *et al.*, 2001). Thimerosal was used in childhood vaccines such as Hepatitis B, influenza, DPT (diphtheria, pertussis and tetanus) and MMR (measles, mumps and rubella). Mercury is known to cause neurological problems in animals (Davidson *et al.*, 2004). Although public fears about vaccines may be dispelled by showing the benefits of modern vaccinations in reducing or preventing viral and bacterial infections, others have linked vaccinations to the development of illnesses in Gulf War veterans (Unwin *et al.*, 1999; Hotopf *et al.*, 2000). Thus, acceptance of "toxic" adjuvants to vaccines may not be desirable in some instances due to the negative publicity in developed countries by those opposed to vaccines. Oral immunity is known to be effective for the cholera, polio and typhoid vaccines (Sabin, 1965; Ogra *et al.*, 2001), but plant-derived antigens may increase the number of available oral vaccines and thus eliminate toxic adjuvants. This will help dispel public fears associated with vaccine additives. This motivation will drive the development of low-cost, edible-plant based vaccines in the future (Mason *et al.*, 2002; Arakawa *et al.*, 1998a). Therefore, an economic alternative for production and delivery of vaccines at a relatively low cost is required for the developing countries. Plants may become the economic

alternative. A study of the hepatitis B virus core particles now follows so that the reader can gain more insight into the usefulness of the hepatitis B virus core particles as carriers for foreign epitopes.

Hepatitis B virus core particles

Hepatitis B virus core particles or hepatitis B core antigens (HBcAgs) are composed of 90 or 120 dimeric subunits that assemble to form mainly spherical 28-32 nm diameter HBcAgs. The secondary structure of the HBcAg has been determined to be mainly α-helical with a small β-sheet component (Wingfield *et al.*, 1995; Bottcher *et al.*, 1997). The HBcAg subunits are each composed of 183 amino acids of which most are either hydrophobic or charged. The 21 kDa HBcAg polypeptides are synthesized in the cytoplasm where pairs dimerize before assembly into HBcAg (Bottcher *et al.*, 1997). Although four cysteine residues occupy positions 48, 61, 107, and 183, mutagenesis studies have shown that they are not essential for dimerization (Nassal *et al.*, 1992). When expressed in *E. coli*, two distinct HBcAgs were observed by electron cryomicroscopy. The smaller particle is composed of 180 subunits (90 dimers) while the larger, more abundant type is composed of 240 subunits (Crowther *et al*, 1994). Assembly of the HBcAgs was shown to occur in a wide range of bacterial, yeast and mammalian recombinant systems indicating the flexibility of the particle (Schodel *et al.*, 1996). Assembly of the HBcAg appears to depend on four critical hydrophobic residues that have a strict spatial relationship to each other (Yu *et al.*, 1996). They are located seven residues apart (leucine 101, leucine 108, valine 115, phenylalanine 122). Mutagenesis analyses showed valine 115 or phenylalanine 122 could be replaced with a leucine residue but not both at the same time. During packaging, four arginine clusters located beyond the 144th amino acid residue appear to function in the binding of nucleic acids. Truncation beyond the 144th amino acid does not lead to loss of assembly or decrease in the size of HBcAgs but it

eliminates the packaging of nucleic acids (Gallina *et al*, 1989). The HBcAg is not glycosylated in its natural human host and contains strong B and T cell epitopes making it a highly immunogenic particle to stimulate both humoral and cellular immunity (Schodel *et al.*, 1996; Pumpens and Grens, 2001). The versatile and highly immunogenic nature of the HBcAg makes it a good candidate for multivalent vaccinations.

Developments of effective vaccines that can induce neutralizing antibodies as well as result in cytotoxic T lymphocyte (CTL) responses are important in offering protection against infective organisms. Several groups have used short peptides to identify the regions of the HBcAg that were recognized by human CD4+ T-cells from HBV infected patients. It appeared that there were multiple T-cell recognition sites that almost covered the whole 183 amino acids with several immunodominant epitopes localized between amino acids 1-25 and 50-85 (Ferrari *et al.*, 1991; Jung *et al.*, 1995). On the other hand, only few CD8+ CTL epitopes have been identified (Missale *et al.*, 1993). Importantly, the amino acid residues 88–96, recognized by CD8+ CTL were located near the immunodominant c/e1 loop where peptides may be inserted. Therefore, it may be possible that the inserted peptide fragments will be presented by major histocompatibility complex I and in turn, could be targeted by CD8+ MHC class I restricted CTL responses. It appears that for B cell recognition, only two peptide fragments have been identified and both are conformational epitopes. Very significant is that one of the B-cell epitopes is located in the immunodominant c/e1 loop region between amino acid residues 74–83 (Salfeld *et al.*, 1989). This epitope is always exposed on the surface of the viral capsid. Interestingly, analyses revealed that another B-cell epitope was located near amino acid 130 that protrudes through the surface antigen. Therefore, many epitopes exist for the induction of

strong neutralizing antibody as well as cytotoxic T lymphocyte (CTL) responses. This makes the HBcAg an excellent immunogenic particle.

Insertion of peptides at the N or C terminal as well as within the region of the major immunodominant epitope (c/e1) has been reviewed by Pumpens *et al.* (1995). However, there exist differences in the degree of insert exposure such that there is complete exposure at the c/e1 loop but only partial exposure at the N-terminal and C-terminal. The N-terminal appears to be less flexible, accepting up to 50 amino acids while the C-terminal appears more flexible in accepting about two times this number of amino acids (Pumpens *et al*, 1995). The c/e1 immunodominant epitope is of particular interest and is localized near the 80^{th} amino acid, forming a non-structural loop that appears as a protruding spike on the surface of the assembled core particle (Figure 3) (Salfeld *et al.*, 1989; Pumpens and Grens, 1999). In the duck hepatitis B virus core antigen, Argos and Fuller (1988) discovered a nonhomologous insertion of 39 amino acid residues. This discovery has led to numerous studies on insertion of peptides in HBcAg that ranged from 5 to 238 amino acid residues (Kratz *et al*, 1999). Displaying foreign epitopes in a polymeric array may indicate foreignness to the host defence system making HBcAgs very immunogenic (Pumpens *et al*, 1995; Bachman and Zinkernagel, 1996). Therefore, the HBcAg is a good candidate for presenting foreign epitopes to the immune system.

Recently, Karpenko *et al.* (2000) found that the length of the inserted peptides in the c/e1 region did not affect the assembly of the HBcAgs. It was suggested that any peptide/protein that can fold independently might be engineered into this c/e1 region. Successful insertions have been reported for GP41 from HIV-1 (Clark *et al.*, 1991), HBsAg from HBV (Borisava *et al.*, 1993), hepatitis C virus (Yoshikawa *et al.*, 1993), human papilloma virus surface antigen (Tindel *et al.*, 1994), complete GFP (Kratz *et al.*, 1999) and

Figure 3. The three-dimensional HBcAg subunit and the possible peptide insertion sites. The diagram hypothetically depicts the HBcAg subunit secondary structure where cylinders represent the alpha-helical regions and ribbons show the polypeptide folds. The numbered amino acids reflect their location within the secondary structure as well as the N and C termini. The first 144 amino acids from the N-terminal sequence are required for subunit dimerization and assembly into HBcAgs. Antigenic peptides from various pathogens can be inserted into the N and C termini or within the immunodominant c/e1 loop. Hence, the immunodominant loop (amino acid residues 78-84) of the H4 plant codon-optimized sequence was designed to incorporate a unique *Hinc* II restriction site for future peptide insertions. This model was adapted from Borisova *et al.* (1996) and Bottcher *et al.* (1997).

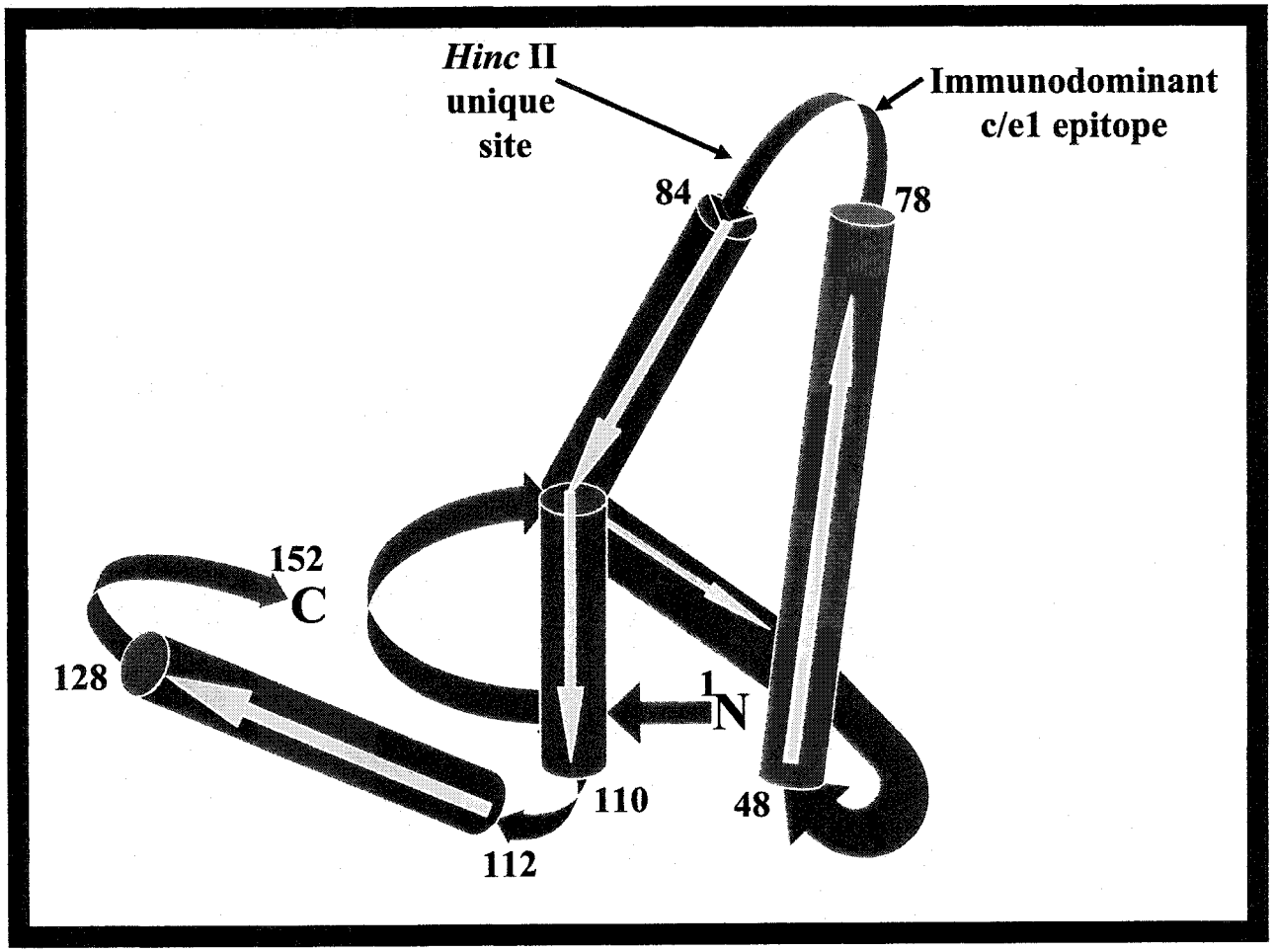

Hinc II
unique
site
Immunodominant
c/e1 epitope
84
78
152
C
128
1
N
110
48
112

Figure 3

hantavirus (Ulrich *et al.*, 1999). Table 2 shows various peptides that were inserted into the HBV core subunit. For example, Kratz *et al.* (1999) have reported that up to 238 amino acids could be engineered into the c/e1 region which resulted in fully assembled chimeric native green fluorescent protein (GFP)/HBcAg (Figure 4). These chimeric particles were also shown to be immunogenic. These observations showed that foreign epitopes displayed on the self-assembled chimeric VLPs can still ellicit strong B-cell and T-cell responses that make the HBcAg an ideal particle for the presentation of candidate vaccines to the immune system (Kratz *et al*, 1999; Schodel *et al*, 1996; Pumpens *et al*, 1995). Not all inserts resulted in successful assembly of the chimeric HBcAgs (Karpenko *et al.*, 2000). Such setbacks may be avoided altogether by engineering a short hydrophobic area at each end of the insertion point in the c/e1 loop (Kratz *et al.*, 1999). Therefore, to establish a solid edible vaccine foundation, the stuffed HBcAgs with foreign inserts could be expressed in transgenic plants for presentation to the mucosal immune system. To lead into the rationale and hypotheses, the disadvantages and advantages of plant systems are now presented below.

Table 2. Antigens inserted into HBV core subunit c/e1 loop

Peptide	Peptide Length	Core Particle Assembly	Reference
Gp 41 from HIV-1	20 a.a	Yes	Clark *et al.*, 1991
HBsAg from HBV	41 a.a	Yes	Borisava *et al.*, 1993
Human Papilloma Virus surface antigen	25 a.a.	Yes	Tindel *et al.*, 1994
Complete GFP	238 a.a	Yes	Kratz *et al.*, 1999

The figure shows the lengths of various peptides that were tested within the immunodominant loop for assembly. The antigenic source, the peptide length and whether the peptide had a positive effect (assembly of the core particle) are also noted.

Figure 4. The stuffed HBcAg subunit with the green fluorescence protein does not prevent dimerization of the subunits and assembly of the HBV core particles. The picture shows an HBcAg with the green fluorescence proteins displayed on each of its constituent subunit at a resolution of 7.2A. The expression construct was used to transform *E. coli* and the harvested chimeric HBcAgs were purified by sucrose density gradient ultracentrifugation (Kratz *et al.*, 1999). The green fluorescence proteins consist of 238 amino acids and are displayed on the immunodominant c/e1 epitope of the HBcAg subunits. This picture was adapted from source http://www2.mrc-lmb.cam.ac.uk/research/SS/CrowtherT/tc1.gif.

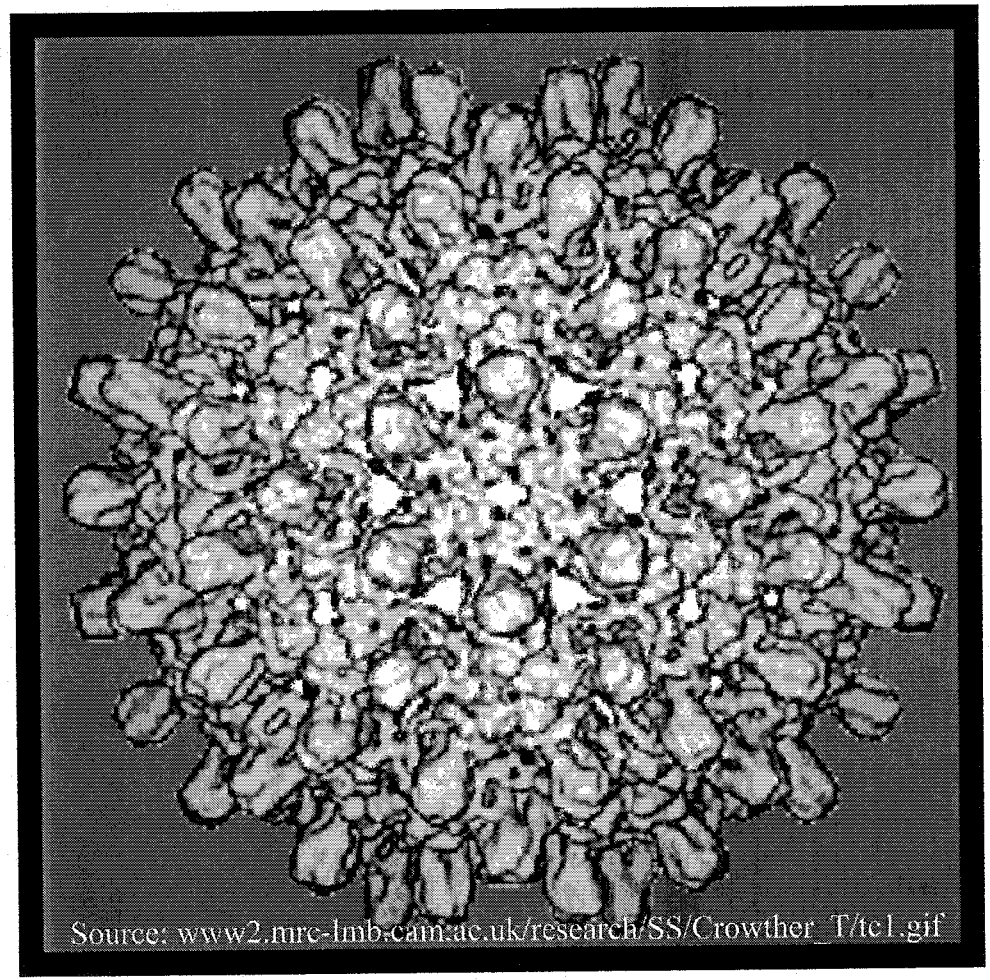


Figure 4

Disadvantages and advantages of using plants as bioreactors

All bioreactor systems have disadvantages as well as advantages. Transgenic plant systems may have the following disadvantages. (1) Expression of transgenes may be low or not detected due to gene silencing (Callaghan, 2001). (2) Carefully designed expression constructs require additional costs. (3) There is no certainty that the predicted expression level will be achieved. (4) Each plant expression vector must be studied on a case-by-case basis. (5) Plant cells multiply slowly when compared to bacterial cells. (6) Concerns have been raised about the effects of transgenic crops on human and animal health including the toxic effects of insecticidal proteins on non-target insects. (7) Concerns have also been raised about the possibility of transgenic pollen contaminations. (8) In a few cases, transgenic plants may become sterile or show morphological abnormalities. (9) At present, acceptance of transgenic crops may not be desirable in certain geographical regions of the world. These demerits are by far outweighed by the merits discussed below. There is no scientific evidence that non-target insects are killed by insecticidal proteins expressed in transgenic crops (De Cosa *et al.*, 2001). Further, advanced molecular biology can effectively eliminate some of the demerits and with the dissemination of the information on the benefits of transgenics, unfavourable opinions may be reduced.

The potential benefits of producing transgenic crops may lead to the following advantages. (1) Stable transgenic plant lines are possible that are cheap, efficient and simple to generate. (2) Increased yields are possible by the addition of a viral leader sequence and/or a plant signal sequence, increased (G + C) content of the coding sequence (Horvath *et al.*, 2000), use of plant codon preferences and addition of a C-terminal ER retention sequence (Schouten *et al.*, 1996). In addition, the ever-increasing biomass of plants leads to increased production. (3) A variety of edible fruits, roots and leaves could be targeted for strict tissue-specific accumulation (Albani *et al.*, 1992; Rascon-Cruz *et al.*,

2004). Thus, a blood protein could be produced in the leaves while the seeds may be further packed with a vaccine. (4) Edible tissues may allow for taste and colour preferences. This will be almost impractical when bacterial, viral or yeast cells are used. (5) Plants lack human pathogens such as HIV-1, HBV, smallpox virus, SARS virus, etc. This decreases the risks associated with obtaining human therapeutics from their natural source organism. Plants have proven effective and safe as recombinant protein expression systems for the production of medically important proteins (Sijmons *et al.,* 1990; Mason *et al.,* 2000). (6) Plant tissues are amenable to production of several recombinant proteins in different compartments and require only a short regeneration time. (7) The start-up costs for transgenic plants are relatively low when compared to industrial fermentation, cell culture, or blood fractionation facilities. (8) Plants have the ability to process recombinant protein in an authentic fashion as found in eukaryotic systems (Cramer *et al.,* 1999). (9) Several antigens could be produced in a single tissue for multi-purpose vaccinations. (10) Edible plant-derived vaccines may result in reduced purification costs. (11) Oral administration of vaccines without adjuvant may lead to decreased public fears of vaccines. (12) Plants are modifiable to produce almost any kind of recombinant proteins. (13) Made-in plants therapeutics are inexpensive due to the "free" inputs (air, sun, soil, water), minimal or no purification costs, and lower costs associated with orally administered vaccines. After careful experimentation, the plant system may become the preferred system for producing oral vaccines for protection against common infectious agents. Taken together, these merits bode particularly well for global administration of vaccines and dissipate the disadvantages associated with plant expression systems. Therefore, the augmentation of processing and assembly of a C-terminal truncated HBcAg in transgenic plants was attempted in tobacco plants and extended into edible carrot and rice plants.

RATIONALE AND OBJECTIVES LEADING TO HYPOTHESES

Rationale

Edible vaccines are based on the expanded notion of creating transgenic plants that can be packed with epitopes from different pathogens instead of generating transgenic plants expressing a single antigen. Considering the advantages mentioned above for the use of plant systems, this novel platform is based on the HBcAg which can self assemble into non-infectious VLPs. A single HBcAg can carry up to 240 epitopes of another peptide that is inserted within the c/e1 immunodominant region. These VLPs can be used as universal carriers for vaccines as well as a supplement to the current hepatitis B vaccinations (Karpenko *et al.,* 2000). Consequently, future insertion of peptides derived from the HBV surface antigen may allow for better protection than the current HBV vaccine that is based only on the surface antigen. During vaccination, the VLPs allow for effective stimulation of both B-cells and T-cells that can later target specifically the natural pathogen (Shiau and Murray, 1997; Murray and Shiau, 1999). Hence packing the HBcAg with 240 epitopes may lead to better protection against a specific pathogen due to the strong humoral and cellular responses against the HBcAg and the inserted epitope (Lazinski, 1998).

Edible transgenic plants, including rice and carrots, can stably store antigens in specialized compartments. Upon administration via the oral route, the vaccine may mobilize the immune system to challenge the natural infectious agent. It has been shown that transgenic potatoes, when fed to mice, can elicit an immune response against the challenge pathogen (Tacket *et al.,* 2000). Oral immunization may result in broad mucosal protection and a systemic response (Clough, 2002). Plants have the ability to produce large quantities of biomass for vaccinations. In feeding trials, several antigens, produced in

plants, were equivalent as immunogens to their counterparts derived from other commercial sources (Mason *et al.*, 1998; Modelska *et al.* 1998). The long-term goal using this strategy is to deliver multivalent edible vaccines using a combination of antigens harvested from different plants. The benefits of a low cost immunization program will have a significant global impact on the cost of health care.

Given the urgent need to develop cheap, safe and effective vaccine to patients, I set out to study the possibility of expressing the HBcAg gene abundantly in transgenic plants by using a strong viral promoter, a viral untranslated leader sequence or a plant signal sequence and plant codon preferences for the HBcAg sequence. The strong CaMV-35S promoter is active in every plant tissue. The AMV-RNA4 untranslated leader sequence should be able to compete efficiently for plant ribosomes. The extensin signal sequence was chosen because extensins are major cell wall protein components and hence it would be very efficient in protein excretion. Plant-codon optimization of the HBcAg sequence will have the right percentages of codons present in the plant to allow more efficient translation. The NOS-TER sequence will allow for efficient termination of transcription and polyadenylation thereby increasing the half-life of the mRNAs. All of these components are predicted to augment the HBcAg level in transgenic plants. Therefore, the expression of the HBcAg was first tested in the tobacco plant system and later extended into edible plant systems.

Hypothesis 1: Augmentation studies in tobacco plants

The first postulate is that transgenic tobacco plants transformed with various expression vectors encoding the C-terminal truncated form of the HBcAg subunit will correctly assemble HBcAg, notwithstanding the fact that the C-terminal truncated form of

the HBcAg subunit, to the best of our knowledge, has not been shown to assemble in plant systems.

Specific objectives

1. To produce four different vectors containing the HBcAg under the control of the CaMV d35S promoter.

2. To optimize the expression levels of HBcAg in transgenic tobacco plants by using the AMV RNA4 translational enhancer or extensin signal sequence fused to the wild-type or plant codon-optimized HBcAg coding sequence and/or addition of the C-terminal encoded KDEL tetra peptide.

3. To determine if the extensin signal sequence is better than the AMV RNA4 translational enhancer for increased HBcAg expression.

4. To determine if the synthetic codon-optimized cDNA and or the KDEL encoded sequence can lead to increased expression of the recombinant HBcAgs.

Hypothesis 2: Edible plant expression studies

The second postulate is that transgenic carrot and rice plants transformed with a wild-type HBcAg expression vector will correctly assemble HBcAgs in a similar manner as that observed in the model tobacco plant system. A future postulate is that immunity will be possible when the HBV core particles are fed to animals or subcutaneously administered. Strong immune responses against challenge by the natural pathogen are predicted in vaccinated animals. The specific objective is to test one or two expression constructs in these plant systems.

Chapter 2

MATERIALS AND METHODS

Materials

Tobacco, carrot and rice tissues for transformation

Young tobacco leaves from non-transformed (NT) tobacco plants (*Nicotiana tabacum* L. cv. Xanthi) were available in the laboratory. Carrot (*Daucus carota* L. cv. Scarlet Nantes) hypocotyls were obtained from germinating commercially available carrot seeds on semi-solid medium (0.8% agar, 3% sucrose). After one week in the dark, carrot hypocotyls were harvested under sterile conditions in a Laminar flow hood for transformation. Chinese variety rice (*Oryza sativa* L. cv. Xiushui 11) seeds were obtained from the Institute of Nuclear Agricultural Sciences, Zhejiang University, Hua Jia Chi, Hangzhou 310029, China. Seeds were sterilized and placed on MS calli induction medium before harvesting the calli tissue as was previously described (Hiei *et al.,* 1994; Cheng *et al.,* 1997, 1998; reviewed by Komari *et al.,* 1998). Sterilized plant tissue sections were transformed with the generated expression constructs as described below.

Tobacco and carrot transformation vectors

The Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan, provided the plant transformation vector pRD 400 that contained the neomycin phosphotransferase II gene (*npt* II) for resistance to the antibiotic kanamycin (Datla *et al.,* 1992).

Rice transformation vector

The pCAMBIA 1303 vector was obtained from the Center for the Application of Molecular Biology to International Agriculture, Canberra, Australia.

Chemicals and enzymes

Solutions were prepared with chemicals from the following suppliers: Sigma, Fisher Scientific, Promega and Gibco BRL. Restriction and modifying enzymes were obtained from Boehringer Mannheim, Gibco BRL, Promega, and New England Biolabs. Antibiotics {ampicillin, carbenicillin, cefotaxime, geneticin (G418), gentamicin hygromycin, kanamycin, rifampicin, ticarcillin, and vancomycin} were obtained from Boehringer Mannheim, Gibco BRL and Sigma. Radionucleotides (deoxycytidine triphosphatase: ^{32}PdCTP) were from Amersham. The University of Ottawa Biotech Institute or Synthaid Biotechnologies Inc., Nepean, Ontario, synthesized customized oligonucleotide primers for sequencing reactions.

METHODS

Generally, experimental techniques were conducted using the previously published standard procedures (Sambrook *et al.,* 1989). The tobacco transformation and regeneration protocol of Horsch *et al.* (1985) was used with a slight modification for the generation of transgenic tobacco plants. Instead of using leaf disks, leaf sections were generated by cutting sterile leaves with a sterile surgical blade. Carrot hypocotyls were transformed and embryonic induction from somatic cells was conducted with the protocol of Hardegger and Sturm (1998). Rice transformation and regeneration was conducted with modifications using the protocol of Cheng *et al.* (1997) and is described later under the rice transformation section. Briefly, the *Hind* III/*Eco* RI fragments from the respective expression constructs were ligated into independent binary vectors for *A. tumefaciens*

mediated transformation of tobacco leaves, carrot hypocotyls and rice calli. The ligation ratios were adjusted such that the insert to vector ratios were three: one. Continuous selection of antibiotic resistant callus tissues was conducted to regenerate transgenic tobacco and rice plants. The antibiotic resistant carrot calli were used to generate single cells that eventually differentiated into embryos. Transgenic plants were regenerated and analysed for HBV core particle expression as described below.

Construction of a C-terminal truncated wild type HBV core particle expression vector

For routine subcloning, standard procedures were used as previously described by Sambrook *et al.* (1989) and Alli (2001). The wild-type coding sequence (C-terminal truncation consisted of the first 153 amino acids), of the hepatitis B virus core particle subunit was contained in an *E. coli* expression plasmid pKK233-2. This plasmid was obtained from Dr. Anton Andonov (Health Canada, Bureau of Microbiology and Immunology, Winnipeg, Manitoba). The C-terminal truncated coding sequence was inserted into another vector (pBI 525) that was obtained from the National Research Council (NRC), Canada (Datla *et al.,* 1993). The pBI 525 plasmid contained the strong Cauliflower Mosaic Virus 35S promoter (Covey *et al.,* 1981; Guilley *et al.,* 1982; Condit *et al.,* 1983; Odell *et al.,* 1985) for constitutive tissue expression, a transcriptional enhancer element (d35S) (Kay *et al.,* 1987), an AMV RNA4 translation enhancer sequence and the nopaline synthetase terminator and polyadenylation sequence (NOS-TER) from *A. tumefaciens*. The pBI 525 plasmid was digested with *Nco* I and *Bam* HI restriction enzymes that cleaved the plasmid after the AMV RNA4 leader sequence. A 462 bp DNA fragment, containing the coding sequence for HBV C-terminal truncated core particle subunit, was excised from the vector pKK233-2 using the same enzymes as above. The fragment of interest was ligated into the pBI-525 vector in-frame with the AMV RNA4 leader sequence.

Transformation of *E. coli* with these plasmids and verification by restriction analyses was conducted to determine their authentic nature. Clones were analyzed by restriction analyses to confirm their authenticity. Further, the HI construct was sequenced using both forward and reverse primers to confirm that the right fragments have been correctly ligated together in-frame. The deduced HBcAg coding sequence is shown in Figure 5. The resultant plasmid as shown in Figure 6 was designated as the H1 expression construct. Since the pBI 525-backbone vector was not a plant transformation vector, further subcloning was required to insert a complete H1 expression construct into a dicot transformation vector.

Figure 5. The coding sequence of the H1 expression construct. The HBV coding sequence contained the first 459 nucleotides from the N-terminal of the wild-type HBV coding sequence and was followed by a stop codon (3 bp). The coding sequence was deduced from DNA sequencing analyses. This sequence was obtained from Health Canada and was removed from the pKK233-2 plasmid using the enzymes *Nco* I and *Bam* HI. Subcloning was conducted to insert the HBV coding sequence into a pBI525 vector that contained the d35S promoter, an AMV-RNA4 translational enhancer sequence and the NOS-TER sequence. The HBV coding sequence was ligated between the AMV-RNA4 translational enhancer and the NOS-TER sequence. The complete H1 expression construct is shown in Figure 6 and was further subcloned into the pRD 400 and pCAMBIA 1303 vectors for *A. tumefaciens* mediated transformation of plant tissues.

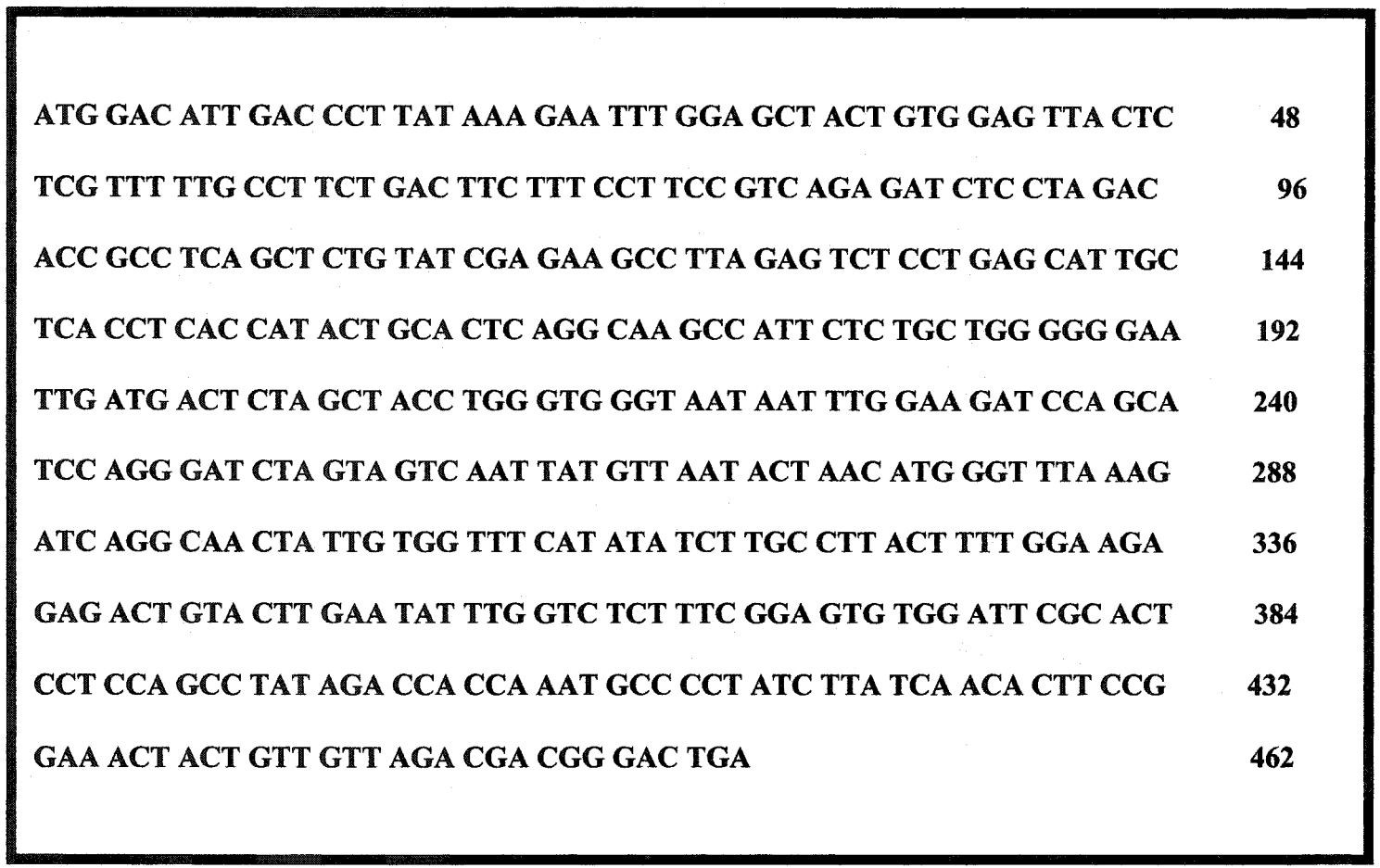

ATG GAC ATT GAC CCT TAT AAA GAA TTT GGA GCT ACT GTG GAG TTA CTC 48
TCG TTT TTG CCT TCT GAC TTC TTT CCT TCC GTC AGA GAT CTC CTA GAC 96
ACC GCC TCA GCT CTG TAT CGA GAA GCC TTA GAG TCT CCT GAG CAT TGC 144
TCA CCT CAC CAT ACT GCA CTC AGG CAA GCC ATT CTC TGC TGG GGG GAA 192
TTG ATG ACT CTA GCT ACC TGG GTG GGT AAT AAT TTG GAA GAT CCA GCA 240
TCC AGG GAT CTA GTA GTC AAT TAT GTT AAT ACT AAC ATG GGT TTA AAG 288
ATC AGG CAA CTA TTG TGG TTT CAT ATA TCT TGC CTT ACT TTT GGA AGA 336
GAG ACT GTA CTT GAA TAT TTG GTC TCT TTC GGA GTG TGG ATT CGC ACT 384
CCT CCA GCC TAT AGA CCA CCA AAT GCC CCT ATC TTA TCA ACA CTT CCG 432
GAA ACT ACT GTT GTT AGA CGA CGG GAC TGA 462

Figure 5

Figure 6. The four different HBcAg expression constructs used for the protein augmentation studies in tobacco. These expression constructs were based on the HBV wild-type codons (H1) and the *Arabidopsis thaliana* genomic codon bias were used to generate the plant codon-optimized (CO) expression constructs H2, H3 and H4. Each complete expression construct was subcloned into independent plant transformation vectors (pRD 400) using the restriction endonucleases *Hind* III and *Eco* RI. The pRD 400 backbone vectors with independent inserts H1-H4 were used to transform *A. tumefaciens* strain LBA 4404 for subsequent transformation of tobacco leaf sections. The figures are not drawn to scale and are further discussed in the text.

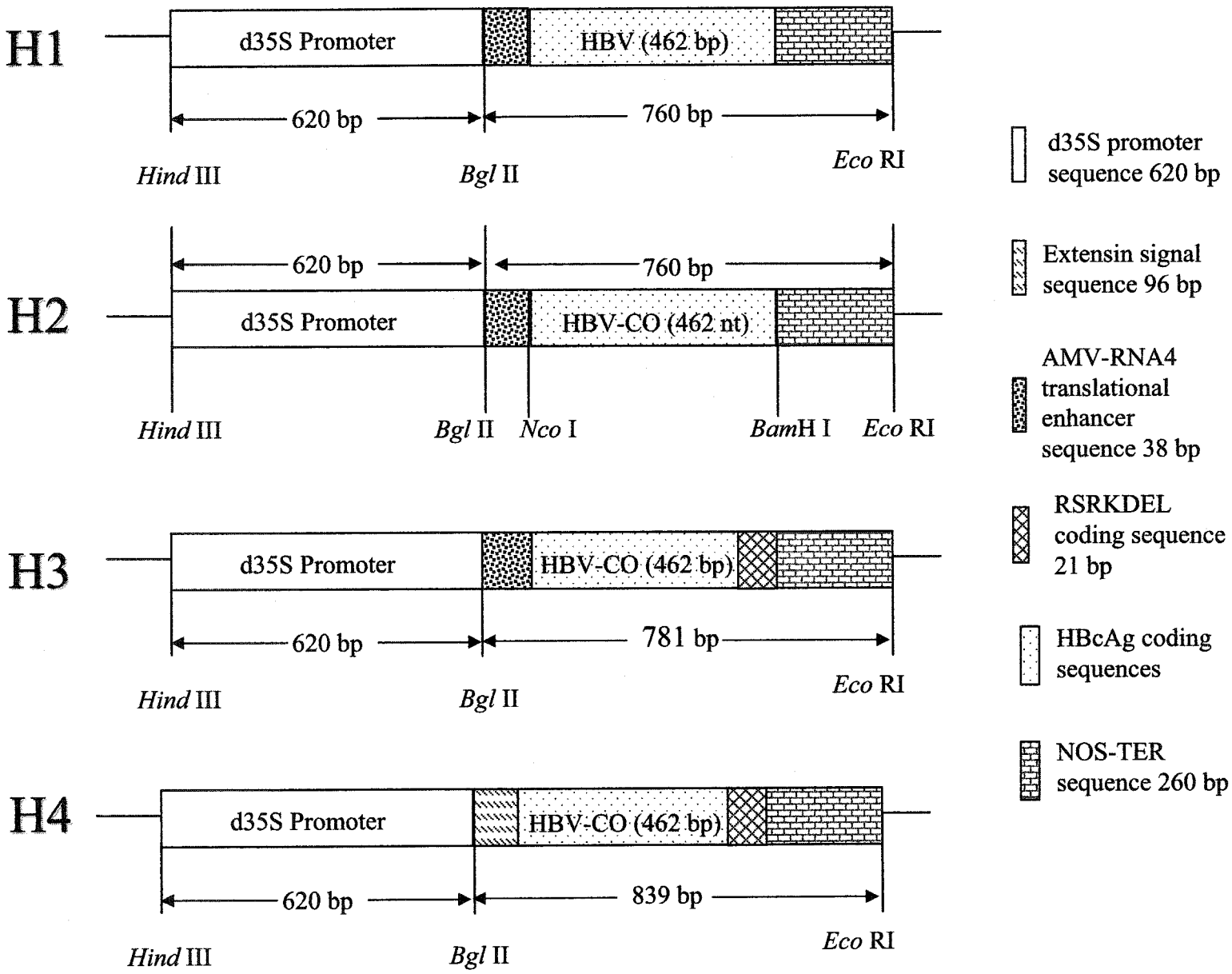

H1
d35S Promoter
HBV (462 bp)
620 bp
760 bp
Hind III
Bgl II
Eco RI
H2
620 bp
760 bp
d35S Promoter
HBV-CO (462 nt)
Hind III
Bgl II
Nco I
BamH I
Eco RI
H3
d35S Promoter
HBV-CO (462 bp)
620 bp
781 bp
Hind III
Bgl II
Eco RI
H4
d35S Promoter
HBV-CO (462 bp)
620 bp
839 bp
Hind III
Bgl II
Eco RI
d35S promoter sequence 620 bp
Extensin signal sequence 96 bp
AMV-RNA4 translational enhancer sequence 38 bp
RSRKDEL coding sequence 21 bp
HBcAg coding sequences
NOS-TER sequence 260 bp

Figure 6

Design and generation of codon-optimized HBV core particle expression constructs

The nucleotide sequence from the H1 expression construct that encoded the hepatitis B viral core particle subunit was codon-optimized to match that of the *Arabidopsis thaliana* plant codon usage (*Arabidopsis* Genome Initiative, 2000). Further work, plus or minus the extensin signal sequence and/or the endoplasmic retention sequence (KDEL) was initiated with the codon-optimized sequence. The H2 codon-optimized expression construct was produced from the H3 expression construct. The KDEL sequence was flanked by both a 5' and a 3' *Xba* I restriction endonuclease site. The H3 expression construct was digested with the restriction enzyme *Xba* I. This was followed by blunting of the vector ends and subsequent self-ligation to generate the H2 expression construct (Figure 6). The H2 expression construct mimics the H1 expression construct but the coding sequence in the H2 construct is plant codon-optimized (Figure 6).

The H3 expression construct was placed into the pBI 525 vector and 3` of the AMV-RNA 4 translational enhancer sequence. The H3 construct mimics the codon-optimized H2 expression construct but had an additional 3` KDEL encoded component at the end of the HBV core particle coding sequence. Since the H3 expression construct was designed with an easily removable KDEL sequence to produce the H2 construct, three additional amino acids were encoded (RSR) between the HBcAg coding sequence and the KDEL sequence. Thus, the H3 construct contained the HBcAg codon-optimized coding sequence and the RSRKDEL coding sequence (Figure 6). All the coding sequences for the H3 expression construct were contained in a second vector supplied by Health Canada following codon-optimization and synthesis (5` extensin signal sequence, C-terminal truncated HBV core particle subunit and RSRKDEL sequence 3`). The extensin signal sequence was flanked by both a 5` and a 3` *Nco* I restriction endonuclease

site. The desired H3 coding sequence was removed by digestion with *Nco* I and *Bam* HI from the second vector obtained from Health Canada. The H3 coding sequence was inserted into the original pB1 525 vector from the NRC that was similarly digested. Hence, ligation of these two fragments yielded the H3 vector as shown (Figure 6).

The extensin signal sequence was included in the fourth (H4) expression construct for targeting the recombinant protein to the cell wall (Chen and Varner, 1985). This signal sequence preceded and was fused in-frame with the codon-optimized coding sequence of the C-terminal truncated HBV core particle subunit. However, in the H4 expression construct, the AMV-RNA 4 translation enhancer sequence was not included after the d35S promoter. The RSRKDEL coding sequence was synthesized at the time of codon-optimization and remained at the C-terminal ends of both the H3 and H4 expression constructs (Figure 6). The entire H4 coding sequence (extensin signal sequence, C-terminal truncated HBV core particle subunit, RSRKDEL sequence) was flanked by a 5' *Bgl* II site and a 3' *Bam* HI site. These sites were designed for easy removal of the H4 coding sequence and insertion into these same two sites that were present in the pBI 525 vector. The H4 expression coding sequence is shown in Figure 7. The alignment of the H1 and H4 amino acid sequences is shown in Figure 8.

Figure 7. The plant codon-optimized coding sequence of the H4 expression construct. The HBV coding sequence contains the extensin signal sequence (96 bp, green), the plant codon-optimized HBV coding sequence from the N-terminal of the wild-type HBV coding sequence (459 bp, red), three extra codons due to the incorporation of an *Xba* I restriction site (9 bp for the RSR peptide, orange), the KDEL encoded endoplasmic reticulum retention sequence (12 bp, red) and a stop codon (3 bp, black). The coding sequence was deduced from DNA sequencing analyses. The entire DNA sequence was obtained from Health Canada following synthesis. The HBV coding sequence was inserted between the d35S promoter and the NOS-TER sequence of a pBI525 vector. The complete H4 expression construct is shown in Figure 6 and was further subcloned into the pRD 400 and pCAMBIA 1303 vectors for *A. tumefaciens* mediated transformation of plant tissues.

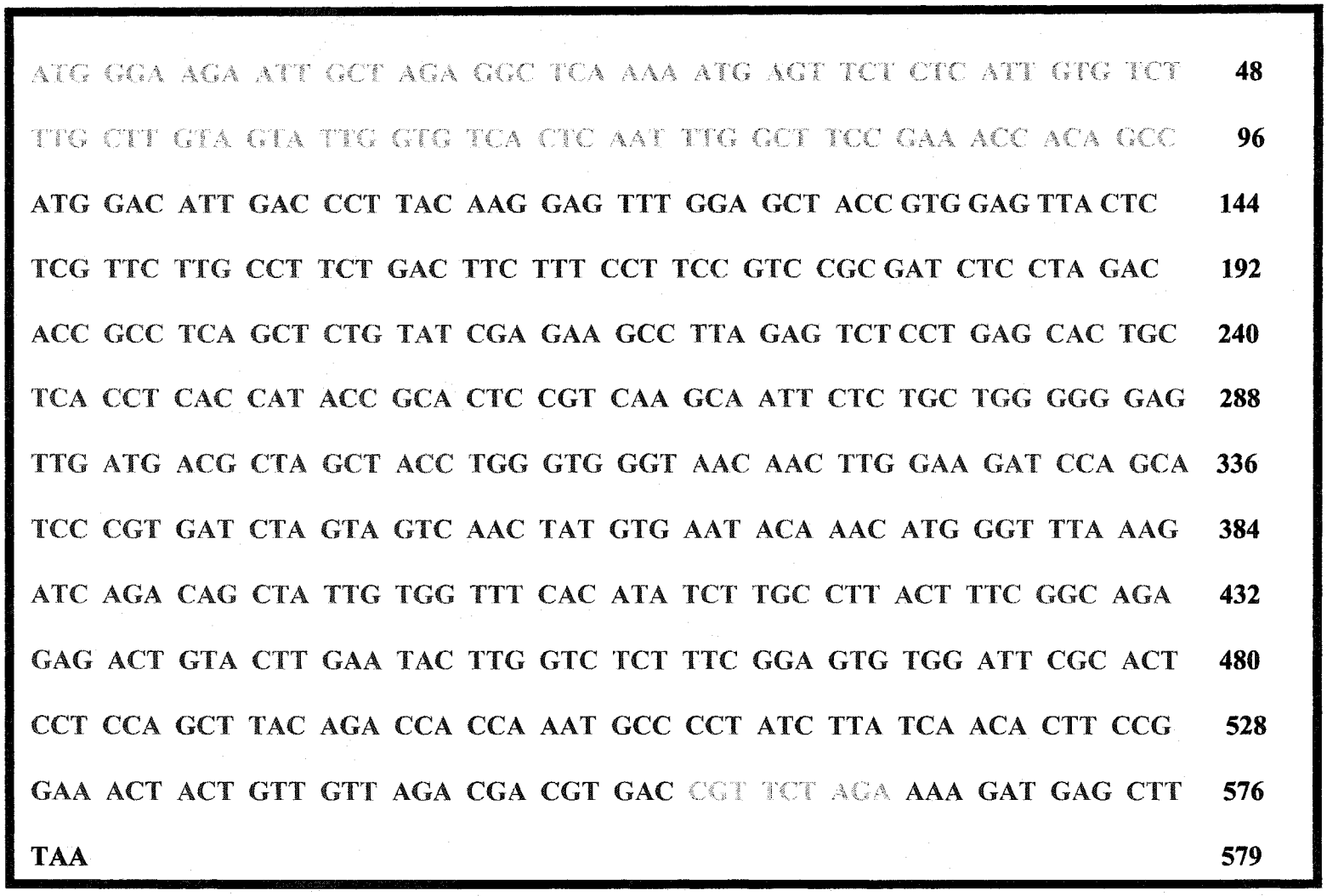

ATG GGA AGA ATT GCT AGA GGC TCA AAA ATG AGT TCT CTC ATT GTG TCT 48
TTG CTT GTA GTA TTG GTG TCA CTC AAT TTG GCT TCC GAA ACC ACA GCC 96
ATG GAC ATT GAC CCT TAC AAG GAG TTT GGA GCT ACC GTG GAG TTA CTC 144
TCG TTC TTG CCT TCT GAC TTC TTT CCT TCC GTC CGC GAT CTC CTA GAC 192
ACC GCC TCA GCT CTG TAT CGA GAA GCC TTA GAG TCT CCT GAG CAC TGC 240
TCA CCT CAC CAT ACC GCA CTC CGT CAA GCA ATT CTC TGC TGG GGG GAG 288
TTG ATG ACG CTA GCT ACC TGG GTG GGT AAC AAC TTG GAA GAT CCA GCA 336
TCC CGT GAT CTA GTA GTC AAC TAT GTG AAT ACA AAC ATG GGT TTA AAG 384
ATC AGA CAG CTA TTG TGG TTT CAC ATA TCT TGC CTT ACT TTC GGC AGA 432
GAG ACT GTA CTT GAA TAC TTG GTC TCT TTC GGA GTG TGG ATT CGC ACT 480
CCT CCA GCT TAC AGA CCA CCA AAT GCC CCT ATC TTA TCA ACA CTT CCG 528
GAA ACT ACT GTT GTT AGA CGA CGT GAC CGT TCT AGA AAA GAT GAG CTT 576
TAA 579

Figure 7

Figure 8. The amino acid sequence alignment of the H1 and H4 coding sequences. The deduced amino acid sequences as shown in Figures 6 and 8 were obtained using the DNA Star software. The protein sequence alignment was obtained from the website http://xylian.igh.cnrs.fr/bin/nph-align_query.pl. The upper panel represents the H1 amino acid sequence while the bottom panel represents the H4 amino acid sequence. The extensin signal sequence consists of 32 amino acid residues (96 bp) and is shown in green, the RSR peptide is shown in orange and the KDEL peptide is shown in red. Two threonine residues (174 and 178) predicted to be O-linked glycosylated are shown in blue. The parent nucleotide sequences were incorporated into plant expression constructs for *A. tumefaciens* mediated transformations of tobacco, carrot and rice tissues.

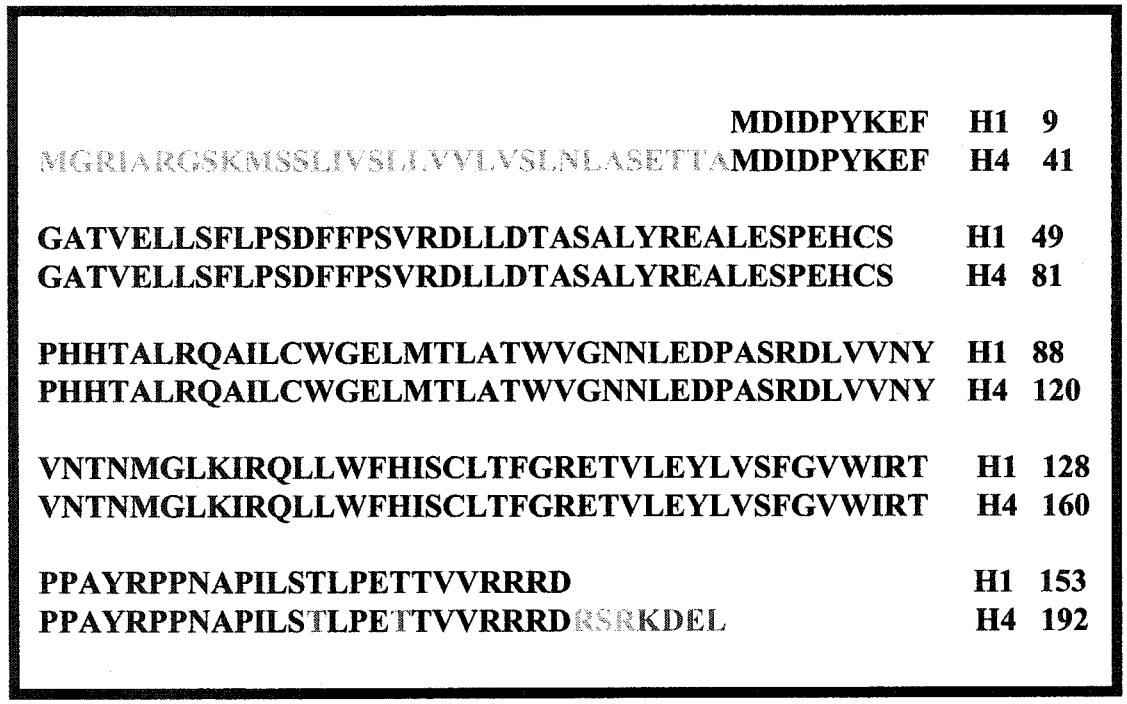

MDIDPYKEF H1 9
MGRIARGSKMSSLIVSLLVVLVSLNLASETTAMDIDPYKEF H4 41
GATVELLSFLPSDFFPSVRDLLDTASALYREALESPEHCS H1 49
GATVELLSFLPSDFFPSVRDLLDTASALYREALESPEHCS H4 81
PHHTALRQAILCWGELMTLATWVGNNLEDPASRDLVVNY H1 88
PHHTALRQAILCWGELMTLATWVGNNLEDPASRDLVVNY H4 120
VNTNMGLKIRQLLWFHISCLTFGRETVLEYLVSFGVWIRT H1 128
VNTNMGLKIRQLLWFHISCLTFGRETVLEYLVSFGVWIRT H4 160
PPAYRPPNAPILSTLPETTVVRRRD H1 153
PPAYRPPNAPILSTLPETTVVRRRDRSRKDEL H4 192

Figure 8

Restriction analyses and DNA sequencing of expression constructs

Each expression construct was analyzed via restriction analyses and verified via DNA sequencing. Once restriction analyses were completed, the coding sequences and flanking regions were sequenced at the University of Ottawa DNA Sequencing and Synthesis Core Facility. For the HBV wild-type expression construct (H1), the forward and reverse primers were 5` CCT GAT CTT TAA ACC CA 3` and 5` TGA CTC TAG CTA CCT GGG 3`. These two primers bound the DNA between +278 bp and +294 bp for the forward sequencing reaction and between +197 bp and +214 bp for the reverse sequencing reaction. For the HBV plant codon-optimized expression constructs (H4), the completely synthetic expression construct obtained from Health Canada was sequenced using the following forward and reverse primers: 5` GGT GTC ACT CAA TTT GGC 3` and 5` TGA GGC GGT GTC TAG GA 3`. These two primers bound the DNA between +72 bp and +89 bp located in the extensin signal sequence for the forward sequencing reaction and between +194 bp and +214 bp for the reverse sequencing reaction. Since these two primers allowed for the complete sequencing of all the coding sequences present in the H4 expression construct and that contained in the H3 expression construct (no extensin signal sequence present), only one additional primer was used to sequence the 3` end of the H2 expression construct. This primer bound the DNA between +451 bp and +471 bp and allowed for determining whether the RSRKDEL encoded sequence was removed from the H2 expression construct: 5` CTT GAA TAC TTG GTC TCT TTC 3`.

Generation of tobacco, carrot and rice transformation vectors

For dicot transformations, the H1, H2, H3 and H4 expression constructs contained in independent pBI 525 vectors were each digested with *Hind* III and *Eco* RI to release the desired sequences (Figure 6). The fragments of interest were purified after electrophoresis

on 0.8% agarose gels and ligated into similarly digested independent pRD 400 vectors. Screening for the H1/pRD 400, H2/pRD 400, H3/pRD 400 and H4/pRD 400 vectors followed the transformation of competent *E. coli* cells with the ligation reactions. The transformation reactions were plated on YEP/agar plates (10 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl, 1.5% agar, pH 7.0) containing 50 μg/mL kanamycin. Restriction analyses were performed on extracted DNA from bacterial clones from each transformation reaction to determine whether the H1, H2, H3 and H4 inserts were correctly ligated into each pRD 400 binary vector. After confirmation of the correct clones, transformation experiments involving *A. tumefaciens* were initiated using the harvested DNA plasmids. The expression constructs (H1/pRD 400, H2/pRD 400, H3/pRD 400 and H4/pRD 400) were incorporated into *A. tumefaciens* cells by the freeze-thaw method from Clontech and is briefly described later. For tobacco transformations, the H1/pRD 400, H2/pRD 400, H3/pRD 400 and H4/pRD 400 vectors were used while for carrot transformations, only the H1/pRD 400 and later, the H4/pRD 400 expression constructs were used for transformations.

For rice transformations, the H1 and H4 recombinant expression constructs (Figure 6) were incorporated into independent pCAMBIA 1303 monocot transformation vectors to generate the H1/pCAMBIA 1303 vector and the H4/pCAMBIA 1303 vector. The H1 and H4 expression constructs were released from the non-plant transformation vector pBI 525 using the restriction enzymes *Hind* III and *Eco* RI and these genecleaned fragments were ligated into independent but similarly digested and gel-purified pCAMBIA 1303 vectors. The resulting ligation reactions were used to transform competent *E. coli* cells. The transformation reactions were plated on YEP/agar plates (10 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl, 1.5% agar, pH 7.0) containing 50 μg/mL kanamycin and

50 μg/mL hygromycin). Screening for the H1 or H4/pCAMBIA 1303 vectors was performed on extracted DNA from independent clones. After confirmation of the correct clones for the H1/pCAMBIA 1303 and the H4/pCAMBIA 1303 constructs, they were incorporated into *A. tumefaciens* (strain LBA 4404) cells as described later.

Transformation, selection and growth of competent *E. coli* cells

For routine subcloning and restriction analyses, plasmid DNA or ligation reaction mixture was used to transform 50 μL of competent cells. Competent *E. coli* DH5 α-cells (Gibco BRL) were transformed using the protocol provided by the supplier. Briefly, competent cells were thawed on ice for 30 min and 1 μg of the plasmid DNA was added. The content was gently pipetted five times and left on ice for 30 min. After a heat shock treatment at 37°C for 30 s, one mL of Luria-Bertani (LB) medium (10 g/L bactotryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0) was added. The mixture was inverted five times followed by incubation at 37°C for 1 h. Following a 30 second-centrifugation step (10,000 x g), the pelleted cells were resuspended in approximately 200 μL of LB medium. A 20-μL aliquot was spread on LB/1.3% agar plates containing 100 μg/mL ampicillin or 50 μg/mL kanamycin. After incubating overnight at 37°C, the antibiotic resistant colonies were picked and grown in LB medium containing 100 μg/mL ampicillin or 50 μg/mL kanamycin. Bacterial cultures were used for DNA isolation of the various expression constructs as described later.

Generation of competent *A. tumefaciens* cells

About 30 μL of *A. tumefaciens* cells were plated on YEP/agar plates containing no antibiotics (LBA 4404) or 100 μg/mL gentamicin and 150 μg/mL rifampicin (GV 3101). These plates were incubated at 28°C for 2-3 days, within which time colonies appeared. Several single colonies were each grown in 5 mL of YEP medium for 15-20 hours. Each

overnight culture was transferred to 45 mL of fresh YEP medium (without agar) contained in 500 mL sterile flasks. Cultures grew until an O.D. of approximately 0.5 was achieved. The bacterial cultures were poured into sterile 50-mL centrifuge tubes and centrifuged (1,520 x g, 4°C, 5 min). After discarding the supernatants, the pellets were resuspended in 1 mL of 20 mM $CaCl_2$. Aliquots of 100 µL competent cells were dispensed in pre-chilled 1.5 mL sterile microfuge tubes. Competent *A. tumefaciens* cells were stored at -70°C until needed for plasmid uptake.

Transformation of *A. tumefaciens* cells

The freeze-thaw method from Clontech was used to transform an aliquot of competent *A. tumefaciens* cells (100 µL) with the desired expression constructs (H1, H2, H3 or H4) contained in either the pRD 400 or pCAMBIA 1303 binary vector. After adding the plasmid DNA (1 µg), the cells were gently mixed by pipetting and then were incubated on ice for 30 min. After the incubation period, liquid nitrogen was used to freeze the cells. The cells were thawed at 37°C for 10 min, afterwards, 1 mL of YEP medium was added and the tubes were inverted several times to mix. The mixtures were incubated at 28°C for 4 hours. Following a 30 s - centrifugation step, the pelleted cells were resuspended in approximately 60 µL of the remaining YEP medium and 30 µL of each was spread on YEP/agar plates containing 50 µg/mL kanamycin (for tobacco expression constructs H1-H4 using the LBA 4404 strains of *A. tumefaciens*). These plates were incubated at 28°C for 2-3 days to allow for antibiotic resistant colonies to appear. Transformed *A. tumefaciens* clones were identified after growth on YEP/agar plates containing 50 µg/mL kanamycin selection medium. However, for carrot transformations with the H1/pRD 400 and the H4/pRD 400 expression constructs, selection for transformed *A. tumefaciens* clones in the GV 3101 strain was identified after growth on 100 µg/mL gentamicin, 50 µg/mL kanamycin and 150 µg/mL rifampicin. Further, for rice transformations with the

H1/pCAMBIA 1303 and the H4/pCAMBIA 1303 expression constructs (contained in the LBA 4404 strains of *A. tumefaciens*), selection for *A. tumefaciens* cells carrying the H1/pCAMBIA 1303 and the H4/pCAMBIA 1303 vectors were conducted on YEP/agar plates containing 50 μg/mL kanamycin and 50 μg/mL hygromycin. These colonies were used to prepare the inoculants for transformation of the different plant tissues.

Transformation of plants via *A. tumefaciens*

The expression constructs (H1-H4) were incorporated into *A. tumefaciens* cells by the freeze-thaw method from Clontech. Once *A. tumefaciens* colonies were identified as having specific expression constructs, each was used to transform tobacco (H1/pRD 400, H2/pRD 400, H3/pRD 400 and H4/pRD 400), carrot (H1/pRD 400 and H4/pRD 400) and rice (H1/pCAMBIA 1303) tissues. To initiate transformation of plants, *A. tumefaciens* cells carrying binary vectors were co-cultivated with cut tobacco leaf sections (1.0 X 0.5 cm) and carrot hypocotyls (1.0 cm long). In the case of rice, transformation required rice calli derived from rice seeds. Selection for antibiotic resistance leaf cells utilized kanamycin (for tobacco), geneticin (for carrot) and hygromycin (for rice). The antibiotics carbenicillin, cefotaxime or ticarcillin were used to kill *A. tumefaciens* cells after a two-day co-cultivation period.

Inoculant preparations for tobacco, carrot and rice

Transformed *A. tumefaciens* clones in independent pRD 400 vectors were used to inoculate 5 mL YEP medium containing the appropriate antibiotics. For pRD 400 clones in LBA 4404 strains (for tobacco transformations), growth and replication was conducted in 50 μg/mL kanamycin while clones in GV 3101 (for carrot transformations) used three antibiotics: 100 μg/mL gentamicin, 50 μg/mL kanamycin and 150 μg/mL rifampicin. Gentamicin and rifampicin allowed for maintenance of the helper plasmid and

chromosomal DNA in the *A. tumefaciens* GV 3101 strain. Transformed *A. tumefaciens* clones in independent pCAMBIA 1303 vectors (for rice transformations) were used to inoculate 5 mL of AB medium composed of three solutions. The three solutions were prepared separately and then combined in fixed proportions immediately before use (**20 X AB buffer:** 60 g/L K_2HPO_4, 20 g/L NaH_2PO_4; **AB medium without salt and buffer:** 5g/L glucose; **20 X AB salts**: 20 g/L NH_4Cl, 6g/L $MgSO_4.7\ H_2O$, 3 g/L KCl, 0.2 g/L $CaCl_2$, 50 mg/L $FeSO_4.7H_2O$). To 900 ml of the AB medium without salt and buffer were added 50 mL of 20 X AB buffer and 50 mL of 20 X AB salts. The final AB mixture was supplemented with 100 µM acetosyringone, 50 µg/mL kanamycin and 50 µg/mL hygromycin.

After shaking (260 rpm) at 28°C for 24 h, a second set of inoculations using 45 mL of fresh media (YEP or AB supplemented with the appropriate antibiotics and/or acetosyringone) were initiated using the first 5 mL of inoculated cultures. The second inoculated cultures were incubated as above but only for 4 h. Sterile 50-mL centrifuge tubes were used to collect the cultures (O.D. ~ 0.5) and for centrifugation (1,520 x g, 4°C, 7 min, Beckman TJ-6 centrifuge). The supernatants were discarded and the tubes were inverted to remove most of the antibiotic medium. After resuspending the pellet in 5 mL medium (YEP or AB) without antibiotics, the culture was used for transformation of the sterile plant tissues as described below.

Sterilization of plant tissues

Young tobacco leaves *(N. tabacum* L. cv. Xanthi) as well as carrot (*D. carota* L. cv. Scarlet Nantes) and rice (*O. sativa* L. cv. Xiushui 11) seeds required surface sterilization prior to conducting the desired transformation experiments. All surface sterilizations were conducted in a sterile laminar flow hood at room temperature.

Tobacco tissues

Tobacco leaves were freshly harvested and placed into sterile Petri dishes. Leaves were surface sterilised for 30 s in 70% ethanol followed by a 10 min soak in 6% sodium hypochlorite. After three washes with sterile dH_2O (15 min each time), the leaves were placed into a second sterile Petri dish containing sterile dH_2O. The leaves were cut with a sterile surgical blade into small sections approximately 0.5 mm X 0.5mm. The *A. tumefaciens* cells harbouring the H1-H4 expression constructs were used to transform leaf sections as described below.

Matured tobacco seeds harvested from Ro transgenic tobacco plants or from NT plants were surface sterilized in a 6% sodium hypochlorite solution for 30 minutes on a shaker (40 rpm). After pouring off the bleach, the seeds were washed 3 times with sterile distilled water and placed on ½ strength Murashige and Skoog (MS) medium (½ MSO) contained in Petri dishes {1X MS medium (Murashige and Skoog basal medium), 30 g/L sucrose, 3 g/L PhytaGel™, pH 5.8}. Selection of transgenic tobacco plants required that the ½ MSO medium be supplemented with 300 mg/L kanamycin. Approximately 20-30 seeds from NT or transgenic plants were placed into each Petri dish before sealing with Parafilm. The seeds were placed at 24°C and a photoperiod of 16-h light and 8-h dark.

Carrot tissue

Carrot seeds were surface sterilized by sequentially soaking in two different solutions. The first solution was composed of 70% ethanol while the second solution was a 6% sodium hypochlorite solution. Seeds were placed for 15 min in each solution. After discarding the solutions, surface sterilized carrot seeds were soaked twice in sterile distilled water for two 15-min intervals. Surface sterilized carrot seeds were then transferred to 3%

sucrose semi-solid medium (0.8% agar) for germination in the dark. Carrot hypocotyls were observed after 7 days and harvested for *A. tumefaciens*-mediated transformations.

Rice tissue

Rice seeds (~500) were surface sterilized in 100 mL of a 3% sodium hypochlorite solution that contained one drop of Tween 20. After 45 minutes on a shaker (40 rpm), the seeds were washed 3 times with sterile distilled water (15 min each wash) and placed on Murashige and Skoog (MS) calli induction medium contained in Petri dishes {1X MS medium (Murashige and Skoog basal medium), 30 g/L sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 3 g/L PhytaGel™, pH 5.8}. Approximately 10-20 seeds were placed into each Petri dish before sealing with Parafilm. The plates were immediately incubated in the dark at 24°C until calli tissue became visible.

Co-cultivation of sterile plant tissues with *A. tumefaciens*

The tissues described above were used for *A. tumefaciens*-mediated transformations of tobacco (Horsch *et al.*, 1985), carrot (Hardegger and Sturm, 1998) and rice (Cheng *et al.* 1997 and 1998). All steps were conducted under sterile conditions.

Tobacco tissue

Distilled water from surface sterilized tobacco leaf sections was removed by briefly blotting the leaf sections onto sterile filter papers. The leaf sections were immediately transferred into the *A. tumefaciens* suspension prepared as above. After 5 min, the leaf sections were blotted onto sterile filter papers before placing onto tobacco co-cultivation medium {1X MS, 3% sucrose, 1.0 mg 6-benzyladenine [BA], 0.1 mg/L alpha-naphthalene acetic acid [NAA], and pH 5.8, 0.8% agar}. Approximately seven leaf sections were

placed into each Petri dish before sealing with Parafilm. Subsequently, the plates were incubated in the dark at 20°C for two days.

Carrot tissue

Briefly, carrot hypocotyls, prepared as above, were co-cultivated with the prepared *A. tumefaciens* suspension by incubating in a Petri dish for 5 min. The hypocotyls were then placed on carrot co-cultivation medium {Gamborg B5 medium, 0.25 mg/L NAA, 0.25 mg/L 6-benzylaminopurine (BAP), 30 g/L sucrose and 8 g/L select agar} without antibiotics for a 2-day period in the dark.

Rice tissue

Rice calli were gently scraped away from the semi-solid MS calli induction medium using a sterile spatula. The calli were then broken into smaller pieces (3-5 mm in diameter) and the *A. tumefaciens* suspension, prepared as described above, was added to a 50 mL tube until all the calli were completely immersed. After a 30 min incubation period with occasional inversion, the *A. tumefaciens* suspension was poured off and the calli tissues were removed aseptically with forceps and transferred onto 2N6-AS (N6 vitamins-acetosyringone) medium {4.3 g/L N6 major salts, 4.3 g/L N6 minor salts, 0.01% N6 vitamins, 1 g/L casamino acids, 30 g/L sucrose, 10 g/L glucose, 100 μM acetosyringone, 2 mg/L 2,4-D, pH 5.2 and 3g/L PhytaGel™} contained in Petri dishes. Approximately five to ten pieces of calli were placed into each Petri dish that was afterwards sealed with Parafilm. Two days of cocultivation in the dark at 28°C were then followed by regeneration of plants. Transgenic tobacco, carrot and rice plants were regenerated as described below.

Calli induction and regeneration of transgenic plants

Tobacco plants

Leaf sections from the above co-cultivation plates were transferred to a tobacco regeneration medium {4.3 g/L Murashige and Skoog (MS) major and minor salts, 3% sucrose, 1 mg/L BA, 0.1 mg/L NAA, pH 5.8, 0.8% agar, 500 mg/L carbenicillin and 300 mg/L kanamycin}. Carbenicillin was used to kill *A. tumefaciens* cells while kanamycin selected for transformed leaf cells. Calli were induced from transformed leaf cells at 25°C and a photoperiod of 16-h light and 8-h dark (General Electric - Wide Spectrum fluorescent lamps). Within one month, calli developed and eventually grew into green shoots. When shoots were at least 1.0 cm long, they were dissected and placed into magenta jars containing a rooting medium (4.3 g/L MS major and minor salts, 2% sucrose, pH 5.8, 0.8% agar, 500 mg/L carbenicillin, 300 mg/L kanamycin). After one month, plantlets developed roots and were transferred into pots containing soil. To prevent desiccation, plants were placed in the greenhouse and covered with transparent plastic bags for the first week. The greenhouse photoperiod was the same as above but the temperatures were different (light: 26°C, dark: 20°C). After one week, plastic bags were removed and plants were allowed to grow to maturity. Plants were watered when needed and fertilized with a water-soluble fertilizer (Plant Product® brand with composition N:P:K; 20:20:20) every two weeks. Transgenic tobacco plants normally matured and flowered within six to nine months.

Carrot plants

After the co-cultivation period, the carrot hypocotyls were transferred to 6-cm Petri dishes containing the same carrot co-cultivation medium with antibiotics for the killing of *A. tumefaciens* {Gamborg B5 medium, 0.25 mg/L NAA, 0.25 mg/L 6-benzylaminopurine (BAP), 30 g/L sucrose, 8 g/L select agar} containing 200 mg/L carbenicillin or ticarcillin

and 250 mg/L vancomycin. After two weeks on this medium at 25°C under a photoperiod of 16 h light and 8 h dark, the above medium containing an additional antibiotic {20 mg/L geneticin (G418)} was used for selection of transformed calli tissue. After one month on the G418 selection medium, carrot hypocotyls had proliferated into well-developed putatively transformed green calli. Green carrot calli (up to 2 g) were removed from the Petri dishes and crushed with a sterile spatula before placing into 250 mL sterile culture flasks containing 100 mL of cellular proliferative medium (Gamborg B5 liquid medium supplemented with 30 g/L sucrose and 0.1 mg/L 2,4-D) containing antibiotics (10 mg/L geneticin and 200 mg/L cefotaxime). The flasks were completely covered with aluminium foil and placed at 24°C in the dark on a shaker (120 rpm) for 1-3 months. Every two weeks, 10 mL portions of the carrot cell suspension cultures were removed and added to 250 mL sterile culture flasks containing the same media (90 mL) as above. After single cells became visible, they were washed three times with growth regulator deficient Gamborg B5 liquid medium supplemented with 30 g/L sucrose and 200 mg/L cefotaxime. The growth regulator free cells were then transferred to 250 mL sterile culture flasks containing the same growth regulator deficient Gamborg B5 liquid medium as above. After 2-4 weeks in the dark (24°C, 120 rpm), embryos became visible. Once embryos developed into tiny carrot plantlets (0.5-1.0 cm in length), they were removed and placed into magenta jars containing Gamborg B5 semi-solid medium, supplemented with 1.5% sucrose, 10 mg/L geneticin and 200 mg/L cefotaxime. These carrot plantlets were placed at 26°C under a photoperiod of 16-h light and 8-h dark. All carrot plants were maintained in the greenhouse until they matured (two months) and set seeds.

Rice plants

The *A. tumefaciens* co-cultivated rice calli were removed from the 2N6-AS medium after two days and placed into a new medium contained in 9-cm Petri dishes {LHT

medium: 4.3 g/L MS major and minor salts, 1 mg/L thiamine.HCl, 100 mg/L myo-inositol, 30 g/L sucrose, 2 mg/L 2,4-D, 3 g/L Phytagel, pH 5.8} containing 200 mg/L ticarcillin antibiotic for *A. tumefaciens* killing without selection for transformed plant cells. After one month in the dark on the above LHT medium, selection for transformed plant cells was conducted on an LHT medium containing two antibiotics (50 mg/L hygromycin and 200 mg/L ticarcillin) that allowed for both *A. tumefaciens* killing and selection for transformed rice calli cells. However, growth of transformed rice calli on this second LHT medium was also conducted in the dark for a further month. After two months on the two different LHT media, the putatively transformed rice calli were transferred onto a plant regeneration medium {LRHT: 4.3 g/L MS major and minor salts, 1 mg/L thiamine.HCl, 100 mg/L myo-inositol, 20 g/L sucrose, 2 mg/L kinetin, 0.5 mg/L IAA (indole-3-acetic acid), 3 g/L Phytagel, pH 5.8} and placed at 26°C under a photoperiod of 16-h light and 8-h dark. In the LRHT medium, the two antibiotics for bacteria static and cellular selection were retained (50 mg/L hygromycin and 200 mg/L ticarcillin). At this stage, under the normal rice plant photoperiod, hygromycin-resistant microcalli became visible as the white calli became green. After an approximate two months on the LRHT medium, green shoots of transgenic rice plants emerged. These rice plants were then transferred from the 9-cm Petri dishes to 6-inch high magenta jars containing LRHT medium as mentioned above. After two months, well-developed rice plants were transferred from the magenta jars to soil. Rice plants were placed in the greenhouse at 26°C under a photoperiod of 16-h light and 8-h dark.

DNA extraction from tobacco, carrot and rice leaves

Young developing leaves were collected from transformed and non-transformed plants. The leaf surfaces were washed with distilled water and approximately 3 grams of leaves were placed into a sterile mortar that was pre-chilled with liquid nitrogen (LN).

Leaves were ground to a fine powdery white flour. Liquid nitrogen was added several times during the course of grinding to keep the tissues hard and to prevent degradation of the DNA. Each sample was added to a 50-mL centrifuge tube containing 5 mL of buffer S {100 mM Tris.HCl (pH 8.5), 100 mM NaCl, 50 mM EDTA (pH 8.0), 2% SDS, 0.1 mg/mL proteinase K, 10 mM DTT}. The centrifuge tubes were inverted several times to mix their contents and afterwards, they were incubated at 37°C for 1 h. After the incubation period, a 5 mL portion of buffered {100 mM Tris.HCl (pH 8.5), 100 mM NaCl, 50 mM EDTA} saturated phenol was added to each sample and the tubes were placed at 37°C for 30 min. The mixtures were centrifuged (1,520 x g, 4°C, 10 min) and the top aqueous phases were transferred into new 50-mL centrifuge tubes. The DNA/RNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 99% ethanol. The precipitation step proceeded at 20°C on a rotary shaker with gentle rocking. After approximately one hour, nucleic acids settled at the bottom of the tubes and the mixtures were centrifuged (1,520 x g, 4°C, 5 min). The supernatants were removed using clean pipettes that were flame drawn. The pellets were allowed to dry and then were resuspended in 0.5 mL of TE buffer (10 mM Tris.HCl, 1 mM EDTA, pH 8.5). To remove contaminating RNAs, 100 μg of RNase A was added to each sample. After incubating at 37°C for 2 hours, one volume of 1:1 buffered saturated phenol to chloroform was added. The samples were inverted several times to mix and centrifuged (1,520 x g, 4°C, 10 min). The top (aqueous) phases were carefully removed and the DNA was precipitated as above but the samples were placed at 0°C overnight. The DNA pellets were collected after a centrifugation step (1,520 x g, 4°C, 10 min) and each sample resuspended in 200 μL of dH_2O. The concentrations of the DNA samples were determined by measuring the optical densities at 260 nm using a spectrophotometer. The integrity of the genomic DNAs was assessed via electrophoresis on 0.8% agarose gels.

Extraction and purification of DNA from bacteria

Miniprep kits were obtained from QIAGEN (Mississauga, Ontario). The QIAprep Spin Miniprep kit protocol was used to extract and purify plasmid DNA from bacteria. In this procedure, transformed *E. coli* cells carrying the plasmid of interest were grown overnight at 37°C in 3.0 mL of LB or YEP medium supplemented with the appropriate antibiotic (ampicillin 100 μg/mL or kanamycin 50 μg/mL). Cells were poured into 1.5 mL microfuge tubes and centrifuged (10,000 x g) for 1 min to obtain the bacterial pellet. All other centrifugation steps for purification of plasmid DNA were conducted at 10,000 x g. The supernatants were poured off and the pellets were completely resuspended in 250 μL of Buffer P1 (QIAGEN). Then 250 μL of lysis buffer (P2, QIAGEN) was added to each sample. The alkaline lysis was allowed to proceed for 4 min before being neutralized with 350 μL of buffer N3 (QIAGEN). The lysates became cloudy and were centrifuged (10 min at 4°C). The supernatants were applied to QIAprep spin columns and centrifuged for 30 s (10,000 x g). After washing the spin columns, the DNA was eluted with 50 μL of TE (10 mM Tris.HCl, 1 mM EDTA, pH 8.5). Generally, 5 μL of each DNA sample was electrophoresed on 0.8% agarose gels to assess the quality and yield. More accurate quantifications were performed by spectrophotometry using a wavelength of 260 nm. The purified DNA was used for cloning, making probes, or restriction endonuclease analyses and as positive control in Southern blots.

Generation of radiolabelled probes

The original transformation vectors (H1-H4) were digested with *Eco* RI and *Hind* III for making Southern blot probes while *Nco* I and *Bam* HI were used to digest these same vectors for making Northern blot probes. The restriction enzymes *Nco* I and *Bam* HI flanked the HBV coding sequences (wild-type or plant codon-optimized). The digested DNAs were separated on 0.8% agarose gels and the fragments of interest were excised

under UV and recovered from the agarose gel using a Gene-Cleaning kit (QIAEX II) from QIAGEN. Briefly, three volumes (3 mL QX I/g of agarose) of buffer QX I was added to a 1.5 mL microfuge tube containing the agarose/DNA fragment and 30 µL of QIAEX II silica particle mixture. The QIAGEN supplied high chaotropic salt contained in the QX I buffer used large anions to disrupt sugar monomers and polymers of agarose. The mixture was incubated for 10 min at 50°C and vortexed every 2 min. The sample was centrifuged (30 s, 10,000 x g) and the supernatant discarded. The pellet was washed with 500 µL of QX I buffer and centrifuged (30 s, 10,000 x g). Two additional washes followed using 500 µL of the supplied PE buffer. The pellet was air-dried for 15 min and eluted with 50 µL of 10 mM Tris.HCl (pH 8.5). The DNA was recovered after a 30 s centrifugation step (10,000 x g) to pellet the silica particles. The DNA concentration was estimated on an agarose gel (0.8%) and used for the probe labelling reaction.

Radiolabelling of probes for Southern and Northern hybridizations

The probes for Southern and Northern hybridizations were labelled using the Ready.To.Go DNA Labelling Beads (dCTP) kit from Pharmacia. The Manufacturer's Standard Protocol was followed for the labelling reaction. Briefly, boiling for 3 min at 95°C denatured approximately 50 ng of the genecleaned probe (*Eco* R I/*Hind* III fragments for Southern blots or the *Nco* I/*Bam* HI coding sequences for Northern blots). The reaction was placed immediately on ice for 2 min. After a brief pulse centrifugation step, the DNA and 50 µCi of [^{32}P] dCTP were added to the supplied reaction tube. The solution was mixed by vortexing and briefly pulse centrifuged before incubating at 37°C for 15 min. The supplied reaction mixture consisted of the following components: reaction buffer, dATP, dGTP, dTTP, Klenow fragment (7-12 units) and random oligodeoxyribonucleotides (mainly 9-mers). Unincorporated nucleotides were removed from the labelled DNA fragments by centrifuging through a MicroSpin S-300 HR column (Pharmacia).

Prehybridization and hybridization for Southern and Northern blots

The baked nitrocellulose membrane was placed in 5X SSC solution for 5 min and transferred to a tray containing 50 mL of prehybridization buffer. The prehybridization and hybridization buffers were composed of the following components: 50% deionised formamide, 5X Denhardt's solution {0.1% ficoll, 0.1% polyvinyl-polypyrrolidone (PVPP) and 0.1% BSA}, 5X SSC, 0.1% SDS, and 100 pg/mL denatured salmon sperm DNA. The membrane was incubated at 42°C in the prehybridization buffer with constant, gentle shaking (50 rpm). After two hours, the prehybridization buffer was replaced with 30 mL of fresh hybridization buffer. The purified radioactive probe was boiled for 3 min and rapidly cooled on ice. After 5 min on ice, the probe was added to the hybridization solution. The hybridization was allowed to proceed at 42°C for 16 hours with gently constant shaking at 50 rpm. After the hybridization reaction was complete, the nitrocellulose membrane was carefully removed and placed into a tray containing 400 mL of 2X SSC and 0.1% SDS solution. This solution was used for washing the membrane at room temperature (20°C). The second and third washes were conducted at 65°C (15 min each) in 400 mL of a 1X SSC solution containing 0.1% SDS. The final wash was performed at 65°C (15 min) with 400 mL solution (0.4X SSC containing 0.1% SDS). The membrane was then removed and placed onto a piece of filter paper to air dry for half an hour before being wrapped in plastic wrap. The membrane was placed in a cassette and an X-ray film was placed on top of the membrane. Two intensifying screens sandwiched the radioactive membrane and the X-ray film. The film was developed using a standard procedure (Sambrook *et al.*, 1989).

Restriction analyses of DNAs

Plasmid or genomic DNA was digested with the appropriate restriction endonucleases as recommended by the enzyme supplier. For Southern analyses, 40 μg of total genomic DNA was digested with *Eco* RI and *Hind* III overnight at 37°C while plasmid

DNAs were digested for 3 hours at 37°C. Southern blotting identified the molecular basis for antibiotic resistance and recombinant protein expression. The autoradiographs were used to assess the efficiency of transformation and selection of plants.

Agarose gels for DNA electrophoreses

Electrophoreses of the digested genomic DNAs were conducted in 0.8% agarose gels (0.8 g agarose/100 mL 1X TAE) that contained ethidium bromide. Upon solidifying, the gel was submerged in 1X TAE buffer {50X TAE buffer: 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH 8.0)}. After the DNAs were digested, 6X gel loading buffer (30% glycerol, 0.25% xylene cyanol FF, and 0.25% bromophenol blue) was added to obtain a final 1X gel loading buffer concentration. A brief centrifugation step followed and the samples were loaded into the gel lanes. Two markers were loaded as standards (phage lambda DNA/*Hind* III and ΦX 174 RF DNA/*Hae* III). The DNA was electrophoresed at a constant 5 volts per square cm of gel. After the electrophoresis was completed, the gel was UV illuminated (Fotodyne) and photographed using a Polaroid camera (Polaroid MP.4 LandCamera).

Southern analysis on total genomic DNA

After electrophoresis of the restriction-enzyme-digested samples, the DNAs were transferred onto nitrocellulose membranes (High Bond N, Amersham) and probed with the radiolabelled *Eco* RI/*Hind* III fragments derived from the H1-H4 expression constructs. Gels were photographed as stated above and placed into DNA denaturation solution (1.5 M NaCl, O.5 M NaOH). After 1 hour, gels were rinsed with dH_2O and placed into neutralization buffer {1 M Tris.HCI (pH 7.5), 1.5 M NaCl}. During the hour-long neutralization step, the transfer apparatus was set-up as previously described (Sambrook *et al.,* 1989) using 10X SSC (3.0 M NaCl, 300 mM sodium citrate, pH 7.0) as the transfer

buffer. Two pieces of 3MM filter paper were wrapped halfway around glass plates with the ends of the filter papers directly in contact with the transfer buffer. The gels were placed on top of the 3MM filter papers. Nitrocellulose membranes, pre-soaked in dH_2O, were placed on top of the gels. Two additional pieces of 3MM filter papers were pre-soaked in 2X SSC and placed on top of the membranes. A stack of paper towels was placed onto each transfer assembly. A glass bottle weighing ~500 g was placed on top of each stack of the paper towels. The transfers proceeded at 20°C for 18 h. As the paper towels became wet, they were replaced with dry ones. After the transfers were completed, the membranes were soaked in 2X SSC to remove any agarose that were sticking to them. The membranes were allowed to air-dry before the DNAs were fixed by baking under vacuum (80°C, 2 h).

Extraction of total RNA from tobacco leaves, rice leaves and carrot roots

A previously published procedure for the collection of RNA was slightly modified for the collection of RNA from leaves and roots of all transgenic and non-transgenic plants (Verwoerd *et al.,* 1989). Young leaves and roots were carefully collected to prevent contamination of the tissue and to prevent RNA degradation. Gloves and sterile surgical blades were used for different samples. The tissue was frozen with liquid nitrogen immediately after dissecting from the tobacco, carrot or rice plants. The tissue was ground in liquid nitrogen to a fine powdery flour using a sterile mortar and pestle. A pre-weighed 1.5 mL microfuge tube was used to collect approximately 100 mg of ground tissue and, immediately, 500 µL of hot (80°C) extraction buffer was added {(1:1) phenol: 0.1 M LiCl, 100 mM Tris.HCl (pH 8.0), 10 mM EDTA, 1% SDS}. To homogenize the mixtures, the tubes were vortexed for 30 seconds and 250 µL of 24:1 (chloroform: isoamylalcohol) was used to extract the ribonucleic acids from each tube. A five-minute centrifugation step followed and the top aqueous phases were transferred to new tubes. One volume of 4 M

LiCl was added to each sample. The RNAs were allowed to precipitate overnight at 4°C. After centrifuging for five minutes, the pellets were collected and each re-dissolved in 250 μL of diethyl pyrocarbonate (DEPC)-treated water. The samples were then treated with 0.1 volume of 3 M NaOAc (pH 5.2). The RNAs were precipitated using two volumes of 99% ethanol and placed at -70°C for two hours. Following a 10 min centrifugation step, the supernatants were removed and the RNA pellets were washed with 70% ice-cold ethanol (containing 30% DEPC water). The pellets were collected after centrifuging for 10 min and were dried at 37°C. The RNA pellets were then dissolved in 50 μL of DEPC water. The stored 20 μL aliquots (-70°C) were used for Northern blotting experiments.

Preparation of RNA gels, electrophoresis and transfer of RNAs

Gels, buffers and samples were prepared in DEPC water. The integrity of the above extracted RNA was assessed by electrophoresis on a 1.2% agarose gel containing 1.9% formaldehyde. The gel tray, gel comb and electrophoresis chamber were washed with distilled water and presoaked overnight in a DEPC treated dH_2O solution containing 1% SDS. The 1X RNA electrophoresis buffer {MOPS [3-(N-morpholino) propanesulfonic acid]/EDTA} was prepared from a 10X stock solution (10X MOPS/EDTA buffer: 0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0). To each 5 μL of RNA sample was added 25 μL of RNA loading buffer (0.75 mL deionized formamide, 0.15 mL of 10X MOPS electrophoresis buffer, 0.24 mL of 37% formaldehyde, 0.1 mL DEPC treated dH_2O, 0.1 mL glycerol, 0.08 mL of 10% (w/v) bromophenol blue). The RNA samples containing the RNA loading buffer were placed at 65°C for 15 min. After cooling to 50°C, 1 μg of ethidium bromide was added to each sample. Prior to loading the RNA samples, a pipette was used to flush the sample wells with fresh 1X RNA electrophoresis buffer. After a brief centrifugation step, the RNA samples were loaded into the flushed wells and the gel run at a constant 30 V. After electrophoresis, the gel was visualized and photographed under UV

illumination (Fotodyne) as described for DNA agarose gels. Quantification of the RNAs by spectrophotometry (260 nm) was also conducted. Total RNA was used for Northern blotting experiments in which each lane contained equal quantities of RNA (20 μg) and further assessed by ethidium bromide intercalation with the RNA and visualization under UV illumination. The RNAs were electrophoresed along with standard RNA markers (Invitrogen Life Technologies, Cat. No. 15620-016). After the electrophoresis was completed, the gel was UV illuminated (Fotodyne) and photographed using a Polaroid camera (Polaroid MP.4 LandCamera). The gel was placed into an RNA denaturation solution (0.05 M NaOH in 1X sodium chloride and sodium citrate solution (SSC)). After 20 min, the gel was soaked in 10X SSC (3.0 M NaCl, 300 mM sodium citrate, pH 7.0) twice. The incubations were for 20 min each time at room temperature. The nitrocellulose membrane was wet with dH_2O and soaked in 10X SSC for 5 min. The RNAs were transferred and probed as described for Northern blot hybridization. The transfer proceeded overnight at 20°C for 24 hours using 10X SSC transfer buffer. After the transfer was completed, the membrane was allowed to air-dry before the RNAs were fixed by baking under vacuum (80°C, 2 h). Digesting the H1-H4 plasmids with the restriction endonucleases *Nco* I and *Bam* HI to release the HBV wild-type or codon-optimized coding sequences generated the probes for Northern blots.

Extraction of total protein from leaves, roots and seeds

Total protein was extracted using a previously published protocol (Mason *et al.*, 1996). Briefly, approximately 500 mg of plant tissue (tobacco leaves, carrot leaves, rice leaves, tobacco seeds, carrot roots or rice seeds) was placed into a pre-chilled mortar. A pestle was used to grind the sample to a fine powder. The pulverized sample was scooped up using a sterilized spatula and placed into a pre-weighed 1.5 mL microfuge tube. The tube containing the sample was weighed again and the net weight was determined. Protein

extraction buffer (1X PBS: phosphate buffer saline pH 7.2, 50mM sodium ascorbate, 2mM EDTA, 0.2 % (v/v) Triton-X 100, 2mM phenylmethyl-sulfonyl fluoride) was immediately added in a one to one ratio (v/w). For example, 500 µL of protein extraction buffer was added to 500 mg of ground sample. The sample was vortexed to ensure complete mixing and centrifuged (10,000 x g, 4°C, 10 min). The supernatant was transferred to a new tube and the pellet discarded or re-extracted as desired using the same extraction buffer ratio as above. The extracted plant proteins were used for HBV core particle specific ELISA, Western blotting, electron microscopy and immunogenicity studies.

Quantification of the HBcAg subunits

These analyses were performed to gain a better apprecitation of the relative effects of the construct sequences. The relative band intensities were estimated using Western blots of the various plant extracts supplied by Dr. Anton Andonov, Tim Booth and Darryl Falzarano. I performed densitometry analyses in Dr. Fraser Scott's laboratory using the Kodak Image Station 440 CF from Mandel Scientific Company. Standard curves (example Figure B1) were generated and the unknown concentrations of the plant recombinant protein were approximated as previously described by Curtiss and Cardineau (1997).

Statistical methods

Statistical analyses were conducted using Micrsoft Excel software to determine the Chi square test of independence of paired transgenic groups, as well as, to calculate the level of significance (p-values). The Chi square tests were conducted on the various plant expression constructs to detemine if the critical sequences imbedded in each conferred an advantage for assembly of the HBcAg. The ELISA value obtained for each tobacco plant that was above an O.D. value of 1.0 was placed in the group that assembled the HBcAg while those below an O.D. value of 1.0 were taken as negatives (for assembly).

Chapter 3

AUGMENTATION STUDIES OF THE HBV CORE PARTICLE IN TRANSGENIC TOBACCO PLANTS

Results Part I

Generation of HBV core protein expressing constructs

Special thanks go to Dr. Ravinder Sardana, Dr. Johann Schernthaner and Dr. Laurian Robert for their assistance in designing the codon-optimized expression construct. The first 153 amino acids of the HBV core particle subunit was tested for assembly in tobacco plants using four different expression vectors. If this strategy were to work, the truncated version of the HBV core particle subunits would still assemble into HBcAgs. All four expression constructs were analysed via restriction endonuclease digestions followed by agarose gel electrophoreses and all were found to be assembled correctly as indicated in Figure 6. When the H1-H4 expression constructs were digested with *Hind* III and *Bgl* II, the 620 bp promoter was released. Digestion with *Bgl* II and *Nco* I released the 38 bp AMV-RNA4 translational enhancer sequence from the H1-H3 expression construct and the 96 bp extensin signal sequence from the H4 expression construct. Digestion with *Nco* I and *Bam* HI released the 462 bp coding sequences of the H1-H2 constructs. Digestion of the H3-H4 expression constructs with *Nco* I and *Bam* HI released the 483 bp coding sequences. Further, DNA sequencing of the four plasmids was conducted to confirm that there were no mutations in the sequences and the precise ligation points were observed from the sequencing information. No mutations were found and hence, all 4 constructs were ligated into the plant binary vectors pRD400 and pCAMBIA 1303 followed by subsequent mobilization into *A. tumefaciens* for transformation of plant tissues. The DNA fragments encoding the truncated wild-type or the plant codon-optimized HBV core

particle subunits were placed under the control of the d35S promoter for ubiquitous recombinant protein expression.

Generation of transgenic tobacco calli and plants

The *Eco* RI-*Hind* III fragments of these four HBV core protein expression constructs were each placed into an independent plant binary vector pRD 400 (Datla *et al.*, 1992) for *A. tumefaciens* mediated transformation of tobacco leaf sections. The kanamycin resistant calli eventually sprung shoots. It was found that each leaf section co-cultivated with *A. tumefaciens* produced over five independent calli at its edges. Over 20 independent transgenic plants were regenerated from each of the tobacco transformation events involving the four different plant expression constructs. However, not all plants grew to maturity since some died before all the analyses could be conducted on them.

Determination of the transgenic nature of tobacco plants

The transgenic nature of tobacco plants was verified via Southern blots (Figure 9). All transgenic tobacco plants tested contained the complete expression construct (*Eco* RI-*Hind* III fragment) as shown after genomic DNA was digested with *Eco* RI and *Hind* III restriction endonucleases and analysed via Southern blots. Figure 9 shows four representative Southern blots of three plants from each transformed group. A total of 26, 15, 8 and 20 plants were analysed from the H1 to the H4 groups respectively. The radiolabelled probes used were made from the H1-H4 expression constructs indicated in Figure 6. Bands at 1.380 kb were observed for the H1 and H2 plants while bands at 1.401 kb and 1.459 kb were observed for the H3 and H4 plants respectively (Figure 9). All the other transgenic plants which are not shown in Figure 9 also tested positive for the respective H1-H4 expression constructs (data not shown). Further, Northern blotting experiments were conducted on the best three plants from each transformation event and all

Figure 9. Southern blots generated from genomic DNA of four different transgenic plant lines H1-H4. Genomic DNA (40 μg) from each tobacco plant was digested with the restriction enzymes *Eco* RI and *Hind* III and electrophoresed on 0.8% agarose gels. After electrophoresis, the DNA was transferred to nitrocellulose membranes, probed with gel purified and radiolabelled *Eco* RI-*Hind* III fragments as shown in Figure 6, and described in the "Materials and Methods" section on Southern blots. Lane 1: the *Eco* RI-*Hind* III insert released from the different plasmids containing the different HBV core protein expression constructs; lane NT: DNA cleaved with *Eco* RI and *Hind* III from a non-transformed plant (NT); lanes 1 – 3: DNA cleaved with *Eco* RI and *Hind* III from three transgenic plants from each of the different H1-H4 plant lines.

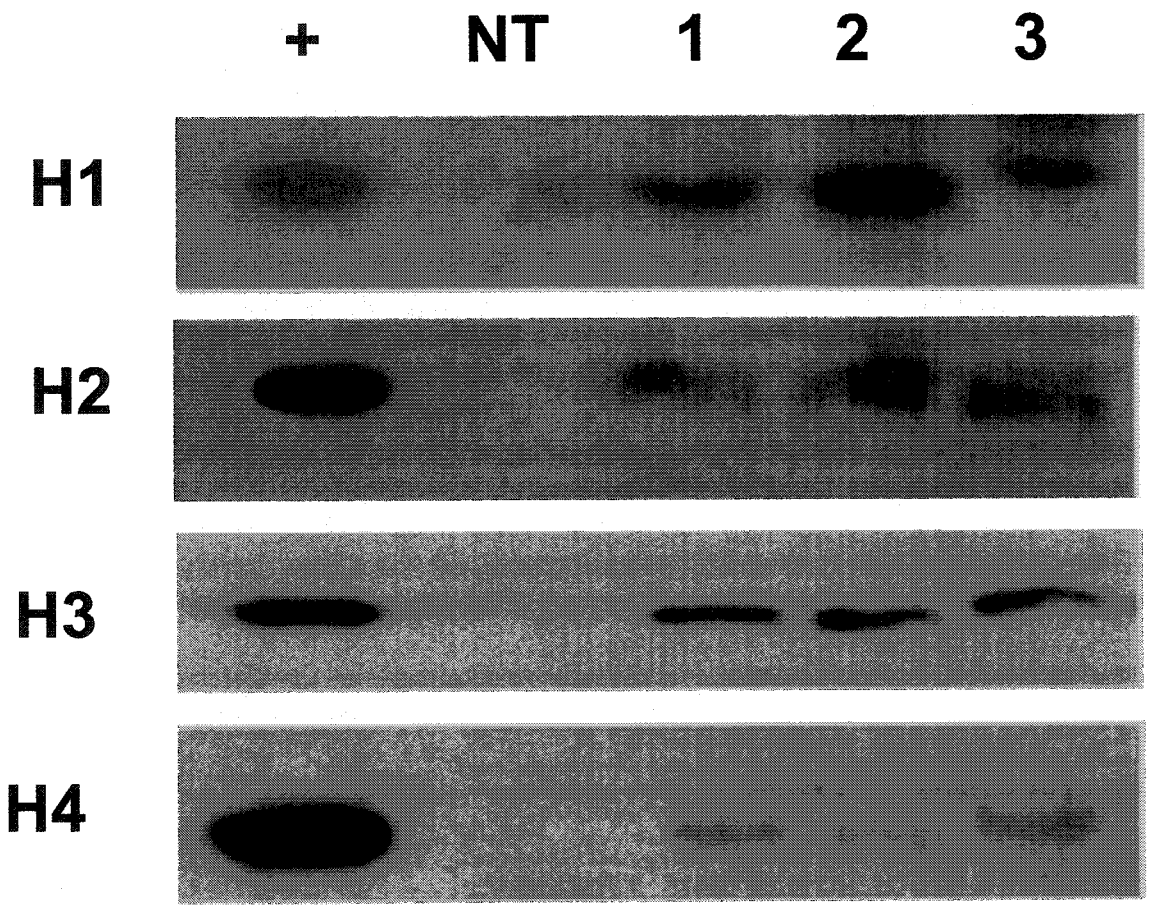


Figure 9

analysed plants were positive for a 0.560 kb transcript (H1-H2), a 0.590 kb transcript (H3) or a 0.665 kb transcript (H4) (Figure 10). No bands were visible in any lane that contained non-transformed plant DNA or RNA during Southern or Northern blot analyses. No DNA or RNA molecular weight standards were labelled and so did not appear on the autoradiograms.

Analyses of the acquired transgenic trait(s)

To determine if the matured seeds from transgenic tobacco plants were viable and able to pass on the acquired characteristic to successive generations, seeds were surface sterilized and plated on ½ MSO medium containing 300 mg/L kanamycin. When NT tobacco seeds were placed on ½ MSO medium without selective antibiotic, 100% of the NT seeds germinated; however, when 300 mg/L kanamycin were added to the ½ MSO medium, 0% of NT tobacco seeds germinated. Further experiments were conducted on the matured seeds obtained from the three best plants for each group (H1-H4), which showed the highest HBV core particle production. Further, these first regenerants (R1) obtained after plating on ½ MSO medium, were grown to maturity and analysed in a similar manner as their parental lines (R0). These R1 plants have been transferred to Health Canada in Winnipeg for further analyses.

Figure 10. Northern blots generated from total RNA of four different transgenic tobacco plant lines H1-H4. Equal quantities of total RNA (20 μg) from each tobacco plant was electrophoresed on denaturing 1.2 % agarose gels. After electrophoresis, the RNA was transferred to nitrocellulose membranes and each gel was probed with the corresponding gel purified and radiolabelled HBV core particle subunit coding sequence. The probe from each expression construct (H1-H4), as shown in Figure 6 was obtained by digesting each with the appropriate restriction enzymes as described in the "Materials and Methods" section on Northern blots. Lane 1: total RNA from NT plants; lanes 2-4: total RNA from three independent transgenic plants from each of the different H1-H4 plant lines and correspond to the plants analyzed via Southern blots in Figure 9. Additional details are presented in the text.

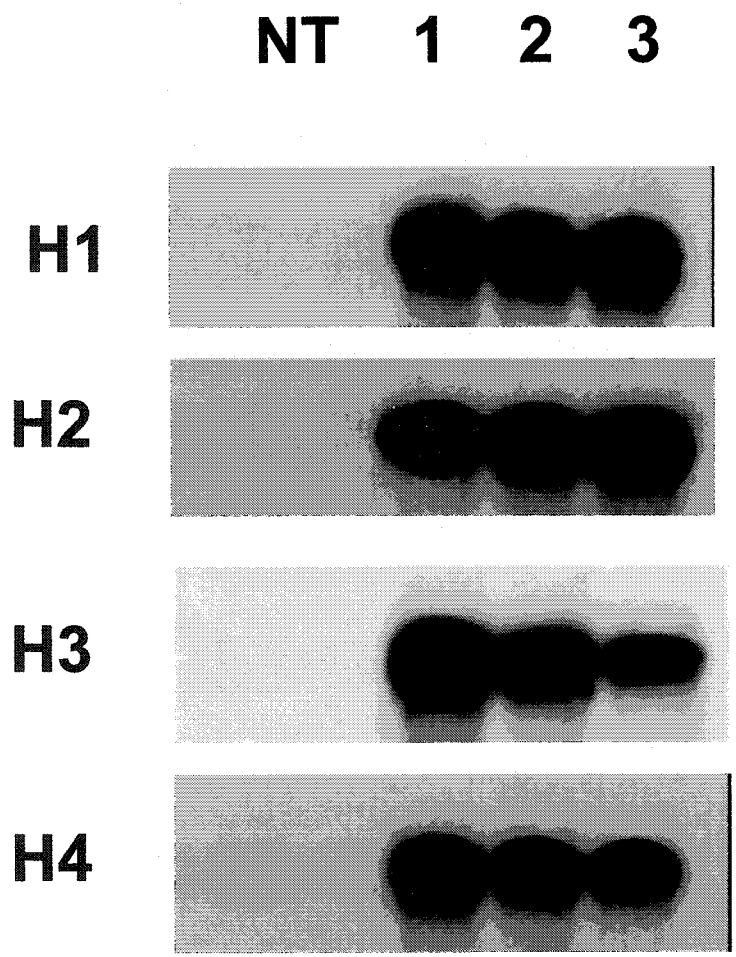


Figure 10

Tobacco derived HBV core protein analyses

ELISA on crude protein extracts from tobacco leaves

Thanks go to Dr. Anton Andonov, Timothy Booth, Lynne Burton, Tim Booth and Darryl Falzarano from Health Canada for their contributions to this thesis. The plant lysates (1 mg/mL) from tobacco were prepared and submitted to Health Canada, Bureau of Microbiology and Immunology, Winnipeg, Manitoba for ELISA, Western blots, electron microscopy, and subcutaneous immunization analyses. The ELISA values and Western blots were returned to me for Statistical and densitometry analyses. To characterize the HBcAgs in the H1-H4 transgenic tobacco plants, ELISAs were used. The recognition site of the monoclonal antibody (MAB842) that detects only assembled HBcAgs was not disclosed by the supplier. Data from 26, 15, 8 and 20 plants from the H1 to the H4 groups are presented in Figures A1-A4 respectively. Chi square analyses were conducted on the ELISA values obtained for these four groups of tobacco plants, and are presented in Table 3. Each plant ELISA value is the mean of two measurements. To clearly establish a positive plant where assembly of the HBcAg had occurred, an arbitrary O.D. value of 1.0 and above was assigned for transgenic tobacco plants (Table 3). Based on the Chi square assignments, the H1-H4 expression constructs led to 30.8%, 20.0%, 75.0% and 90.0% positive plants for assembly of the HBcAg respectively. Therefore, the difference in percentages for these four groups of transgenic plants is significant χ^2 (4, N = 69), p = 7.85E-05. Further Chi square test of independence of paired groups showed that the groups H1 and H4, groups H2 and H4, and groups H2 and H3 were significantly different with p-values of 3.13E-04, 1.58E-04 and 3.64E-02 respectively. The p-values obtained for Chi square test of independence of paired groups H1 and H2, groups H1 and H3 and groups H3 and H4 were 7.55E-01, 8.45E-02 and 5.91E-01 respectively and hence were not considered significantly different.

Table 3. Chi square test of independence of ELISA data obtained from the H1-H4 tobacco plant lines.

Tobacco Plant Group	Number of positives	Number of negatives	Totals
H1	8 (13.18)	18 (13.18)	26
H2	3 (7.60)	12 (7.39)	15
H3	6 (4.06)	2 (3.94)	8
H4	18 (10.14)	2 (9.86)	20
Totals	35	34	69
Chi square = 24.04 Degrees of Freedom = 4		p = 7.85X10E-5	

The table was prepared using the ELISA data obtained from all tobacco plant lines (H1-H4, Appendix Figures A1-A4) using an arbitrarily assigned O.D. value of 1.0 and above to represent a "positive" plant in which the HBcAg had assembled. All values below an O.D. of 1.0 were arbitrarily assigned as negative. The tobacco plant groups, the number of positives, negatives and the totals of each column or row were also calculated. The expected positive or negative values are also shown in parenthesis. The Chi square value, the degrees of freedom and the resultant p-value are also shown.

Western blot analyses on tobacco leaf proteins

To further characterize the HBcAg, Western-blotting experiments were conducted on soluble protein extracts from all transgenic plant lines. The leaf extracts of transgenic plants were analysed while the seeds were kept for feeding trials. Representative Western blots are shown in Figures A5-A8 for the leaf extracts of the H1-H4 plant lines respectively. The H1 tobacco plants produced bands on Western blots of 16.5 kDa that was similar to the *E. coli* derived positive control (Figure A5). Further, no bands were observed in the NT plant lane (Figure A5). Similarly, Western blots for the H2, H3 and H4 plants are shown in Figures A6-A8 respectively. Most of the transgenic plants tested on Western blots were positive for the HBcAg when 10 μL of leaf protein extract was analysed. Distinct bands ranging in size from 16.5-19.5 kDa were observed for extracts of the plant-codon-optimized lines, H2-H4 (Figures A6-A8). For the H3 and H4 transgenic plants that carried the KDEL tetra peptide sequence, bands on Western blots (Figures A7 and A8) appear to run at an apparent higher MW (17.5-19.5 kDa) than those seen for the H1 and H2 plant samples. Higher MW bands (33, 44, and 47 kDa Figures A7 and A8) were also observed for the H3 and H4 plant lines.

The protocol of Curtiss and Cardineau (1997) was used to further estimate the amount of HBcAgs produced by transgenic plants. The use of Dr. Fraser Scott's densitometer is acknowledged for the Western blot quantifications. The estimated amount of HBcAg that were produced in the leaves of the H1-H3 plants is shown in Tables 4-6 respectively. The highest amount of HBcAg observed for the H1-H3 lines were 0.6%, 3.4% and 3.8% respectively in the fresh leaves. However, the estimated amount of HBcAg in an H4 plant (#10) reached 9.8% in the leaf extract. These data indicate that the H1-H4 plants were transgenic and the transgenes were being actively transcribed and translated into stable recombinant proteins.

Table 4. Quantification of the plant leaf HBcAg subunits in three H1 plants

Plant I.D. from transgenic H1 tobacco line	**#1**	**#2**	**#3**
Microgram of soluble HBcAg subunit per milligram of tobacco seed	5.11	6.30	4.58
Percentage of HBcAg in the fresh leaf tissue	0.51%	0.63%	0.46%

The table was prepared by quantifying via Western blots the amount of soluble HBcAg in the protein extracts of H1 transgenic plants. Known dilutions of prepared weighed HBcAg subunits from *E. coli* were loaded as standards and the various plant protein extracts were loaded on SDS-PAGE gels. The protein quantities were estimated from the standard curve or estimated via band intensities when the unknowns were outside the standard curve range. The values are based on the highest quantity of HBcAg found in each transgenic plant analysed. The percentage of HBcAg (per gram fresh weight) is also shown.

Table 5. Quantification of the plant leaf HBcAg subunits in three H2 plants

Plant I.D. from transgenic H2 tobacco line	#1	#3	#5
Microgram of soluble HBcAg per milligram of tobacco leaf	302.02	337.54	2.38
Percentage of HBcAg in the fresh leaf	3.0%	3.4%	0.02%

The table was prepared by quantifying via Western blots the amount of soluble HBcAg in the protein extracts of H2 transgenic plants. Known dilutions of prepared weighed HBcAg subunits from *E. coli* were loaded as standards and the various plant protein extracts were loaded on SDS-PAGE gels. The protein quantities were estimated from the standard curve or estimated via band intensities when the unknowns were outside the standard curve range. The values are based on the highest quantity of HBcAg found in each transgenic plant analysed. The percentage of HBcAg (per gram fresh weight) is also shown.

Table 6. Quantification of the plant leaf HBcAg subunits in three H3 plants

Plant I.D. from transgenic H3 tobacco line	#1	#2	#3
Microgram of HBcAg per milligram of tobacco leaf	325.82	59.77	380.94
Percentage of HBcAg in the fresh leaf	3.3%	0.6%	3.8%

The table was prepared by quantifying via Western blots the amount of soluble HBcAg in the protein extracts of H3 transgenic plants. Known dilutions of prepared weighed HBcAg subunits from *E. coli* were loaded as standards and the various plant protein extracts were loaded on SDS-PAGE gels. The protein quantities were estimated from the standard curve or estimated via band intensities when the unknowns were outside the standard curve range. The values are based on the highest quantity of HBcAg found in each transgenic plant analysed. The percentage of HBcAg (per gram fresh weight) is also shown.

Western blot analyses on tobacco seed proteins

To determine whether tobacco seeds also produced the HBV core particle subunits, Western blots were conducted on protein extracts from transgenic tobacco seeds. Figure A9 shows a Western blot obtained for the seed extracts of three independent H4 transgenic plants. As the positive control from *E. coli* increased to the highest concentration as shown (Figure A9, leftmost "+" lane), so did higher molecular weight bands, especially the band at 33 kDa. Further, no higher molecular weight bands were previously shown for the H1 plant line (Figure A5). Co-incidentally, the 33 kDa band from the *E. coli* derived positive control appeared at a somewhat similar position to the 33-35 kDa bands observed for the plant samples on several Western blots (Figures A6- A9).

Quantification of HBV core particle subunits

These data were estimated using the protocol of Curtiss and Cardineau (1997) that used densitometric analyses of Western blots for quantification of recombinant plant proteins. As previous reports in the literature hinted at the possibility of obtaining higher yields of the transgenic protein in the seeds as opposed to the leaves of the same plant, the data from three plants were analysed and presented in Table 7. The H4 transgenic tobacco plant #10 produced up to 9.8% and 20.0% of HBcAg in the leaves and dry seeds respectively. However, H4 plants #4 and #5 produced 4.2% and 0.6% of HBcAg in the leaf extracts and 8.5% and 1.3% in the seed extracts respectively. As shown in Table 7, there was an approximate two-fold higher recombinant protein yield observed for the seeds as opposed to the leaves of these plants that were derived from the H4 plant line. To this effect, the amounts of seeds produced for several tobacco plants were obtained for a three-month period. Under conditions used in these experiments, each plant produced between 53.45g to 105.67g of fresh weight seeds for the three-month period.

Table 7. The plant seed to leaf HBcAg ratios found in H4 plants

Plant I.D. from transgenic H4 tobacco line	#4	#5	#10
Micrograms of soluble HBcAg per milligram of tobacco seed	84.5 (8.5%)	13.0 (1.3%)	200.3 (20.0%)
Micrograms of soluble HBcAg per milligram of tobacco leaf	42.1 (4.2%)	6.3 (0.6%)	98.7 (9.8%)
Plant seed to leaf HBcAg ratios	2	2	2

The table was prepared by quantifying via Western blots the amount of soluble HBcAg subunits in the protein extracts of H4 transgenic plants. Known dilutions of prepared weighed HBcAg subunits from *E. coli* were loaded as standards and the various plant protein extracts were loaded on SDS-PAGE gels. The protein quantities were estimated from the standard curve or estimated via band intensities when the unknowns were outside the standard curve range. The leaf and seed quantities are presented for H4 tobacco plants. The values are based on the highest quantity of HBcAg subunits found in each transgenic plant line and indicated that the antigen levels were generally poorer in leaves but higher in dry seeds of tobacco plants. The percentage of HBcAg is also shown in brackets.

Electron microscopy of viral particles from tobacco leaves

Timothy Booth and Lynne Burton at Health Canada conducted electron microscopy analyses to determine whether the HBV core particles were assembled as well as the particle diameters. From the negatively stained grids, correctly assembled virus-like particles of 25 nm to 30 nm in diameter were observed in the purified sucrose density gradient fraction of the leaf extracts of an H1 plant or from the crude leaf extracts from H2 to H4 plants (Figures A10- A13 respectively). No virus-like particles were detected from protein extracts on NT plants on the negatively stained grids (data not shown).

Discussion Part I

This is the first report of the successful generation of transgenic tobacco plants that specifically showed assembly of the truncated HBcAg subunits into virus like particles. Thanks go to Dr. Anton Andonov, Timothy Booth, Lynne Burton, Tim Booth and Darryl Falzarano for their contributions to the ELISA, Western blots, electron microscopy and subcutaneous immunization analyses. Electron micrographs showed similar sized particles in all plant extracts. The ELISA data helped to determine if the HBV core particles were produced in a high enough concentration for assembly to occur and did not necessarily allow quantification of the amount of proteins. This was a setback since Western blots had to be used to estimate the amount of HBcAgs produced in transgenic plants. The trimmed subunit as well as the addition of the RSRKDEL protein sequence did not affect assembly of the HBV core particles (the extensin signal sequence was predicted to have been cleaved by a plant protease for subsequent entry into the ER). Thus, this indicated the versatility of

the chosen antigen carrier to form correctly assembled virus-like particles. Insertion of other antigens into the HBV core particle is possible to produce customized multivalent vaccines for each age, economical, geographical, social, or risk group. These data have important implications on producing edible vaccines for strategic low-cost global vaccination programs.

The d35S promoter, the AMV-RNA 4 translational enhancer and the NOS-TER terminator sequence have been studied extensively in the construction of plant expression vectors. The first two components have been reported to increase HBV core protein expression to varying degree. The HBV C-terminal truncated wild-type expression construct was previously fused to the CaMV 35S promoter to generate transgenic tobacco plants (Callaghan, 2001). However, although these plants were transgenic as assessed by PCR on genomic DNA, they failed to produce sufficient HBV core particles or subunits to be observed in electron microscopy or on Western blot.

Considering the previous study of Callaghan (2001), it was reasoned that by using a stronger promoter such as the CaMV d35S promoter coupled with the AMV-RNA4 translational enhancer, detectable amounts of HBV core particles and subunits would result. Based on previous assessments, the d35S promoter was predicted to increase the mRNA transcripts approximately 2X and the AMV-RNA4 translational enhancer would stabilize the mRNA for increased translation of the message into protein (Datla *et al.*, 1993). The results reported here for the H1 expression construct did indeed result in detectable amounts of HBV core particles and the protein expression increased in transgenic plants from the H1 to the H4 transgenic lines as shown for the best expresser plants in Tables 4-7. The Western blots and ELISA assays used equal amount of total protein extract (volume) to determine the amount of HBcAgs in the fresh weight samples.

Further, tobacco seeds showed even higher amounts of (~2X) HBV core particle subunits than the leaves of the same transgenic plants in the H4 transgenic line. Representative data are presented in Table 7 that specifically show the results for three independent plants from the H4 transgenic line.

The Western blot data showed several bands for the plant codon-optimized expression constructs (H2-H4) (Figures A6-A9). These higher MW bands seem to occur as the concentration of HBV core particle subunits increased (*E. coli* positive control or plant derived). Such findings have also been reported for the rthGM-CSF (Alli, 2001) and the rthIGF-1 (Panahi, 2002) expressed in plant systems. The transgenic tobacco plants of Curtiss and Cardineau (1997) which produced the *Streptococcus mutans* (serotype g) surface protein antigen A (SpaA) also showed dimer formations on Western blots. Further, evidence for the formation of dimers of bovine cholesterol esterase (CE) in SDS-PAGE gels had been linked to increase in boiling time of the samples as well as increase in concentrations of the cholesterol esterase (Vakos *et al.,* 1997). Thus, Vakos *et al.* (1997) reported the observation of dimeric 116 kDa CE as well as the 58 kDa monomeric CE subunit. The reason for dimer formation was indicated as deriving from hydrophobic interactions of the subunits.

One possibility for the small differences (~1 kDa) observed for the H4 sucrose density purified fractions on the SDS-PAGE gel is the presence of O-linked glycosylation on serine or threonine residues. However, this is highly unlikely if there is no signal peptide synthesized at the 5` end as was the case with the H1-H3 plant lines. Therefore, this may occur only in the H4 plant line. Although the natural HBcAg is not normally glycosylated, O-linked glycosylation was predicted to occur in the HBV core particle on threonine 174 and 178 from the NetOGlyc 3.0 Server of the Technical University of

Denmark (http://www.cbs.dtu.dk/services/NetOGlyc/). Indeed threonine 174 had a higher score (0.581) which was above the threshold of 0.5 for O-linked glycosylation to occur. These small differences in molecular weights (~1 kDa) have also been reported for the rthGM-CSF produced in yeast (Ernst *et al.,* 1987; 1992) and plants (Alli, 2001). When the rthGM-CSF from yeast cells was analysed, both O-linked and N-linked glycosylations were reported for the secreted recombinant protein. Western blots showed 14.5 kDa, 15.5 kDa, and 18 kDa bands. To gain insight into the mechanisms at work in yeast cells, endoglycosidase H treatment or site directed mutagenesis of both N-linked glycosylation sites were conducted and resulted in only two observable bands at 14.5 and 15.5 kDa. It was concluded that the 15.5 kDa band was an O-linked glycosylated species and both O-linked and N-linked glycosylations were present in the higher MW species. Further, the rthGM-CSF obtained from tobacco seeds appeared as distinct bands of 19, 21, and 50 kDa on Western blots (Sardana *et al.,* 2002). Thus the ~1 kDa differences reported here for the HBcAg subunits of transgenic tobacco plants may result from one or two sites on the HBV core particle subunit being O-linked glycosylated. In any case, assembly was not affected for the HBV core particles.

If variations in glycosylation patterns were only true for the yeast system, the expression of hGM-CSF gene in *Spodoptera frugiperda* (Sf9) cell culture using viral infection would not have resulted in 14.5 kDa, 15.5 kDa and 16.5 kDa bands on Western blots (Chiou and Wu, 1990). Although baculoviral expression systems require signal sequences for secretion of recombinant proteins into the cell culture medium, these peptides were much larger than 1 kDa and the uncleaved signal sequence could not account for the observed differences (Chiou and Wu, 1990; Shi *et al.,* 1996). Others have reported that baculoviral infection of *Bombyx mori* (silkworm) nuclear cells with an rthGM-CSF

expression vector yielded three different molecular masses of the rthGM-CSF (15 kDa, 18 kDa and 20 kDa) (Shi *et al.,* 1996). Thus, differences in glycosylation patterns may be universal for eukaryotic systems.

Codon-optimization of the cDNA obtained from non-plant source may result in increased expression of the recombinant protein in various plant hosts (Cheng *et al.,* 1998; Horvath *et al.,* 2000). The intended use of these codons in designing the H2, H3 and H4 expression constructs with the codon preference found in the *Arabidopsis thaliana* genome was to ensure: (1) a ready supply of tRNA molecules for increased translation rates in plants; (2) to use codons that the plant system expects to find in its natural frequency; and (3) to obtain higher levels of HBV core particle synthesis due to the efficient use of plant codons. The constructs H1, H2, H3 and H4 (Figure 6) were also designed to decipher the contribution of each component (AMV RNA4, extensin signal sequence, codon-optimization, and KDEL) towards augmented recombinant protein accumulation in plants. The results of the best H2 transgenic tobacco line showed a definitive increase in the HBcAg subunits in the leaves than that obtained from the H1 plant line. These two expression constructs (H1 and H2) were identical except that the *A. thaliana* genomic codon bias was present in the H2 coding sequence. Hence, the increase in recombinant protein subunits and particles was only due to the contribution of the plant codon bias for designing the coding sequence of the truncated HBV core particle subunit. It is interesting to note that the H2 line showed either high O.D. ELISA values or low O.D. ELISA values but no intermediate O.D. ELISA values were observed. The Chi square test of independence of the ELISA data from paired groups H1 and H2 showed no significant difference ($p = 7.55E-01$).

For retention of normal ER resident proteins, either HDEL or KDEL is found on these plant proteins. In the case of the default cytosolic pathway, proteins lacking a targeting signal may be degraded and hence may not be observed (Callaghan, 2001). Further not all proteins are processed in the same manner regardless of the presence of similar regulatory components in the expression construct. Therefore, each protein must be studied on a case-by-case basis to determine the right combination of regulatory sequences that will contribute to overall increase in yields. Thus, the C-terminal KDEL sequence was intended for retention of the HBV subunits and their subsequent assembly in the ER (Schouten *et al.,* 1996). This strategy of using the ER retention signal, should also lead to augmentation of recombinant proteins in plants and animals (Richter *et al.,* 2000). The results confirmed this since the best H3 and H4 plants with the KDEL sequence showed up to 3.8% and 9.8% HBcAg in the leaf extracts respectively (Tables 6-7). Although densitometric analysis is based on band intensities and is only semi-quantitative, the highest observed values allowed a ratio to be calculated for the best plant from each of the four different plant lines. The H1: H2: H3: H4 ratio is 1.0: 5.4: 6.0: 15.6 and indicates that indeed the extensin signal sequence was the strongest contributor to HBcAg accumulation followed by plant-codon optimization. However, the increase observed for addition of the KDEL sequence was milder when compared to plant-codon optimization in the best plant of each group.

In recombinant expression constructs, signal sequences are necessary for the stability of proteins and/or to prevent degradation, thereby increasing the recombinant protein yields (Sardana *et al.,* 1997, 2002). Further, signal sequences may lead to precise targeting of proteins into specific protein storage compartments as was shown for the glutelin signal sequence (Wright *et al.,* 2001). In the case of the extensin signal sequence,

proteins will follow the secretory pathway to the cell wall (Chen and Varner, 1985). This strategy of augmented protein yields was demonstrated when the H4 expression construct was used to generate transgenic tobacco plants. The extensin signal sequence, plant-codon optimization of the coding sequence and addition of the KDEL sequence contributed to increased protein expression that resulted in up to 9.8% and 20.0% in the leaves and dry seeds of the best transgenic H4 tobacco plant. This of course may be exceptionally higher because of the limitations of scanning band intensities. Only a few other plant reports have claimed high expression levels of recombinant proteins. The work of Nawrath *et al.* (1994) that used a pea transit peptide to target three enzymes involved in the polyhydroxybutyrate synthetic pathway to the chloroplast resulted in up to 14% of this polymer in chloroplast but in the absence of the pea transit peptide, only up to 100 μg/g of this polymer was observed in the cytoplasm of transgenic leaves (Poirier *et al.*, 1995). Thus it is believed, that the presence of the plant extensin signal sequence was the major contributing factor to the increased level of HBcAg.

Based on the Chi square assignments, the H1-H4 expression constructs led to frequencies of 8, 3, 6 and 18 respectively (frequency of positive plants that assembled the HBcAg). Although the exact epitope recognized by the MAB842 is not disclosed by the manufacturer, this antibody can only detect assembled HBV core particles. This antibody cannot bind the hepatitis B core subunits (linear epitopes) run on Western blots (personal communication of Dr. Anton Andonov). Further Chi square test of independence of paired groups showed that the H1 and H4 groups and the H2 and H4 groups were significantly different with p-values of 3.13E-04 and 1.58E-04 respectively. However, groups H3 and H4 were not significantly different ($p = 5.91E-01$). Further, the percentage of H4 plants showing assembly of the HBcAg was 90.0% indicating that this expression construct may

lead to decreased screening for plants that express the recombinant protein at very high levels. Based on the Western blot estimation of the best H3 and H4 plant, it may be concluded that the extensin signal sequence will result in approximately 2-3 times more recombinant protein when the AMV-RNA4 translational enhancer is substituted for this signal sequence.

The data obtained here for the HBV core particle expressed in transgenic tobacco plants are for the C-terminal truncation of the HBV core particle subunit. This truncation did not affect assembly into virus-like particles and the particle diameters of 25-30 nm are consistent with that observed when the full-length HBV core particle subunit was expressed in transgenic tobacco plants (Tsuda *et al.*, 1998). Therefore, for multivalent vaccines, the truncated HBV core particle subunit may be more energetically favoured when used as an antigen carrier than the full length.

Production of an antigen is of no use if it is not biologically effective in stimulating an immune response and offering protection against the native pathogen. Once it became clear that synthesis and assembly of the HBV core particles in transgenic plants had occurred, the particle's ability to stimulate an immune response was assessed. The *in vivo* subcutaneous immunizations were conducted by Dr. Anton Andonov and Darryl Falzarano at Health Canada. The purified HBV core particle subunits appeared homogeneous on the silver stained gel (Figure A14) (Falzarano *et al.*, 2003, 2004). Hence, the purified HBcAgs (3 μg/dose of plant-derived or *E. coli* derived HBcAgs containing aluminium hydroxide and QuilA saponin as an adjuvant at a concentration of 0.05% w/v) that were injected subcutaneously into mice on days 0 and 21 would be considered sufficient for this study. The Winnipeg data indicated that there was no difference observed in the serum antibody titres when the *E. coli* derived particles or the plant-derived particles were used for

vaccination of animals. Equal antibody responses were observed for both groups of animals vaccinated with the two different sources of HBV core particles (Falzarano *et al.*, 2004). These results are significant since the HBV core particles retained their antigenicity. As expected, the NT plant extract when used as immunogen did not result in an observable serum antibody response to HBV antigens as assessed by ELISA. Hopefully, the positive observations may be mimicked later during oral administration of the plant derived HBcAgs to mice, and eventually to humans.

Analyses are currently being conducted to determine if oral administration of the transgenic tobacco seed tissue will offer protection to rabbits when challenged with the hepatitis B virus. The successful expression of the HBV C-terminal truncated core particle using plants indicates that the choice of a plant system was excellent for this study. The additional modifications such as codon optimization and the added signal sequence have led to significantly higher recombinant protein expressions. It is postulated that oral immunization may eventually result not only in broad mucosal protection, but also in a systemic response when the pathogen is encountered (Sandhu *et al.*, 2000). Factors such as the effectiveness of an oral response when the transgenic tissues are fed without adjuvants and the ability to offer protection against challenge by the HBV will influence these studies. Further engineered epitopes into the immunodominant c/e1 loop of the HBV core particle may not affect the protein levels as long as the inserted epitopes are plant codon-optimized. Epitopes that are stuffed within the immunodominant c/e1 loop of the HBV core particles should retain their antigenic determinants for effective immunostimulation (Kratz *et al.*, 1999; Murray *et al.*, 1999; Pumpens and Grens, 2001).

Chapter 4

DEVELOPMENT OF A CARROT MODEL SYSTEM FOR THE HARVESTING OF RECOMBINANT PROTEINS

Results Part II

The work conducted in carrot and rice was to determine the effectiveness of the plant expression constructs to function in a similar manner as was found for their expression in tobacco plants. While the H1 expression construct was the first to be assembled, the other three expression constructs (H2-H4) were made within 6-12 months. Hence, the H1 expression construct was used to determine if assembly of the HBV core particle was possible in all three plant systems. Once the other three expression constructs (H2-H4) were analysed in tobacco, a decision was made to use the best expression construct (H4) in edible carrot and rice plants. The two expression constructs (H1 and H4) that were used for the edible plant (carrot and rice) transformation studies are shown in Figure 11. The data gathered for the H4 expression will be briefly compared with the H1 data.

Generation of transgenic carrot plants

Over 27 transgenic H1 carrot plants and over 24 transgenic H4 carrot plants were produced. Matured carrot roots reached lengths of up to 15 cm and some became as wide as 7 cm in diameter. As each independently transformed carrot plant matured, each sprouted additional roots beside its original root. Sometimes, these additional roots were replanted to maintain the same independent plant for up to two years. At maturity, carrot plants reached heights of up to 90 cm in length.

Figure 11. The two different HBV core protein expression constructs used to generate transgenic carrot and rice plants. These expression constructs were based on the HBV wild-type codons (H1) and the *Arabidopsis thaliana* codon bias were used for the H4 plant codon-optimized expression construct. Each complete expression construct was subcloned into independent plant transformation vectors (pRD400 for carrot transformations and pCAMBIA 1303 for rice transformations) using the restriction endonucleases *Hind* III and *Eco* RI. The resultant plant transformation vectors with independent inserts H1 and H4 were used to transform *A. tumefaciens* cells for subsequent transformation of carrot hypocotyls and rice calli. These constructs are not drawn to scale and also appear in Figure 6 but are presented here for clarification.

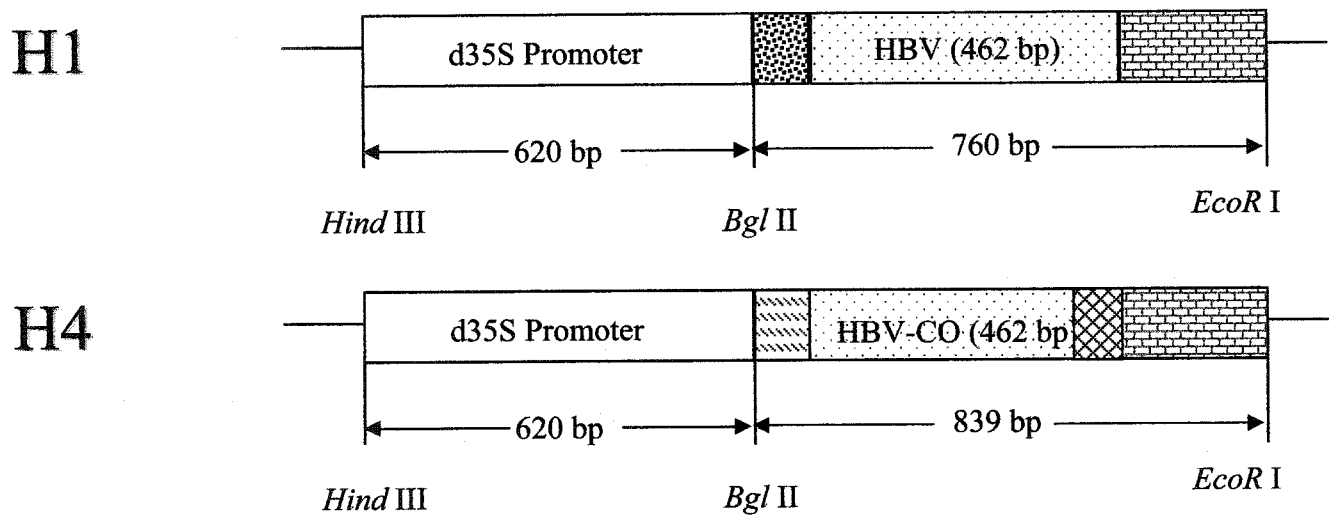

H1
d35S Promoter
HBV (462 bp)
620 bp
760 bp
Hind III
Bgl II
EcoR I
H4
d35S Promoter
HBV-CO (462 bp)
620 bp
839 bp
Hind III
Bgl II
EcoR I
d35S promoter 620 bp
Extensin signal sequence 96 bp
AMV-RNA4 translational enhancer sequence 38 bp
RSRKDEL coding sequence 21 bp
HBcAg coding sequences
NOS-TER sequence 260 bp

Figure 11

Determination of the transgenic nature of carrot plants

Southern blotting experiments were used to assess whether the putatively transformed carrot plants were actually carrying transgenes. All transgenic carrot plants analysed were shown to carry the transgene via Southern blotting experiments. The results of a representative H1 Southern blot are shown in Figure 12, Panel A. The five independently transformed carrot plants shown contained the complete expression construct (*Eco* RI-*Hind* III fragment) that appeared as bands at the same position, 1.380 kb, as the positive control. Similarly, bands at 1.459 kb were observed for all the H4 carrot plants analysed (Figure 12, Panel B). No bands were visible in any lane that contained non-transformed carrot plant DNA during Southern blot analyses (Figure 12, NT lanes, Panels A & B).

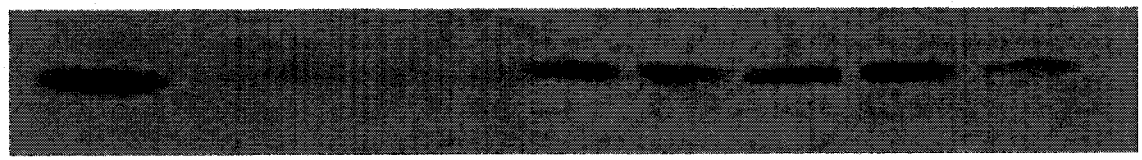
Transgenic carrot derived HBV core protein analyses

Detection of assembled HBV core protein in transgenic carrot root

The plant lysates (1 mg/mL) from carrot were prepared and submitted to Health Canada, Bureau of Microbiology and Immunology, Winnipeg, Manitoba for ELISA, Western blots, electron microscopy, and immunization analyses. The ELISA values and Western blots were returned to me for Statistical and densitometry analyses. To estimate the number of plants that had assembled the HBcAg, an initial test was conducted via ELISA on the protein extracts. Data were gathered for the H1 and H4 carrot root extracts (Figures A15 and A16). For the H1 carrot root extracts, the highest O.D. value observed was 0.852 (plant #2) while several plants of the H4 line showed O.D. values of 4.0. Further, Chi square analyses were conducted for these two groups (Table 8). To clearly establish a positive carrot plant where assembly of the HBcAg had occurred, an arbitrarily O.D. value of three times NT was assigned for carrot plants (Table 8).

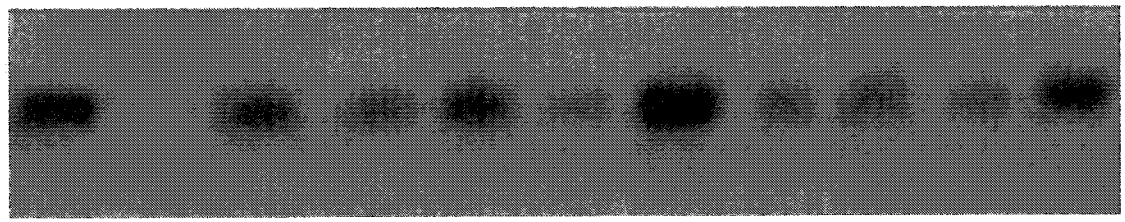
Figure 12. Southern blots generated from H1 and H4 transgenic carrot plant lines. Genomic DNA (40 μg) from each carrot plant was digested with the restriction enzymes *Eco* RI and *Hind* III and electrophoresed on 0.8% agarose gels. After electrophoresis, the DNA was transferred to nitrocellulose membranes, probed with gel purified and radiolabelled *Eco* RI-*Hind* III fragments as shown in Figure13, and described in the "Materials and Methods" section on Southern blots. Lanes +: the *Eco* RI-*Hind* III insert released from the plasmids containing the different HBV core protein expression constructs; lanes NT: DNA from a non-transformed plant cleaved with *Eco* RI and *Hind* III; lanes 1-5 and 5-13: DNA from different transgenic plants cleaved with *Eco* RI and *Hind* III from the H1 and H4 lines respectively.

Panel A



\+ NT 1 2 3 4 5

Panel B



\+ NT 5 6 7 8 9 10 11 12 13

Figure 12

Table 8. Chi square test of independence of ELISA data obtained from the H1 and H4 carrot lines

Carrot Group	Number of positives	Number of negatives	Totals
H1	4 (7.44)	13 (9.56)	17
H4	10 (6.56)	5 (8.43)	15
Totals	14	18	32
Chi square = 6.02 Degrees of Freedom = 2		p = 4.92X10E-2	

The table was prepared using the ELISA data obtained from all carrot lines (H1 and H4, Appendix Figures A15 and A16) using an arbitrarily assigned O.D. value that was three times NT (0.25) and above to represent a "positive" plant in which the HBcAg had assembled. All values below an O.D. of 0.25 were arbitrarily assigned as negative. The carrot plant groups, the number of positives, negatives and the totals of each column or row were also calculated. The expected positive or negative values are also shown in parenthesis. The Chi square value, the degrees of freedom and the resultant p-value are also shown.

This assignment is different from the assigned value used for tobacco plants. The Chi square test assignments for the H1 and H4 expression constructs showed 23.5% and 66.7% positive carrot plants that assembled the HBcAgs respectively. The Chi square test of independence for these two groups of plants showed significant difference, χ^2 (2, N = 32), $p < 0.05$.

Western blot analyses on protein extracts from leaves and roots of carrot plants

Further characterization of the expressed HBV core particle subunits was conducted via Western-blotting experiments on both leaf and root extracts of carrot plants. Representative Western blots are shown in Figures A17 and A18 for the H1 carrot plant line. The transgenic carrot root extracts showed bands of 16.5 kDa that was similar to the *E. coli* derived positive control (Figures A17 and A18). The roots of all H1 plants tested positive for the 16.5 kDa HBV core particle subunit except H1 plant #13. No higher molecular weight bands were observed. Further, no bands were observed in the NT plant lanes (Figure A18, Panels A & B). After concentration of the leaf extract, the highest HBcAg estimated expression in H1 carrot (plant #2) was less than 0.01% in the fresh tissue. However, the HBcAg in H1 plant #2 reached a value of 0.45% in the fresh root tissue.

Similarly, Western blots obtained for the leaf and root extracts from H4 plants are shown in Figures A19 and A20. At least two distinct bands ranging in size from 17.5-19.5 kDa were observed on Western blots for all extracts of the plant codon-optimized H4-line (Figure A19: Panels A & B). In addition to the distinct 17.5-19.5 kDa bands, higher MW bands were observed {33, 44, and 47 kDa, Figure A19, plant #10). The HBcAg in H4 fresh carrot leaves ranged from 0.07% to 1.15% while the fresh roots had a range of 0.11% to 1.73%. When the root to leaf ratio of these three plants was compared, a

ratio of 3:1 for production of the HBcAg subunits was calculated ($p = 0.02$). Further, analyses were conducted to determine the distribution of the HBV core particle subunits throughout the carrot roots. These data indicate that the H1 and H4 carrot plants produced HBV core particle subunits that were higher in the roots than the leaves.

Electron microscopy of viral particles from carrot leaves

To determine whether the HBV core particles assembled as well as the particle diameters, electron microscopy analyses were conducted. From the negatively stained grid, a correctly assembled virus-like particle of 25 nm in diameter was observed for the purified sucrose density gradient fractions of the leaf extract of H1 carrot plant #2 (Figure A21). No virus-like particles were detected from protein extracts of NT plants on the negatively stained grids (data not shown).

Discussion Part II

The successful expression of the HBV C-terminal truncated core particle in transgenic carrot is to the best of my knowledge, the first reported expression of a candidate antigen carrier in transgenic carrot plants that specifically showed assembly via electron microscopy. Carrot plants were transformed with the H1 and H4 expression constructs to determine if the same protein processing and deposition would occur as reported for transgenic tobacco plants. Further, H4 carrot plants were used for comparison with H1 carrot plants to assess the intrinsic qualities of these two different plant lines. Since the original vector pBI 525, containing the d35S promoter, the AMV-RNA 4 translational enhancer and the NOS-TER terminator sequence has been studied in plant systems, it was reasoned that these components would also function in carrot plants. The presence of the stronger d35S promoter coupled with the AMV-RNA 4 translational enhancer would act synergistically to augment recombinant protein accumulation (Datla *et al.,* 1993). Further, modifications of the H1 wild-type construct by plant codon-optimization, addition of the KDEL and the extensin targetting sequences were shown to increase the level of expression as observed by ELISA and Western blots.

The Western blot results obtained for the H1 carrot leaf extracts showed that the HBV core particle subunits were not detectable as the carrot root extracts under identical experimental conditions. All H1 plant roots tested positive for the 16.5 kDa HBV core particle subunit except H1 plant #13. However, it is believed that this plant was also positive for the HBV core particle subunit but the expression level was extremely low in this 10 uL of extract for adequate detection on this Western blot. This indicated that the HBV core particle subunits were produced (or more stable) at a higher level in roots than

that in the carrot leaves. These results have not been reported for carrot plants before in which higher yields of the HBV core protein in the roots as opposed to the leaves of the same plant were seen.

The results of the H1 and H4 carrot lines showed differences in the HBV core particle levels in the leaf protein extract that increased from the H1 to the H4 transgenic lines (Figures A18-A19). Further, the H1 carrot roots showed higher amounts of HBV core particle subunits on Western blots than the leaves of the same independent transgenic plants (Figure A18). Chi square test of independence showed that the H1 and H4 carrot plant lines were different ($p = 0.05$) and the H4 expression construct lead to higher (66.7% fresh weigh) amounts of assembled HBcAg than the H1 expression construct (23.5% fresh weight). The tissue differences observed for carrot plants somewhat mimic the results of the tobacco plants where higher recombinant HBV core particle subunits were observed in the seeds than in the leaves of H4 tobacco plants.

Transgenic H4 carrot plants were shown to produce the HBV C-terminal truncated core particle subunits both in leaves and roots indicating that the plant designed expression constructs were also functional in this system. Edible carrot plants transformed with the H1 expression construct were analysed for self-assembled HBV C-terminal truncated core particles via ELISA and electron microscopy. The electron micrograph obtained from the concentrated leaf extract of H1 carrot plant #2 showed at least one particle with a diameter of 25 nm (Figure A21, data not obtained for the H4 carrot line). As observed for the HBV core particle expression in tobacco, H1 carrot plants also processed the C-terminal truncated HBV core subunit into virus-like particles and this modification did not affect the assembly into particles having diameters of 25-30 nm. Hence trimming of the HBV core

particle subunit did not affect assembly of the particles in this carrot recombinant expression system.

It suffices to mention that the Western blots of the H4 carrot plants were positive when the leaf extracts as well as the root extracts were analysed but when the same volume of H1 leaf protein extracts were used, no HBV core particle subunits were detected. This qualitatively indicated that higher levels of HBV core particle subunits were present in the H4 carrot leaves than the H1 carrot leaves and correspondingly higher levels of HBcAg were observed in H4 carrot roots as opposed to H1 carrot roots. The major contributing factors to the recombinant protein augmentation in the case of the studied tobacco plants were the extensin signal sequence and/or the C-terminal KDEL sequence. From the data gathered so far on both the H1 and H4 carrot lines, there are no data to indicate that the contrary is true. Thus, the signal sequence may allow the recombinant proteins to follow the secretory pathways to the Golgi apparatus but may be returned back to the ER by a tubular transport mechanism that is not fully understood (Stephens and Pepperkok, 2001). This may have prevented degradation that resulted in increased recombinant protein yields in the H4 carrot line.

Further, as with tobacco plants, the C-terminal KDEL sequence is postulated to have no effect on assembly in the H4 carrot line when HBV core particles are produced. If this remains the case when the H4 carrot plants are eventually analysed via electron microscopy, this will serve to confirm the flexibility of the particle to form correctly assembled virus-like particles regardless of multiple site-directed insertions or deletions of amino acids. These observations in carrot plants are consistent with that reported for the full-length HBV core particle produced in transgenic tobacco plants (Tsuda *et al.*, 1998).

This flexibility makes the HBV core particle an ideal antigen carrier that may require less molecular resources for synthesis and expression than the full length HBV core subunit.

The strategy developed here adapted carrot plants, as the vehicle for the next generation of edible vaccines. Production of an antigen is of no use if it is not biologically effective in stimulating an effective immune response and offering protection against the native challenge pathogen. Hence, analyses are currently being conducted to determine if oral administration of the carrot roots will offer protection to rabbits when challenged with the hepatitis B virus. Overall, the carrot plants did produce and assemble the potential oral antigen carrier. The next phase of inserting antigens into the c/e1 immunodominant loop will be critical to the success of carrot based oral immunizations for tactical deployment against infective organisms. These observations have important implications for the use of edible carrot roots in direct oral delivery of vaccines.

Chapter 5

EXPRESSION OF THE HBV CORE PARTICLE IN RICE PLANTS

RESULTS PART III

The two expression constructs (H1 and H4) used for rice calli transformations are shown in Figure 11 and represent the same constructs that were used for the protein expression studies in tobacco and carrot plants. Since H4 rice plants are still being analysed, only the H1 results are presented here.

Generation of transgenic rice calli, plantlets and mature plants

After experimenting on the age of rice calli for *A. tumefaciens* mediated transformation, the calli tissues were found to be at peak competence for transformation after 40 days on the calli induction medium. Within two months the 40-day-old rice calli that were co-cultivated with *A. tumefaciens* showed 100% percent differentiation into dark green calli. The percentage of recovered green calli decreased for rice calli as the age of the calli deviated from 40 days (>40 days or <40 days). However, the older rice calli tissues (>40 days) showed more competence for *A. tumefaciens* mediated transformation than the younger rice calli. A representative Petri dish in Figure 13, Panel B shows 100% of the H4 rice calli as green hygromycin resistant calli. When used as a control, no NT rice calli grew into green hygromycin resistant calli (Figure 13, Panel A). These results were obtained from *A. tumefaciens* mediated transformation of 40-day old rice calli. Eventually, plantlets developed from the green calli. Rice plants took 6 months to mature and a further 2-3 months to produce seeds.

Figure 13. Selection and proliferation of transformed H1 rice calli. The photograph shows green rice calli from putatively transformed H1 line after transfer to a new plate containing a plant growth medium {4.3 g/L MS major and minor salts, 1 mg/L thiamine.HCl, 100 mg/L myoinositol, 20 g/L sucrose, 2 mg/L kinetin, 0.5 mg/L IAA (indole-3-acetic acid), 3 g/L Phytagel} . The rice calli were obtained after one month of selection on 50 mg/L hygromycin or 200 mg/L ticarcillin. Rice calli in Petri dish A were not transformed and therefore necrotized. Plate B shows rice calli that were transformed with the H1/pCAMBIA 1303 vector and hence proliferation into green calli was observed.

A B

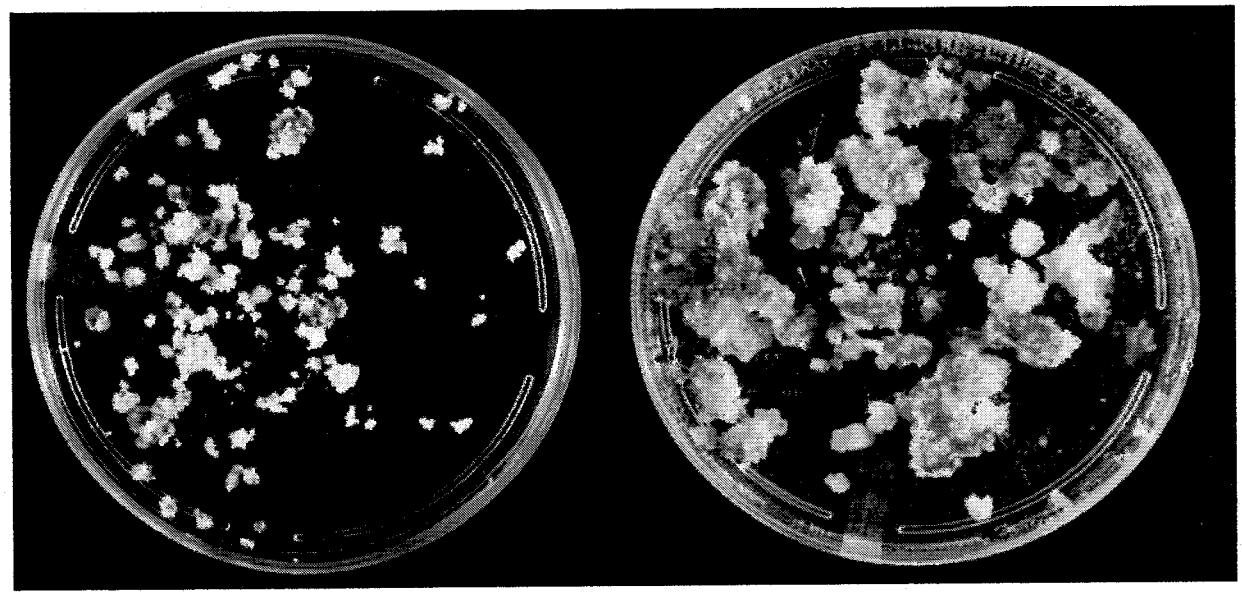


Figure 13

Determination of the transgenic nature of rice plants

Out of 26 transgenic rice plants, only six survived to maturity. Southern blotting experiments were conducted to determine whether the putatively transformed rice plants were actually carrying transgenes. The three transgenic H1 rice plants analysed carried at least one transgene. The number of gene copies was determined directly using the method of Alli (2001) that allowed for the direct counting of the number of transgene copies on autoradiograms. The results of a representative Southern blot are shown in Figure 14. Each of the three independently transformed plants shown contained 2-3-gene copies of the complete expression construct and each gene copy appeared as distinct bands of 1.8, 4.5, and 7.0 kb. The NT rice plant lane did not show any hybridization of the radiolabelled H1 probe (Figure 14, NT lane).

To further analyse the H1 rice plants for H1 mRNA transcripts, Northern blotting experiments were conducted on total RNA obtained from the leaves of transgenic H1 rice plants. The Northern blot shown in Figure 15 was conducted on the same three independent transgenic rice plants analysed by Southern blot in Figure 14. All transgenic H1 rice plants showed the presence of a 0.560 kb transcript (RNA markers do not hybridize with the probe). Densitometry analyses were conducted on the autoradiogram to calculate the ratios of transcripts. The H1 rice plant #1 had very little H1 RNA transcripts while plants #3 and #5 showed 53 and 59 folds higher transcripts than that of plant #1.

Figure 14. Gene copy reconstruction on independent H1 rice plants. Transgenic rice plants were generated via *A. tumefaciens* mediated transformation of calli tissues with the H1/pCAMBIA 1303 vector. Genomic DNA (40 μg) from each rice plant was digested with the restriction enzyme *Eco* RI and electrophoresed on 0.8% agarose gels. After electrophoresis, the DNA was transferred to a nitrocellulose membrane and probed with a gel purified and radiolabelled *Eco* RI-*Hind* III fragment from the H1 construct. Digestion of the genomic DNA with *Eco* RI alone cuts the transgene at the 3` end. The next *Eco* RI site located in the genome 5` of the transgene will then be cut. Therefore, every transgene insertion event in the transgenic plant genome can be reconstructed. Lane NT: DNA cleaved with *Eco* RI from a non-transformed plant; lanes 1-3 DNA cleaved with *Eco* RI from three independent transgenic plants from the H1 line (#1, #3 and #5).

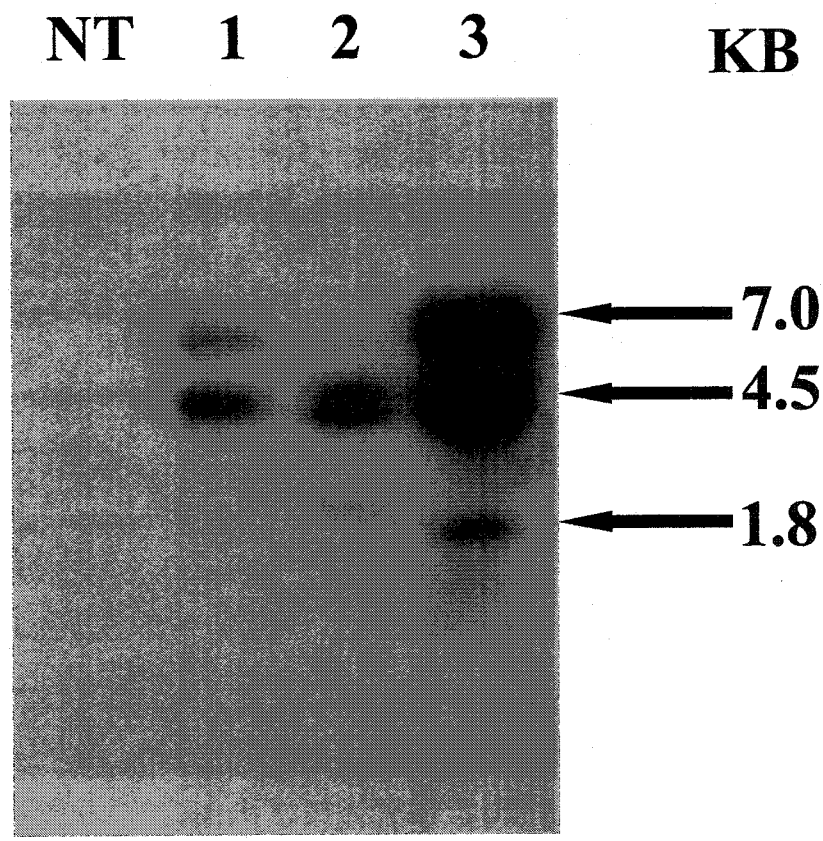


Figure 14

Figure 15. Northern blot of three independent H1 rice plants. Equal quantities of total RNA (20 μg) from each plant was electrophoresed on denaturing 1.2 % agarose gels. After electrophoresis, the RNA was transferred to a nitrocellulose membrane and probed with the corresponding gel purified and radiolabelled HBV core particle subunit coding sequence. No markers were labelled along with the probe. The probe was made from the H1 expression construct as shown in Figure 11 by digestion with restriction enzymes as described in the "Materials and Methods" section on Northern blots. Lane NT: total RNA from an NT plant. Lanes 1-3: total RNA from three H1 independent transgenic plants (#1, #3 and #5) that corresponds to the plants analyzed via Southern blots in Figure 14.

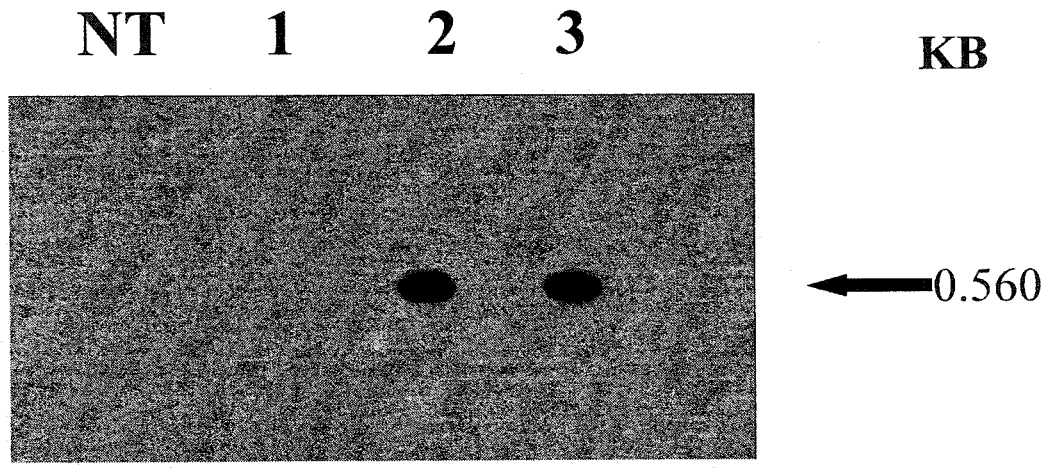


Figure 15

Transgenic rice derived HBV core protein analyses

ELISA on crude protein extracts from rice leaves

The plant lysates (1 mg/mL) from rice were prepared and submitted to Health Canada, Bureau of Microbiology and Immunology, Winnipeg, Manitoba for ELISA, Western blots, electron microscopy, and immunization analyses. The ELISA values and Western blots were returned to me for Statistical and densitometry analyses. Preliminary ELISA data were gathered for three independent plants from the H1 transformed rice line. The total protein extracts were utilized for detection of assembly using a monoclonal antibody directed against the assembled HBV core particle as was done for the tobacco and carrot lines. The H1 rice plant number #3 showed the maximum O.D. ELISA value of 4.0. However, the H1 rice plant #5 showed an O.D. ELISA value of 1.78. The other transgenic H1 rice plant showed an O.D. value below that of the NT plant.

Western blot analyses on transgenic rice plants

Further characterization of the expressed HBV core particle subunits was conducted via Western-blotting experiments on both leaves and seeds of transgenic rice plants (Figures A22-A23). A representative Western blot is shown in Figure A22 for the leaf extract of three transgenic H1 rice plants. When 10 μL of total protein extract was loaded into each well, all of the plants analysed showed a 16.5 kDa band representative of the HBV core particle subunit derived from *E. coli*. The *E. coli* derived positive control on the Western blot appeared as a single band. No band was present in the NT rice plant lane. The estimated amount of HBcAg in the fresh leaves of transgenic H1 rice plants #1, #3 and #5 was 0.22%, 1.44% and 0.84% respectively. No HBcAg subunits were detected in the NT plant leaves. Similarly, a representative Western blot experiment on the seed extracts of H1 rice plant #3 is shown in Figure A23. Equal volumes of protein extracts from the rice

seeds were used on the Western blot in order to quantify the HBV core particle subunits and for comparison with the H1 rice leaf extracts. From the Western blot, a 16.5 kDa band is detected in the first, second, third, and fourth extracts. Similarly, the seeds of plant #5 did produce the HBcAg when assessed via Western blot (data not shown).

Electron microscopy conducted on viral particles from rice leaves

To determine whether the HBV core particles assembled as well as their particle diameters, electron microscopy analyses were conducted on the H1 rice leaf extracts from transgenic plants. From the negatively stained grid, correctly assembled viral-like particles of 25 nm to 30 nm in diameter were observed for H1 rice plant #3 (Figure A24). No virus-like particles were detected from protein extracts of NT plants on the negatively stained grids (data not shown).

Discussion Part III

To the best of my knowledge, this is the first report of the expression of the HBV core particle in transgenic rice leaves and seeds. Transgenic rice plants were shown to produce the HBV C-terminal truncated core particle subunits both in leaves and seeds indicating that the plant designed expression constructs were also functional in this third recombinant expression system. The promoter in the H1 expression construct was the stronger CaMV d35S promoter and this was fused to the AMV-RNA 4 translational enhancer for augmented recombinant protein synthesis. This combination was predicted to increase the mRNA transcripts and lead to efficient translation of the message into protein as opposed to using the the weaker CaMV 35S promoter alone.

The generation of transgenic rice callus tissues and eventually potential edible vaccine plants was achieved using a modified protocol of Cheng *et al.* (1997). The major differences between the modified protocol and the original protocol were: (1) the use of the complete rice grain to generate rice calli that would be conducive to *A. tumefaciens* mediated transformation and (2) the ideal time for *A. tumefaciens* mediated transformation of the rice calli was found to be 40 days post induction. Under these experimental conditions, 100% of the putatively transformed dark green calli were observed (Figure 13). Tissues that were older than 40 days were still competent for *A. tumefaciens* mediated transformation but younger tissues had less chance for growth into green hygromycin resistant calli. The ideal time for the generation of large numbers of transgenic rice calli as reported by Cheng *et al.* (1997) was found to be one month after calli induction. The differences in the methods used in generating the rice calli may be a contributing factor for the 10-day delay period in competence observed for this study.

Since, I (Alli, 2001) and Panahi (2002) have shown that the number of transgenes acquired by each transgenic tobacco plant affects its ability to produce high or low levels of recombinant proteins, transgene copy reconstruction experiments were conducted on rice plants. From the representative blot in Figure 14, high protein expression was observed for transgenic rice plants that had at least three gene copies (e.g. plants #3 and #5). These results serve to confirm the previous results where three gene copies were the ideal number needed to achieve high expression of the recombinant proteins in tobacco plants (Alli, 2001; Panahi, 2002). Further, this may indicate conservation of gene regulatory and protein synthesis pathways within the plant kingdom. To further quantify the relative abundance of the mRNA transcripts present in these three rice plants, Northern blotting experiments were conducted. From the results of Figure 15, differences in the amount of mRNA were found between transgenic rice plants that produced low and high quantities of recombinant proteins. The low expresser plant #1 showed low levels of mRNA as well as low gene copy numbers (two) while the high expresser plants (#3 and #5) showed high levels of mRNA and three gene copies. These results correlate with the results of the gene copy experiment where rice plant #1 had the lowest number of transgenes (2) while rice plants #3 and #5 had at least 3 transgene copies (Figure 14). The preliminary ELISA data showed the maximum O.D. ELISA value of 4.0 was obtained for H1 rice plant #3 with three gene copies. Therefore, the number of transgenes in the genome of transgenic plants affects the amount of transcripts. These parameters influence the recombinant protein expression levels (Panahi, 2002).

Due to the constitutive nature of the CaMV d35S promoter, it was expected that all rice plant tissues (seeds and leaves) would accumulate the HBV core particle subunits. The Western blots are presented in Figures A22 and A23 for the leaf and seed extracts of three

plants. The HBcAg produced in the best H1 plant was estimated at 2.12% of the fresh rice seed. Following successive HBcAg extractions on the same rice seeds (H1 plant #3) a total of 4.04% HBcAg was collected after the fourth extract. Hence the recombinant protein in the seeds of plants is significantly higher than the percentage reported for that obtained from the first protein extract. In the case of the H1 rice plant #3, the total HBcAg collected after the fourth extraction was two-fold higher than that of the first extraction. However, the level observed in the rice leaves (1.44%) of the same rice plant #3 was 67% lower than the level observed in the rice seeds (2.12%). These results are somewhat similar to those obtained for the transgenic tobacco H4 plants where the seeds showed higher levels of recombinant proteins than the leaves of the same plants.

Rice plant #3 transformed with the H1 expression construct also showed self-assembled HBV core particles with diameters of 25 to 30 nm (Figure A24). These results indicated that the HBV core particles were assembled correctly in yet a third plant system and the virus-like particles did not show significant differences in size across plants. The results reported here showed great potential for the expression of edible vaccines in rice grains. Therefore, the trimmed HBV core particle subunits were functional for assembly in this rice system even as shown in the previous carrot and tobacco recombinant systems. Thus, the truncated HBV core particles were easily synthesized and assembled in carrot, rice and tobacco bioreactor systems and may serve as ideal candidates for future multivalent edible vaccine production.

Chapter 6

GENERAL DISCUSSION

Regulatory and targeting sequences

A summary of the experiments conducted is this thesis is presented in Table 9. The postulate on augmentation of the HBcAg in transgenic tobacco plants was that the HBcAg subunits would be observed in transgenic tobacco plants using the combined strong d35S promoter and the AMV RNA4 translational enhancer. This was indeed the case when the H1 plant line showed a 16.5 kDa band on Western blots. The quantitative data for the different transgenic tobacco lines are presented in Table 10. This is notwithstanding the fact that the C-terminal truncated form of the HBcAg subunit to the best of our knowledge, has not been shown prior to assemble in plant systems. Hence, these two components functioned effectively for the recombinant studies. Detection of the HBcAg subunits was further observed in transgenic carrot and rice plants and the quantitative data are presented in Tables 11 and 12. Further to our specific objectives, the CaMV d35S promoter was found to be active in both the leaves and seed/root tissues of transgenic tobacco, carrot and rice plants indicating the conservation of its activities in these expression systems. The tobacco analyses have shown that a plant signal sequence is more effective for augmented recombinant protein accumulation in plants when compared to the use of the AMV-RNA4 translational enhancer element fused to a plant codon-optimized coding sequence.

Table 9. Summary of the various expression constructs used in the tobacco, carrot and rice experiments

Host Plant	Vector	Promoter	TE	SP	CS	RS	NOS-TER	Expression Construct
Carrot	pRD 400	d35S	+	-	HBV	-	+	H1
		d35S	-	+	CO: H4	+	+	H4
Rice	pCAMBIA 1303	d35S	+	-	HBV	-	+	H1
		d35S	-	+	CO: H4	+	+	H4
Tobacco	pRD 400	d35S	+	-	HBV	-	+	H1
		d35S	+	-	CO: H2	+	+	H2
		d35S	+	-	CO: H3	-	+	H3
		d35S	-	+	CO: H4	+	+	H4

The table shows the various regulatory and HBcAg sequences that were used in the different experiments. The abbreviations used are TE: translational enhancer sequence from alfalfa mosaic virus RNA 4 gene; SP: a carrot extensin signal peptide; CS: protein coding sequence; RS: endoplasmic retention sequence (encoding KDEL); NOS-TER: nopaline synthase transcription termination and polyadenylation sequence; CO: plant codon optimized sequence based on the *Arabidopsis thaliana* amino acid codon preferences; d35S: cauliflower mosaic virus double 35S promoter. These experiments and their corresponding results are discussed in the text.

Table 10. Quantification of HBcAg subunits from transgenic tobacco plants

Transgenic Plant Line	Tissue Source	Highest Yield Observed (as a percentage of fresh weight)
H1	Tobacco Leaf	0.63%
H2	Tobacco Leaf	3.4%
H3	Tobacco Leaf	3.8%
H4	Tobacco Leaf	9.8%
H4	Tobacco Seed	20.0%

The table was prepared by quantifying via Western blots the amount of soluble HBcAg subunits in the protein extracts of H1-H4 transgenic plants. Known dilutions of prepared weighed HBcAg subunits from *E. coli* were loaded as standards and the various plant protein extracts were loaded on SDS-PAGE gels. The protein quantities were estimated from the standard curve or estimated via band intensities when the unknowns were outside the standard curve range. The leaf quantities are presented for tobacco plants following densitometry analyses. The data from both the leaf and seed of the H4 tobacco plant is also reported. The values are based on the highest quantity of HBcAg subunits found in each transgenic line of plant and indicate that the antigen levels were generally poorer in leaves but higher in seeds of tobacco. The results are further discussed in the text.

Table 11. Quantification of HBcAg subunits from edible transgenic plant extracts

Transgenic Plant Line	Tissue Source	Highest Yield Observed (as a percentage of fresh weight)
H1	Carrot Leaf	< 0.01%
H1	Carrot Root	0.45%
H4	Carrot Leaf	1.15%
H4	Carrot Root	1.73%
H1	Rice Leaf	1.44%
H1	Rice Seed	2.12%

The table was prepared by quantifying via Western blots the amount of soluble HBcAg subunits in the protein extracts of H1 and H4 transgenic plants. Known dilutions of prepared weighed HBcAg subunits from *E. coli* were loaded as standards and the various plant protein extracts were loaded on SDS-PAGE gels. The protein quantities were estimated from the standard curve or estimated via band intensities when the unknowns were outside the standard curve range. The leaf and root or seed quantities are presented for carrot and rice plants. The data for the H4 rice plants are not reported. The values are based on the highest quantity of HBcAg subunits found in each transgenic line of plant and indicate that the antigen levels were generally poorer in leaves but higher in rice seeds or carrot roots. The results are further discussed in the text.

Table 12. Quantitative data gathered on three plants of the H1 rice line

Plant I.D. from transgenic H1 rice line	#1	#3	#5
Percentage of soluble HBcAg in rice seed	ND	2.12%	1.23%
Percentage of soluble HBcAg in rice leaf	0.22%	1.44%	0.84%
Plant seed to leaf HBcAg ratio	ND	1.5	1.5
Abundance of mRNA transcripts (using plant #1 as base)	1	53	59

The table was prepared by quantifying via Western blots the amount of soluble HBcAg subunits in the protein extracts of leaves and seeds of transgenic H1 rice plants. Known dilutions of prepared weighed HBcAg subunits from *E. coli* were loaded as standards and the various plant protein extracts were loaded on SDS-PAGE gels. The protein quantities were estimated from the standard curve or estimated via band intensities when the unknowns were outside the standard curve range. The leaf and seed quantities are presented for rice plants #3 and #5. These values are based on the highest quantity of HBcAg subunits found in each transgenic plant and indicate that the antigen levels were generally poorer in leaves but higher in seeds of rice plants. The quantitative data for the H1 mRNA transcripts are also shown and is further discussed in the text.

Panahi *et al.* (2004) used the maize ubiquitin promoter for rapid testing of hIGF-I coding sequences that had different codon biases. Transgenic tobacco and rice plants both produced authentic rthIGF-I. The rthIGF-I level observed in rice plants was 4-fold higher than that of transgenic tobacco plants. Although transgene location within the host plant genome can affect recombinant protein levels, this 4-fold difference was significant (P = 0.001) and may be attributed to better functioning of the maize ubiquitin promoter in rice, which is also a monocot (Panahi *et al.,* 2004). In the present recombinant expression studies, the CaMV d35S promoter was of plant viral origin and may have a monocot or dicot preference. Any differential processing of the sequences (AMV-RNA4 translational enhancer, extensin signal, RSRKDEL) may be determined directly by considering each plant system. Thus, differences in the HBcAgs subunit concentrations were observed in the different tobacco lines which showed increases in the levels of recombinant HBcAgs from the H1 to the H4 lines.

Others have investigated monocot-derived promoters in the rapid test plant – tobacco (Leite *et al.,* 2000; Sardana *et al.,* 2002). In those experiments, the rice glutelin-1 and glutelin-3 monocot promoters were both found to be active in transgenic dicot plants. For example, Sardana *et al.* (2002) reported the use of the above mentioned rice derived promoters in tobacco that resulted in up to 0.03 % rthGM-CSF (TSP) in tobacco seeds. However, when this same expression construct was used to generate a transgenic rice line, there was up to 1.3 % rthGM-CSF (TSP) found in the seeds of transgenic rice plants (Sardana *et al.*, 2004). This data indicated that the glutelin promoter was highly active in rice endosperm cells and showed a 43-fold preference for its monocot host than that of tobacco. Therefore, plant promoters are more likely to be monocot or dicot specific than promoters of viral origins. Further, the 4-fold difference in expression level (p = 0.001)

reported by Panahi (2002) might be due to the "native host phenomena" of the maize ubiquitin promoter in rice as opposed to the dicot (tobacco) plant (Panahi *et al.,* 2003, 2004). Further, transacting factors that bind to enhancer elements located on the maize ubiquitin promoter that are present in the "native" rice plant may be absent or have reduced binding affinity for the same promoter in tobacco. The same arguments can be used to explain the increased in yield of the transgenic rthGM-CSF yields in rice seeds.

Tissue-specific distribution of the recombinant HBcAgs

Specific plant tissue such as leaves, seeds or roots may show differences in the distribution of recombinant proteins. For example, an approximate two-fold difference was found in H4 tobacco plants when the seed to leaf ratios were compared (Tables 7 and 10). For the best H1 rice plant and the best H4 carrot plant, the leaves produced lower levels of recombinant proteins than the seeds or roots Tables 11 and 12. These tissue-specific differences will be useful for the production of edible vaccines in carrot roots and rice seeds. Thus plant codon-optimization of the coding sequence, presence of the extensin signal sequence and KDEL encoded sequence lead to the high expression of the HBcAg subunits in H4 tobacco and carrot plants. The major contributing factor appeared to be the presence of the extensin signal sequence in the H4 recombinant expression construct.

Immunological considerations

These studies reported here were first conducted in tobacco and the plant-derived HBcAg was effective in stimulating a systemic immune response in subcutaneously immunized mice. Hence, to advance the notion of an edible vaccine platform, these experiments were extended in edible plants and will be important for assessment of the oral immunization potential of plant-derived antigens. Edible vaccines delivered in packages of

plant tissues should be effective orally as was shown for other antigens produced in transgenic plants (Kong *et al.*, 2001; Lauterslager *et al.*, 2001).

In any case, the development of vaccines needs detailed analyses to determine the best route of administration, the immunizing dose and schedule as well as whether or not adjuvants will be needed. These considerations will become more important as antigenic peptides from mucosal (e.g. HIV-1or hepatitis A) or blood borne pathogens (e.g. malaria or hepatitis B) are inserted into the HBcAg immunodominant loop. For example, if the HIV-1 glycoprotein 120 is inserted into this loop, the best route of administration of this vaccine is predicted to be the oral/nasal route. In this case, a cytokine such as GM-CSF may not serve as a useful adjuvant (Sykes *et al.*, 2002). Indeed the administration of GM-CSF along with a simian immunodeficiency virus (SIV) vaccine resulted in decreased survival rates in SIV challenged monkeys when compared to the group that did not get the GM-CSF adjuvant. Perhaps the experiment was flawed since the genetically live vaccine was subcutaneously administered via particle gun bombardment while SIV or HIV-1 has a usual mucosal route of infection. Thus, if a component of the HIV-1 genome is inserted into our own HBcAg expression vector, a mucosal adjuvant may also need to be encoded by another expression vector. This may be accomplished by the expression of multiple expression constructs in a single transgenic plant (Goderis *et al.*, 2002; Wu *et al.*, 2002b). It has been shown that use of the mucosal adjuvant cholera toxin was effective in stimulating high serum IgG titres that were specific for a plant derived measles virus vaccine in mice (Webster *et al.*, 2002). The measles virus vaccine (50 μg DNA) was delivered on day zero and was followed by boosting by gavage on days 90, 97, 104 and 111 with a plant derived measles virus vaccine (1 g) delivered along with 10 μg of cholera toxin. The cholera toxin is known to bind to M-cells of Peyers patch located on mucosal surfaces, as well as, the GM_1-ganglioside

receptors of epithelial cells. For oral immunizations, when fresh plant tissues are used, the dosage could range from 10-50 μg of the viral antigen and cooking of transgenic potatoes that express the HBsAg resulted in decreased immunogenicity (Kong *et al.,* 2001).

However, bioencapsulation of antigens within plant cells may protect them from complete digestion and allow some antigens to pass through the stomach into the small intestines for processing by the mucosal immune system (MIS). Uptake of antigens arriving at the MIS (digestive tract, respiratory tract, and urogenital tract) will be processed by M-cells and/or dendritic cells for presentation to cells of the immune system (Gewirtz and Madara, 2001; Ogra *et al.*, 2001). Thus, the activated B-cells can travel to the mesenteric lymph nodes for maturation and return back to secrete IgA. The secreted IgAs across the mucosal epithelial should provide protection at the pathogen entry site when the pathogen is encountered. Thus, this process of oral immunity was shown to be active in many studies where, not only did a mucosal immune response result, but also a systemic response that involved the production of serum IgG antibodies (Mason *et al.,* 1996; Brennan *et al.*, 1999; Webster *et al.,* 2002). The mucosal vaccine dose is important since with high dose, tolerance may develop. Immunopathology results when tolerance is not maintained. One possibility for mucosal tolerance involves clonal deletion of antigen sensitized lymphocytes. Low doses of vaccine require one or more mucosal adjuvants to allow for a strong immune response. It has been observed that the cholera holotoxin and *E. coli* heat-labile toxin blocked tolerance when administered with an antigen (Bagley *et al.*, 2003). Hence, the adjuvant properties of the cholera and *E. coli* heat-labile toxins may become more important as edible plant derived vaccines are developed. Since carrots are consumed fresh, development of a carrot edible vaccination system may show more success than other edible plant systems that require prior cooking before administration.

Each of the plant expression systems studied in this thesis has its own merits and demerits for oral vaccination studies.

To further expand the horizon of edible vaccine production and to specifically target any vaccine into edible rice tissue, the glutelin promoter and its associated signal sequence may be used. The spatial and temporal regulation of the glutelin multigene family has been investigated (Takaiwa *et al.,* 1989, 1991, 1996; Leisy *et al.,* 1990; Zhao *et al.,* 1994; reviewed by Okita, 1996). The glutelin-1 (Gt-1) promoter is for seed-specific expression (Zheng *et al.,* 1995) while the Gt-1 signal sequence is for targeting of recombinant protein into seeds (Wright *et al.,* 2001) and specifically in the seed storage vacuoles of rice (Okita *et al.,* 1989). For example, when the Gt-1 promoter was fused to its associated signal sequence and used to drive the expression of rthGM-CSF in tobacco endosperm cells, a specific seed tissue accumulation pattern emerged. This pattern reflected a well-coordinated process during recombinant protein deposition into seed storage vesicles. Therefore, an additional HBV core particle expression study is underway that will use the Gt-1 promoter and its associated signal sequence for high level HBcAg expression in rice endosperm cells. Thus, targeting this HBV core particle subunit and other recombinant proteins specifically in rice seeds is expected to result in: (1) higher yields of the antigens/proteins; (2) coordinated synthesis in a predictable spatial and temporal mode; (3) strict targeted expression only in rice seeds will result in efficient use of the plant's valuable resources. For example, waste occurs when a ubiquitous promoter is used to generate plant bioreactors that results in non-targeted expression in every plant tissue (e.g., CaMV d35S promoter or maize ubiquitin promoter).

Conclusion

In this research, the expression of the HBcAg was studied in the tobacco, carrot and rice systems using the well-characterized CaMV d35S promoter and either the AMV-RNA4 translational enhancer element or the extensin signal sequence. The experimental results suggest that the HBcAg can be made to assemble in all three plant systems regardless of the fact that the subunits were trimmed to only the first 153 amino acids. Further the tobacco analyses have shown that a plant signal sequence is more effective for augmented recombinant protein accumulation in plants when compared to the use of the AMV-RNA4 translational enhancer element fused to a plant codon-optimized coding sequence. Our data also indicated that the plant system processed the HBcAgs in a manner that resulted in an equivalent immunological response in mice as the *E. coli* derived HBcAgs. Therefore, it is feasible to produce authentic candidate vaccines as well as other medical and industrial proteins in plants. This work has important implications for the use of edible plants as future bioreactors and for the delivery of oral antigens.

Future directions

In the future, additional medically important proteins will be expressed and tested in edible plant tissues. The long history of rice research in Dr. Altosaar's laboratory, since 1978, where we have successfully incorporated the genetic and molecular biology advances of Canadian cereal grains into modern biotechnological approaches for global rice improvement, shows that the rice endosperm flour or kernel is an ideal platform for introducing agronomic, nutritional and medical impacts into the human population. Further fine-tuning of the promoter and signal sequences to that of the glutelin gene family will result in seed specific expression in rice and tobacco plants. It is important that other analyses on the plant-produced HBV core particles be conducted (oral immunization and N and C terminal sequencing) to determine the full structural and functional nature of the

recombinant protein. Future studies will insert HIV-1, HBsAg, and other viral and bacterial antigenic peptides into the major immunodominant c/e1 epitope site as well as the N and C terminals of HBcAg subunit for multivalent vaccinations. This study has important implications for augmented recombinant protein production in plants. The extension of this study to produce other therapeutically useful cytokines, vaccine antigens and therapeutic antibodies in plants using different targeting sequences: promoters, signal sequences and ER retention sequences will add to the growing literature on recombinant protein expression. Considering the results of Alli (2001), one question that remains to be answered is whether a syllogistic peptide or a plant signal peptide may serve the same purpose of stabilizing protein accumulation in plant tissues.

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CLAIMS TO ORIGINALITY

1. This is the first report to use the truncated HBcAg subunit (N-terminal 153 amino acids) to produce transgenic tobacco, carrot and rice plants where authentic assembly into virus-like particles was shown.

2. This is the first report of the expression of the candidate HBV core antigen carrier specifically in carrot and rice plants.

3. A novel DNA sequence of the truncated HBcAg subunit incorporating a unique *Hinc* II endonuclease site has been produced for insertion of sequences from viral or bacterial origin.

4. This is the first report of the use of the *Arabidopsis thaliana* codon bias, extensin signal sequence, and the encoded RSRKDEL peptide to achieve augmented expression of the truncated HBcAg in tobacco plants.

PUBLICATIONS

Alli, Z., Falazarno, D., Sardana, R.K., Andonov, A., Robert, L.S., Schernthaner, J.P., Booth, T. and Altosaar, I. Harvesting of recombinant proteins from carrots. In Preparation.

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Appendix 1: Experiments conducted by collaborators

In this section, all experiment conducted by Dr. Anton Andonov, Lynne Burton, Timothy Booth, Tim Booth and Darryl Falzarano are described. These experiments were conducted at Health Canada using the protein extracts of transgenic tobacco, carrot and rice plants sent by Zaman Alli from the University of Ottawa.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), protein transfer and Western blotting were conducted as previously described (Falzarano *et al.*, 2003, 2004).

HBV core particle specific ELISA and electron microscopy

These experiments were conducted as previously described (Falzarano *et al.*, 2003, 2004). For electron microscopy, the viral particles from the H1 transgenic plants (tobacco, carrot and rice) were purified by applying the protein extracts to discontinuous sucrose density gradients (Mason *et al.*, 1996). The purified fractions were applied to grids and negatively stained in uranil acetate for electron microscopy as previously described by Gelderblom *et al.*, 1967. The supernatants of the protein extracts from the H2-H4 plants were used directly without purification for electron microscopy.

Appendix 2: Experimental results (Figures) generated by collaborators

In this section, all results (Figures) generated by Dr. Anton Andonov, Lynne Burton, Timothy Booth, Tim Booth and Darryl Falzarano are shown. The Figures in this Appendix 2 all have an "A" before the actual numbers to clearly identify them as belonging to this Appendix. These Figures were generated using the protein extracts of transgenic tobacco, carrot and rice plants prepared by Zaman Alli and sent to Health Canada for analyses.

Figures A1-A4. Bar graphs of the ELISA values obtained for the HBV core particles derived from H1-H4 transgenic tobacco plants. Transgenic plants were found to carry the H1-H4 expression constructs via Southern blot analyses. The leaf protein extracts were assessed for the presence of the HBcAg via ELISA using a monoclonal antibody directed against a conformational epitope of the HBV core particle. The plants assessed are from the H1-H4 lines as well as non-transformed plants (NT).

Figure A1. ELISA values obtained from the H1 tobacco leaf extracts

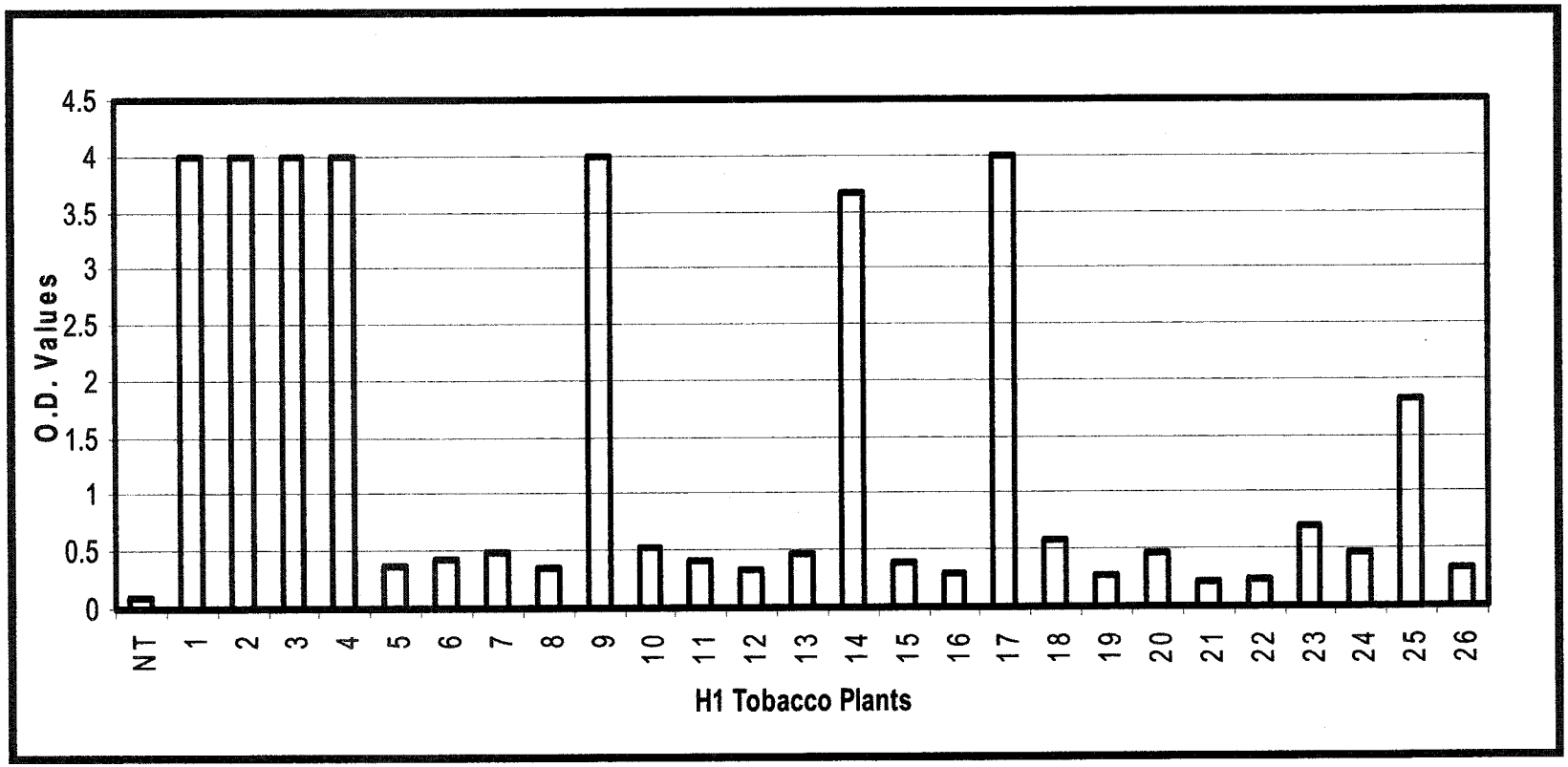


Figure A2. ELISA values obtained from the H2 tobacco leaf extracts

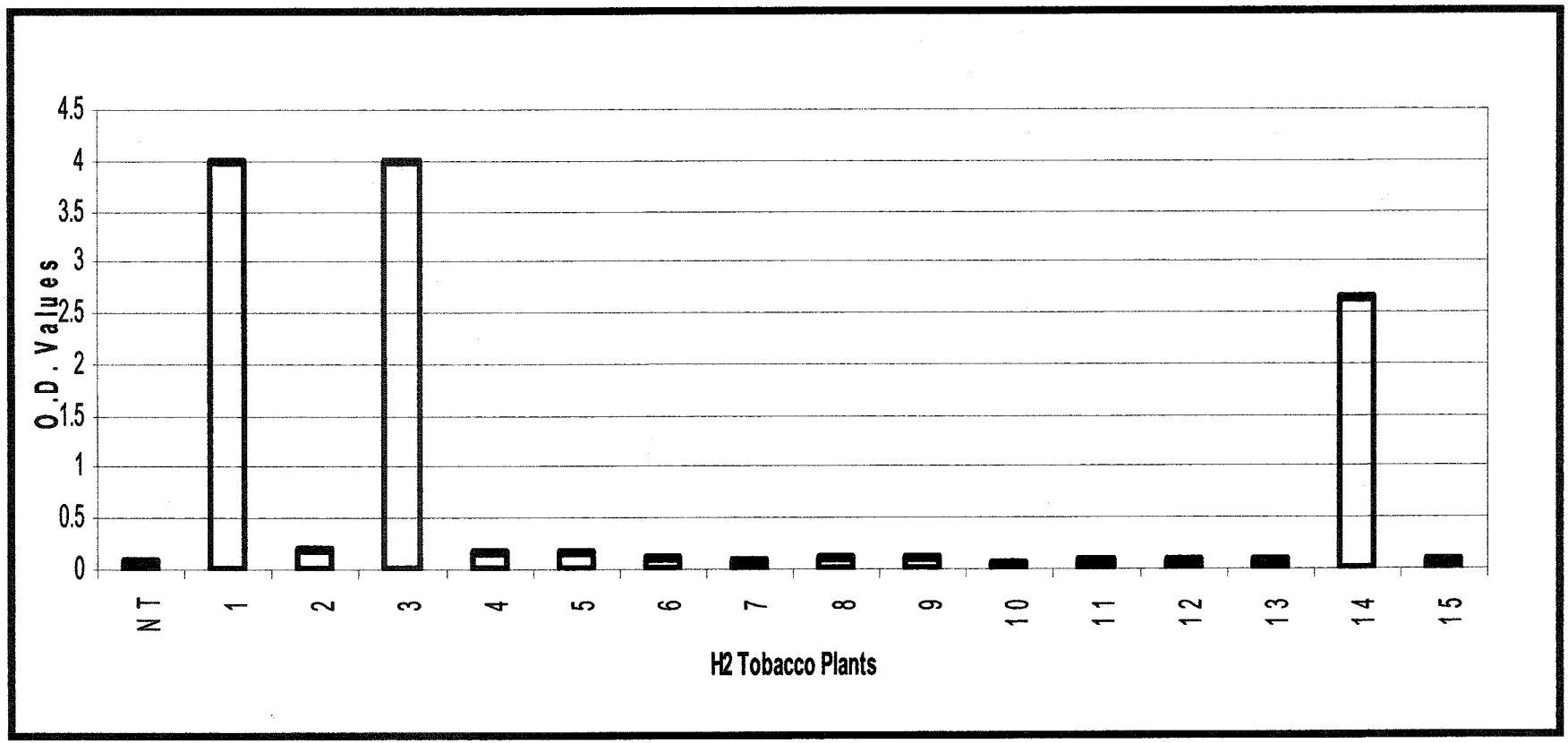


Figure A3. ELISA values obtained from the H3 tobacco leaf extracts

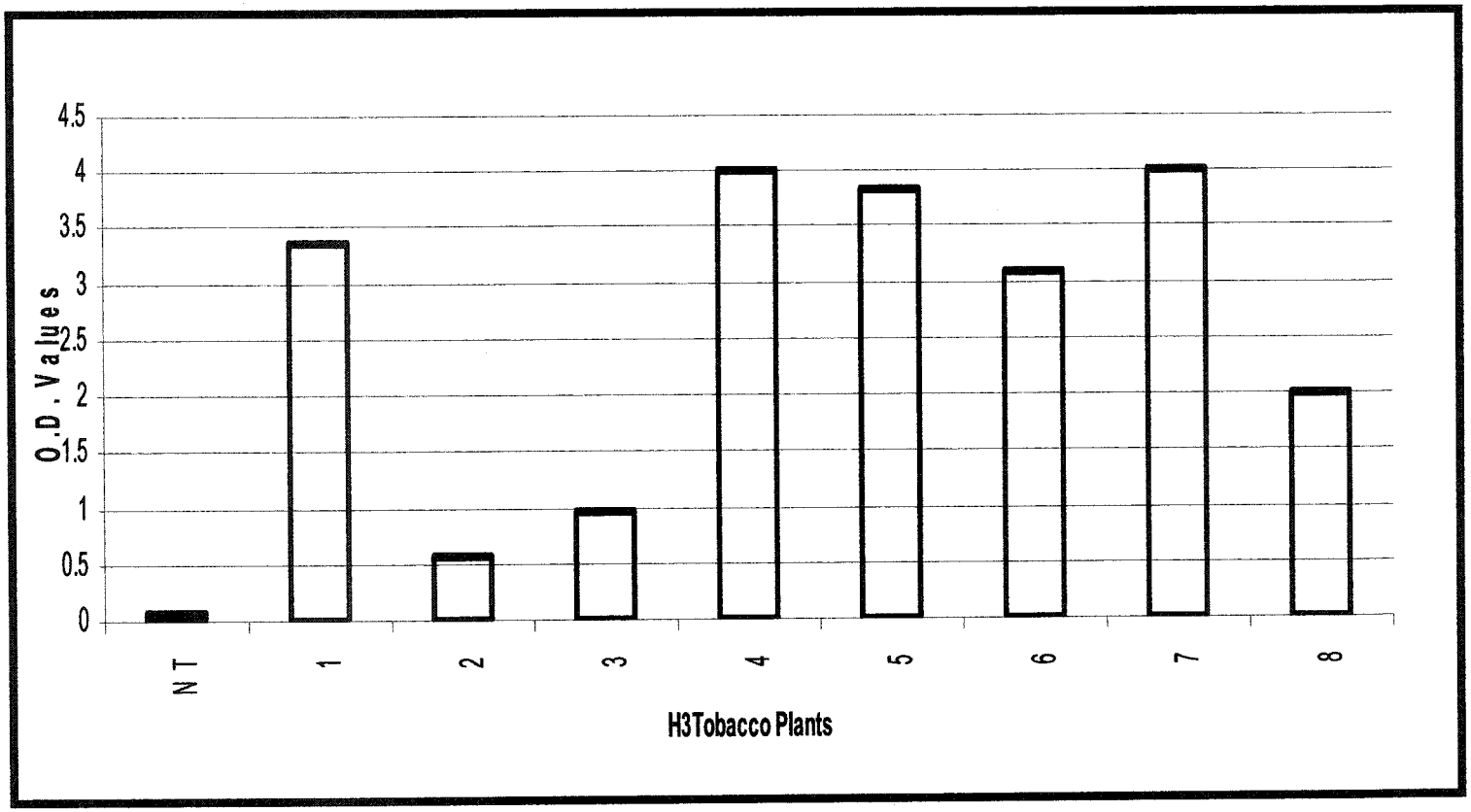


Figure A4. ELISA values obtained from the H4 tobacco leaf extracts

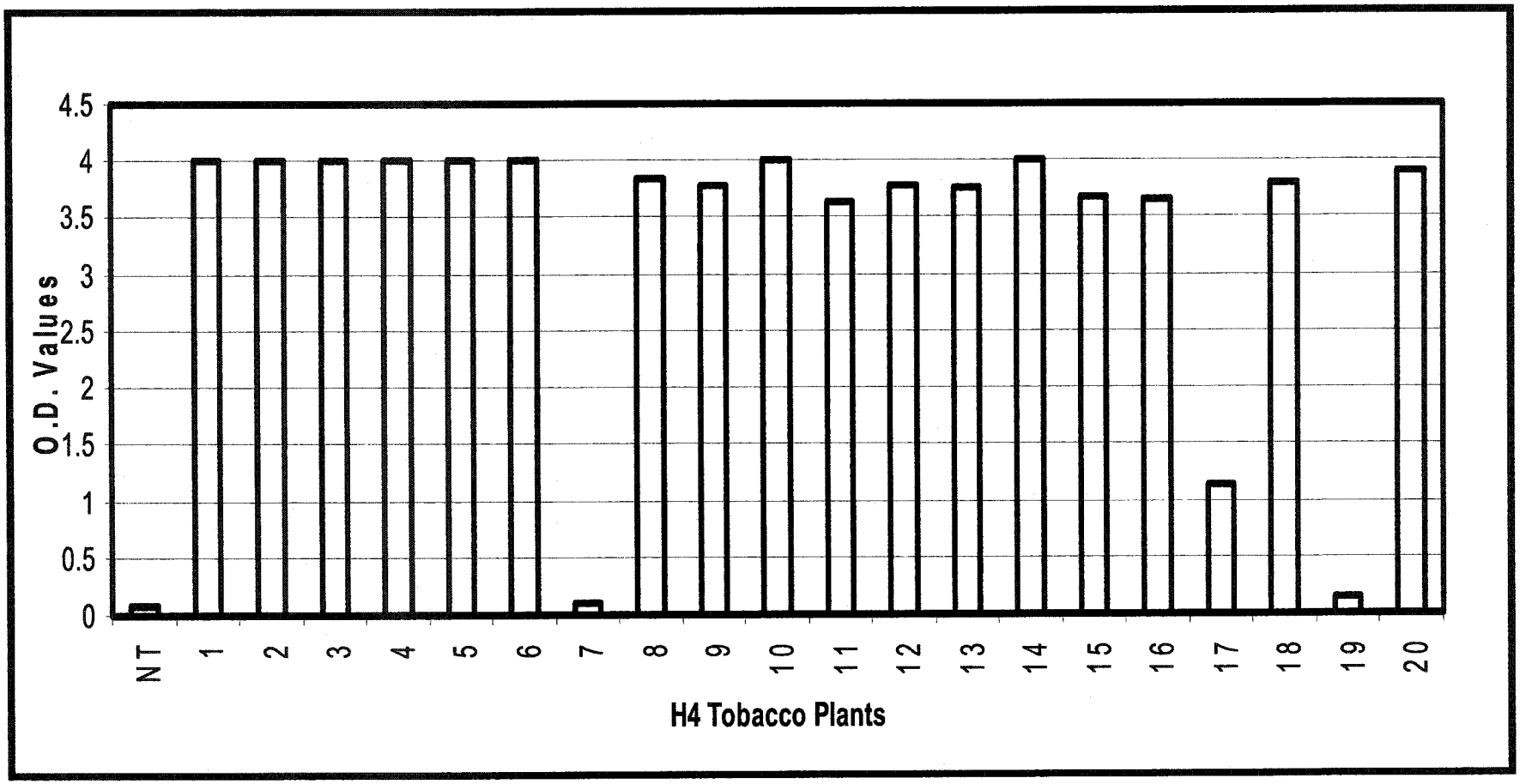


Figure A5. Immunoblot detection of the HBV core particle subunits in H1 tobacco leaf extracts. Each lane contains 10 μL each of total leaf protein extract from H1 transgenic tobacco plants to detect the HBV antigen. The primary antibody used was a polyclonal antibody derived from a patient with chronic hepatitis B. Lanes M: molecular weight protein standards; Lane +: positive control derived from *E. coli*; Lanes 1, 2 and 3: 10 μL each of total leaf protein extract from H1 transgenic tobacco plants #1-3; Lane NT: 10 μL total leaf protein extract from an NT tobacco plant.

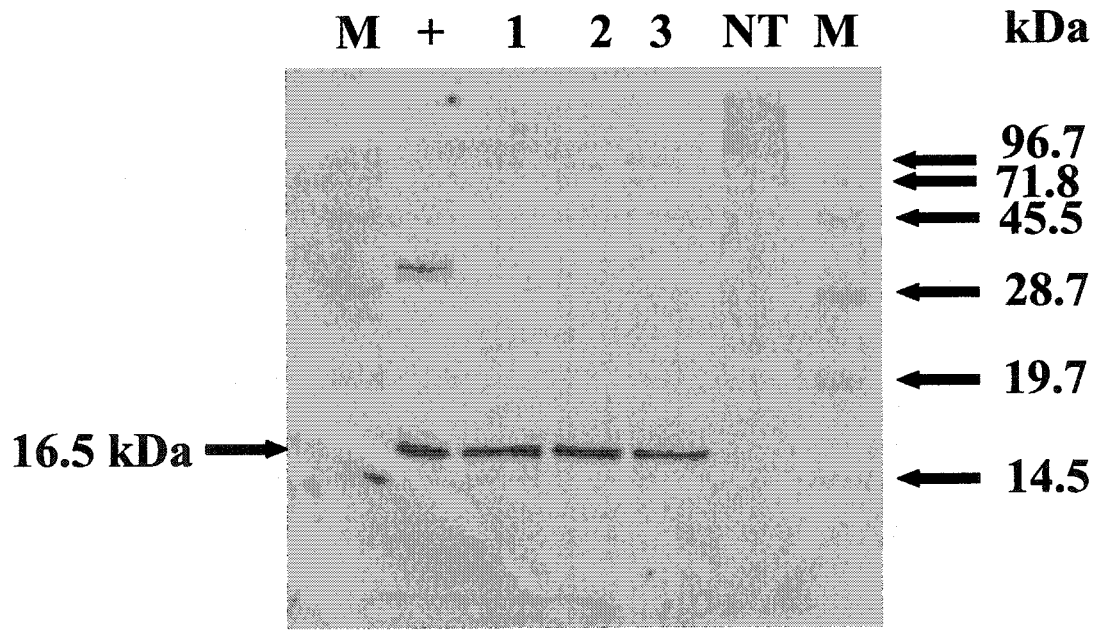


Figure A5

Figure A6. Immunoblot detection of the HBV core particle subunits in H2 tobacco leaf leaf protein extract from an NT tobacco plant. The primary antibody used was a polyclonal antibody derived from a patient with chronic hepatitis B.

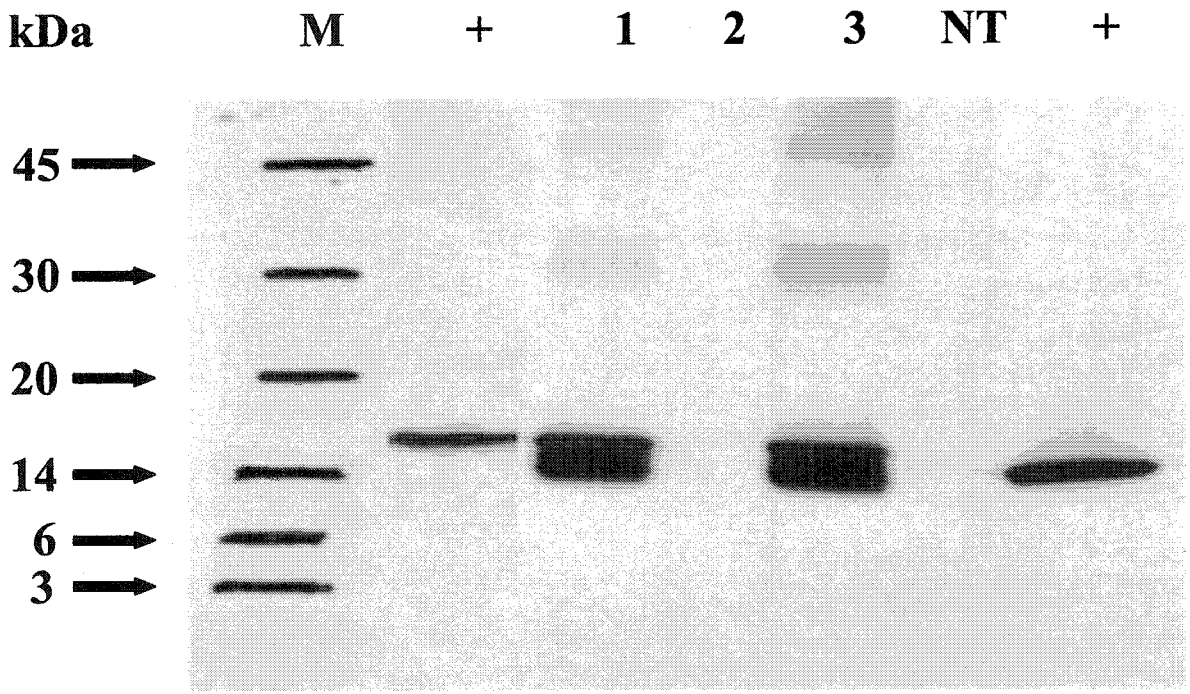


Figure A6

Figure A7. Immunoblot detection of HBV core particle subunits in H3 tobacco leaf extracts. Lane M: ECL plus molecular weight protein standards; Lanes +: positive control derived from *E. coli* and used as standards to quantify HBV core particle subunits, quantity loaded 500 ng, 250 ng, 125 ng, 62.5 ng and 31.3 ng respectively from left to right; Lanes 1-5 represent 10 µL each of total leaf protein extract from five H3 transgenic tobacco plants. The total leaf protein extract from an NT tobacco plant did not show any bands.

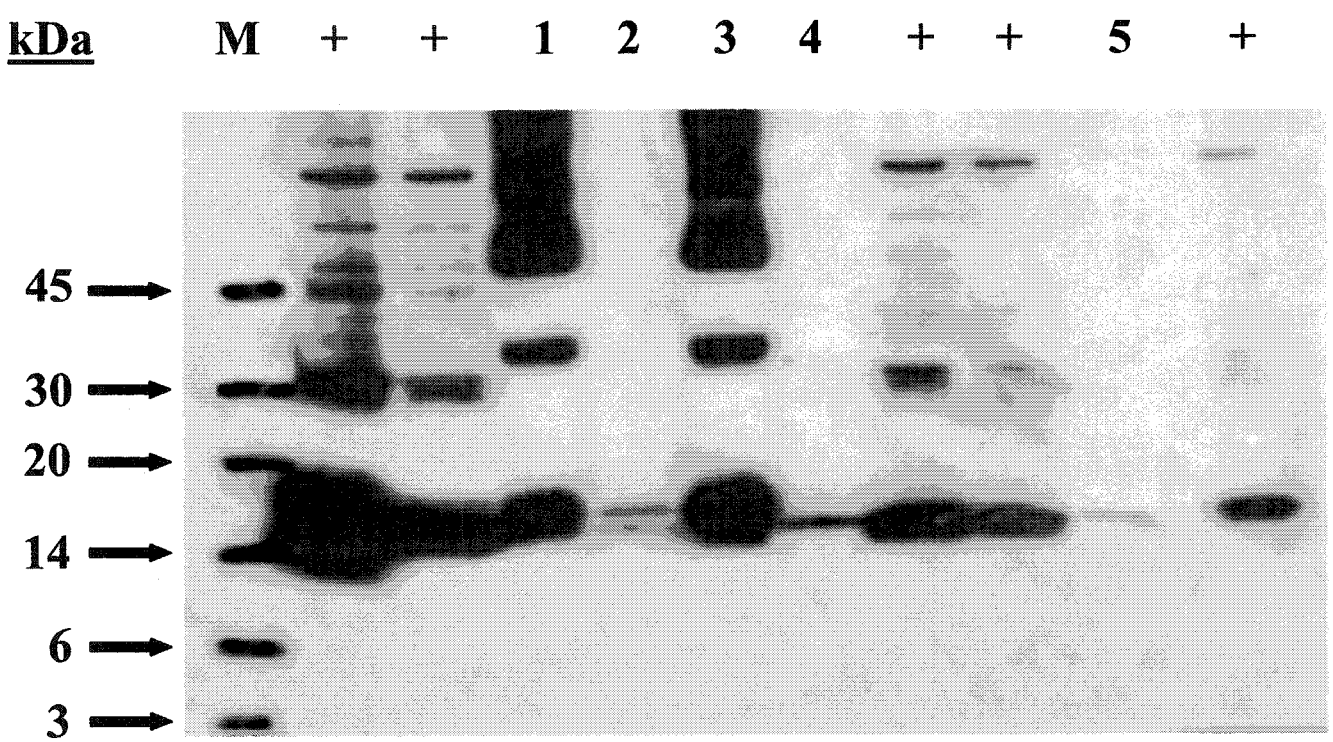


Figure A7

Figure A8. Immunoblot detection of HBV core particle subunits in H4 tobacco leaf extracts. Lane M: molecular weight protein standards; Lanes +: positive control derived from *E. coli* and used as standards to quantify HBV core particle subunits, quantity loaded, 40 ng and 400 ng respectively from left to right; Lanes 2-10 represent 10 μL each of total leaf protein extract from H4 transgenic tobacco plants #2- #10. The total leaf protein extract from an NT tobacco plant did not show any bands.

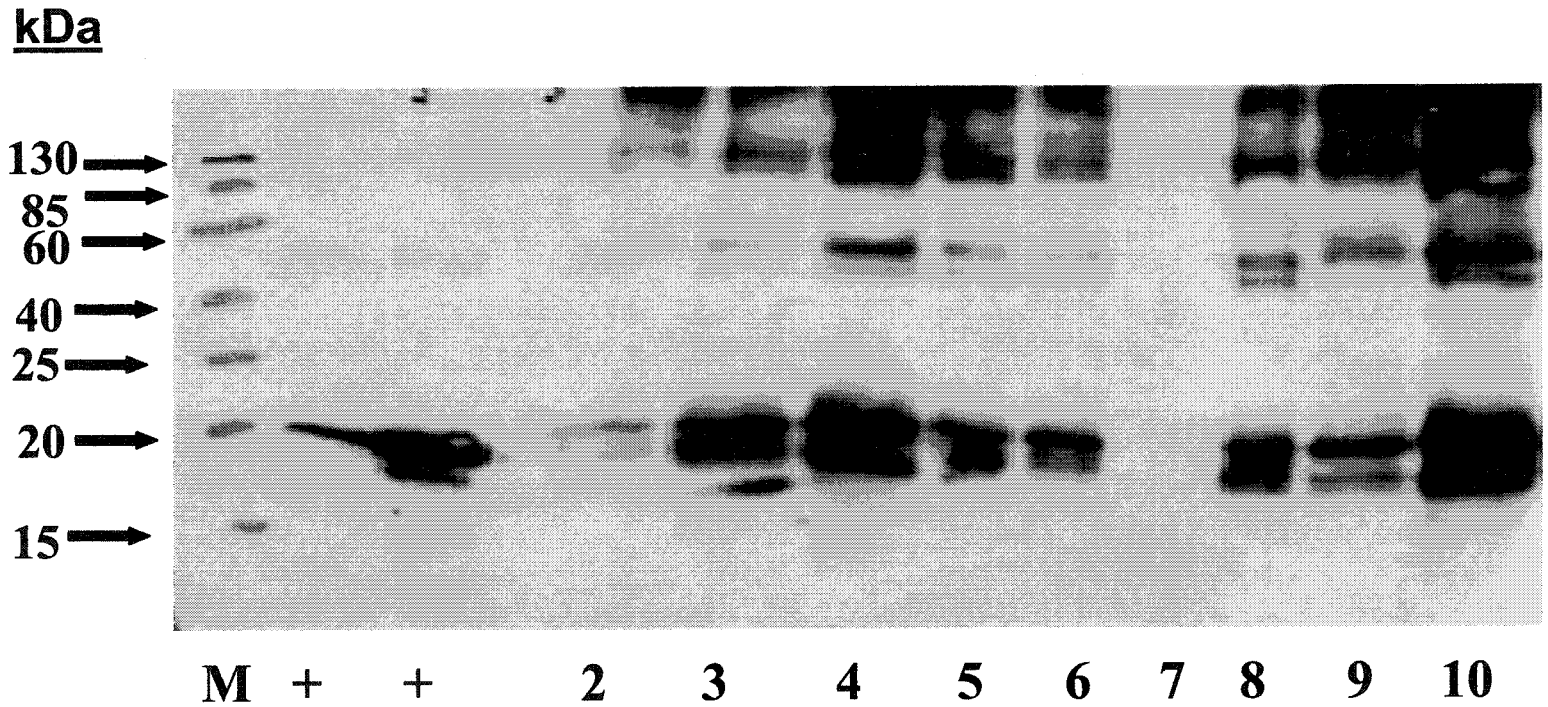


Figure A8

Figure A9. Immunoblot quantification of HBV core particle subunits in H4 tobacco seed extracts. Lane M: ECL plus molecular weight protein standards; Lanes +: positive control derived from *E. coli* and used as standards to quantify HBV core particle subunits, quantity loaded 200ng, 100ng, 50ng, and 25ng respectively; Lanes 2 and 3: ½ and 1 uL each of total seed protein extract from H4 transgenic tobacco plant #4; Lanes 4, 5, and 6: 1, 2, and 5 uL each of total seed protein extract from H4 transgenic tobacco plant #5; Lanes 8 and 9: ½ and 1 uL each of total seed protein extract from H4 transgenic tobacco plant #10. The total leaf protein extract from an NT tobacco plant did not show any bands.

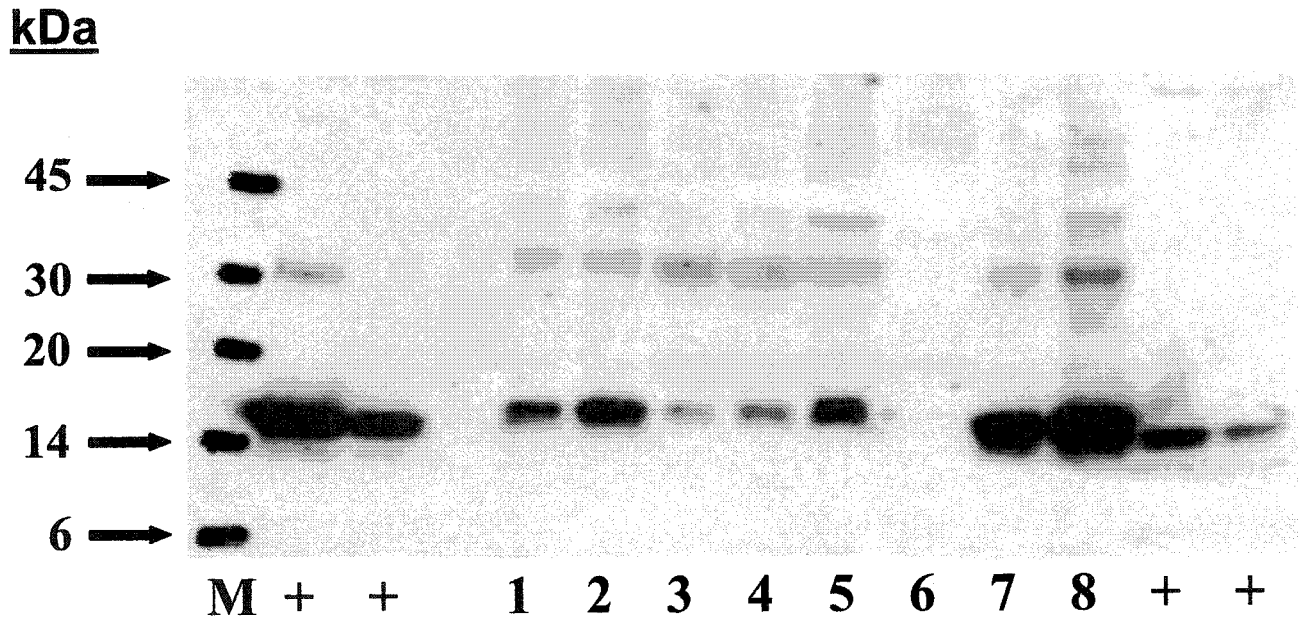


Figure A9

Figures A10-A13. Electron micrographs of assembled HBV core particles from four different transgenic plant lines H1-H4 respectively. Total protein extracts from H1 tobacco plants were purified via sucrose density gradient ultracentrifugation for electron microscopy. The crude protein extracts from H2-H4 plants were used without purification in electron microscopy using negatively stained grids. The assembled HBV core particles detected by electron microscopy varied in size between 25-30 nm in diameter.

H1

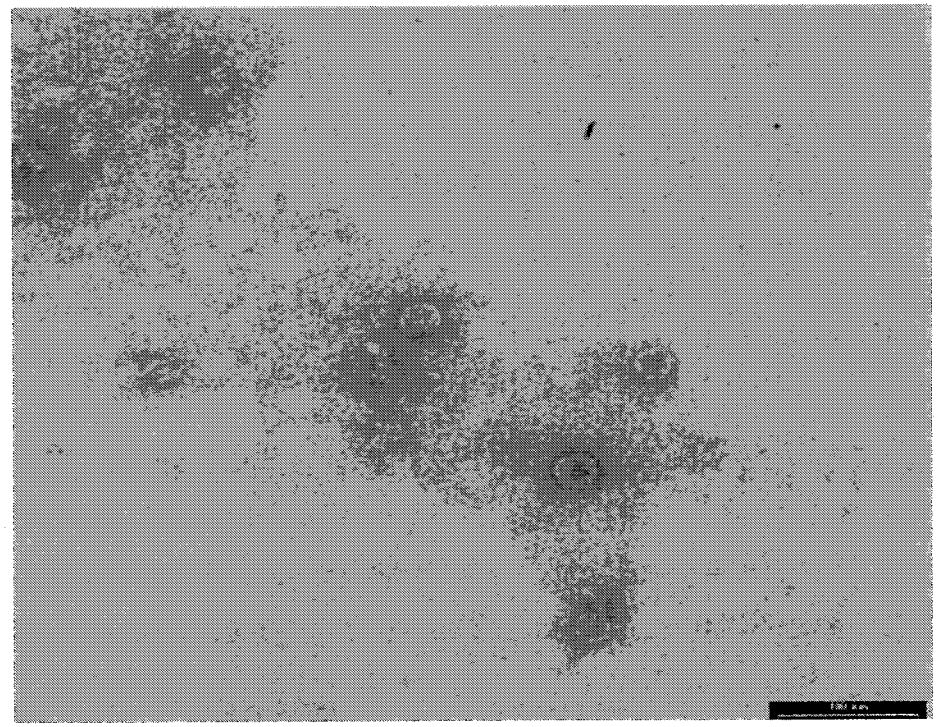
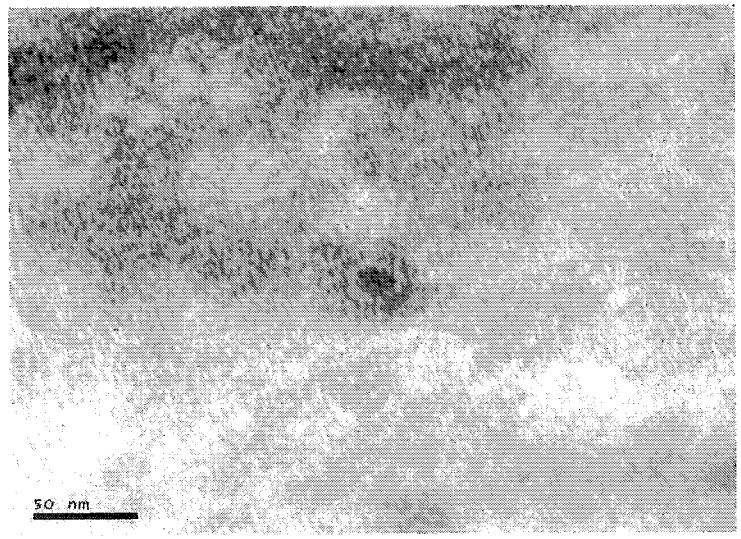


Figure A10

H2



50 nm

Figure A11

H3

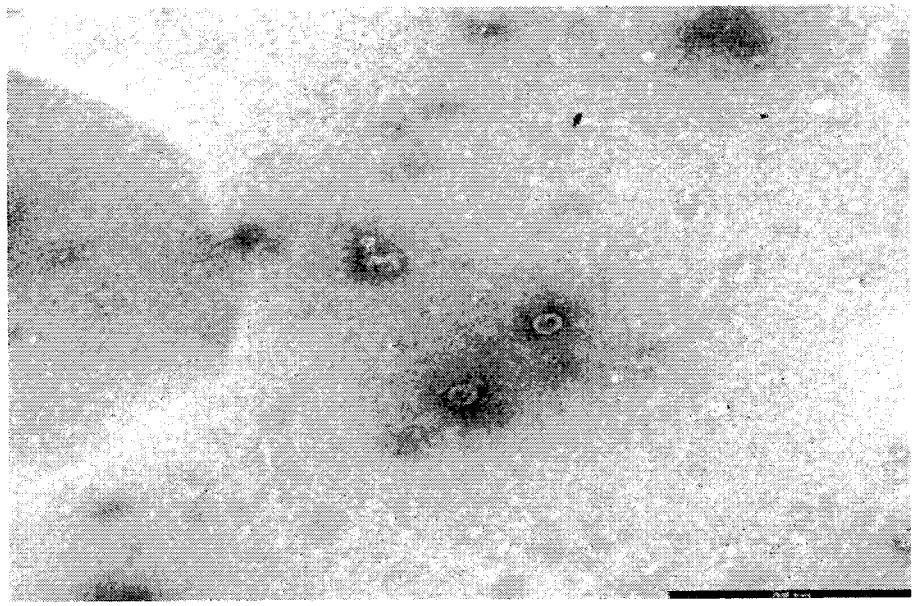


Figure A12

H4

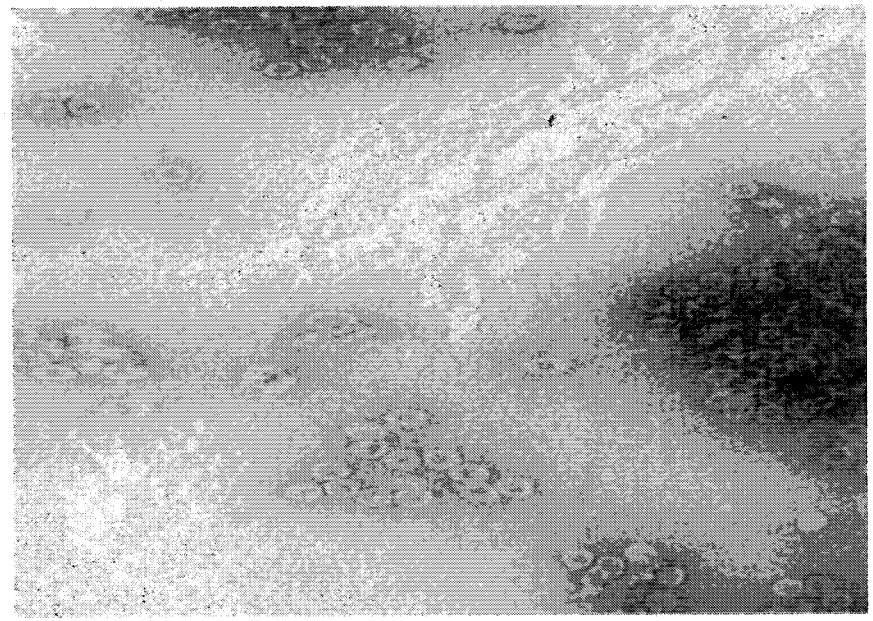


Figure A13

Figure A14. Silver stained SDS-PAGE gel quantification of the HBV core particles purified by sucrose density gradient ultracentrifugation. The crude protein extract from H4 transgenic tobacco plant #10 was purified to homogeneity and protein concentration was determined using known protein standards. Lane 1: molecular weight protein standards; Lane 2: 100 ng of the HBV core particle derived from *E. coli* as positive control; Lane 3: 3 μL of the SDS-PAGE denatured HBV core particle subunits purified by sucrose density gradient ultracentrifugation; Lanes 4-6: lysozyme protein used as a standard and loaded in increasing concentration of 1, 5, and 10 μg respectively.

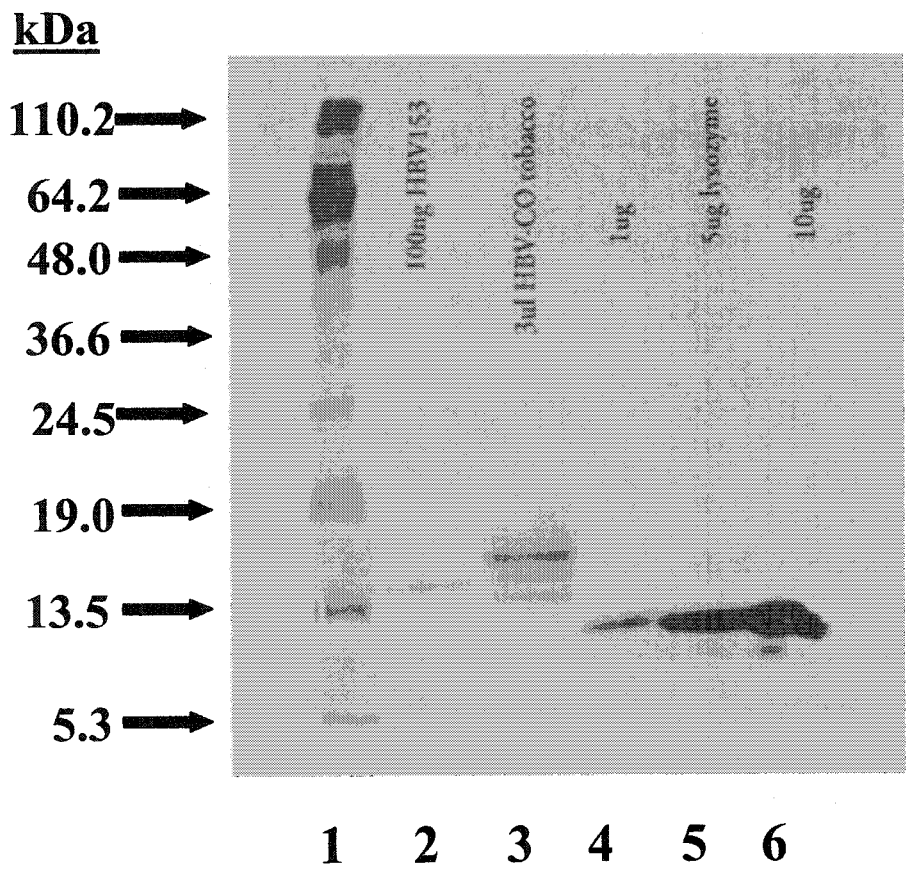

kDa
110.2
64.2
48.0
36.6
24.5
19.0
13.5
5.3
100ng HBV153
3ul HBV-CO tobacco
1ug
5ug lysozyme
10ug
1 2 3 4 5 6

Figure A14

Figure A15. A bar graph of the ELISA values obtained for the HBV core particles derived from H1 transgenic carrot roots. Transgenic plants were found to carry the H1 wild-type expression construct as assessed via Southern blots and further analyses were conducted on their root protein extracts for the presence of the HBcAg via ELISA. The monoclonal antibody used in the ELISA was directed against a conformational epitope of the HBV core particle. The plants assessed are H1 numbers 1-17 and a non-transformed plant (NT).

Figure A15. ELISA analyses on H1 carrot root tissues

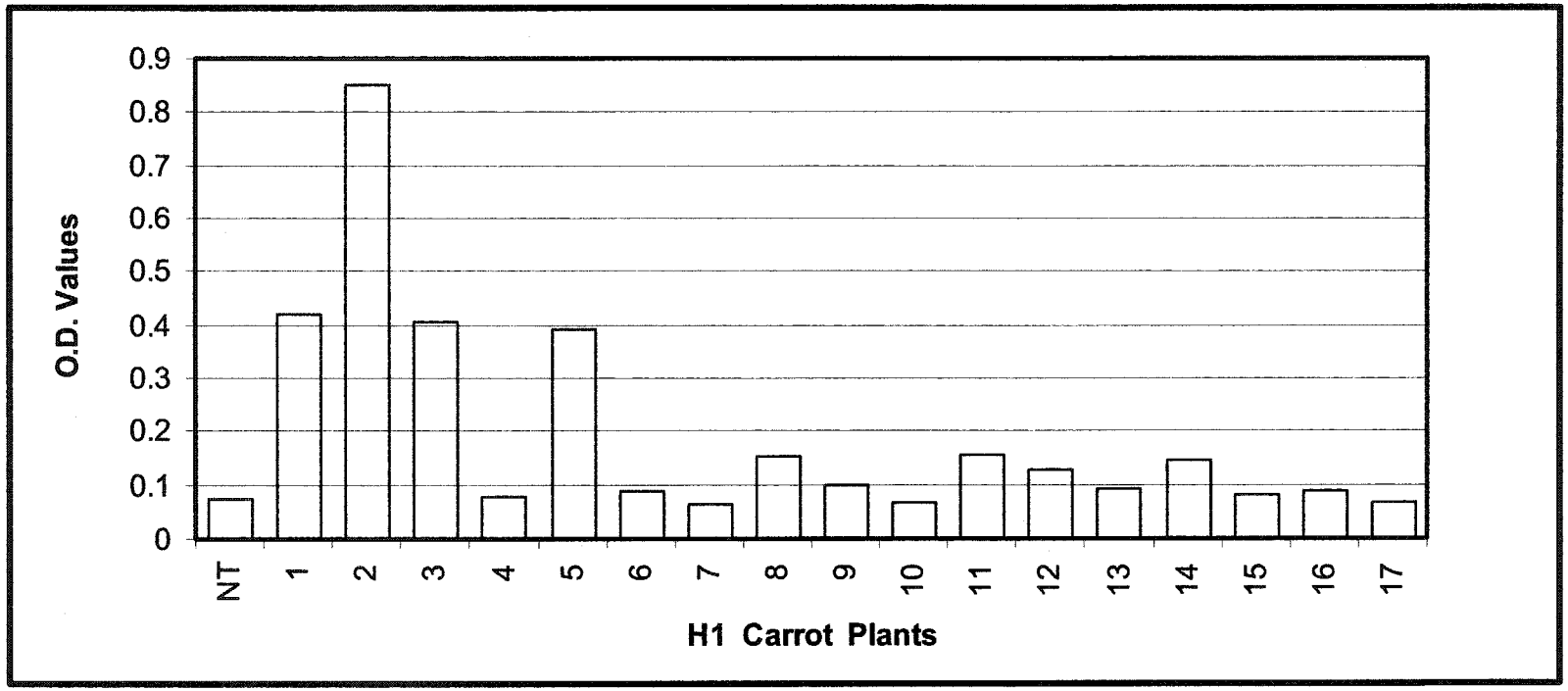


Figure A16. A bar graph of the ELISA values obtained for the HBV core particles derived from H4 transgenic carrot roots. Transgenic plants were found to carry the H4 expression construct as assessed via Southern blots and further analyses were conducted on their root protein extracts for the presence of the HBcAg via ELISA. The monoclonal antibody used in the ELISA was directed against a conformational epitope of the HBV core particle. The plants assessed are from the H4 transgenic line and a non-transformed plant (NT).

Figure A16. ELISA analyses on H4 carrot root tissues

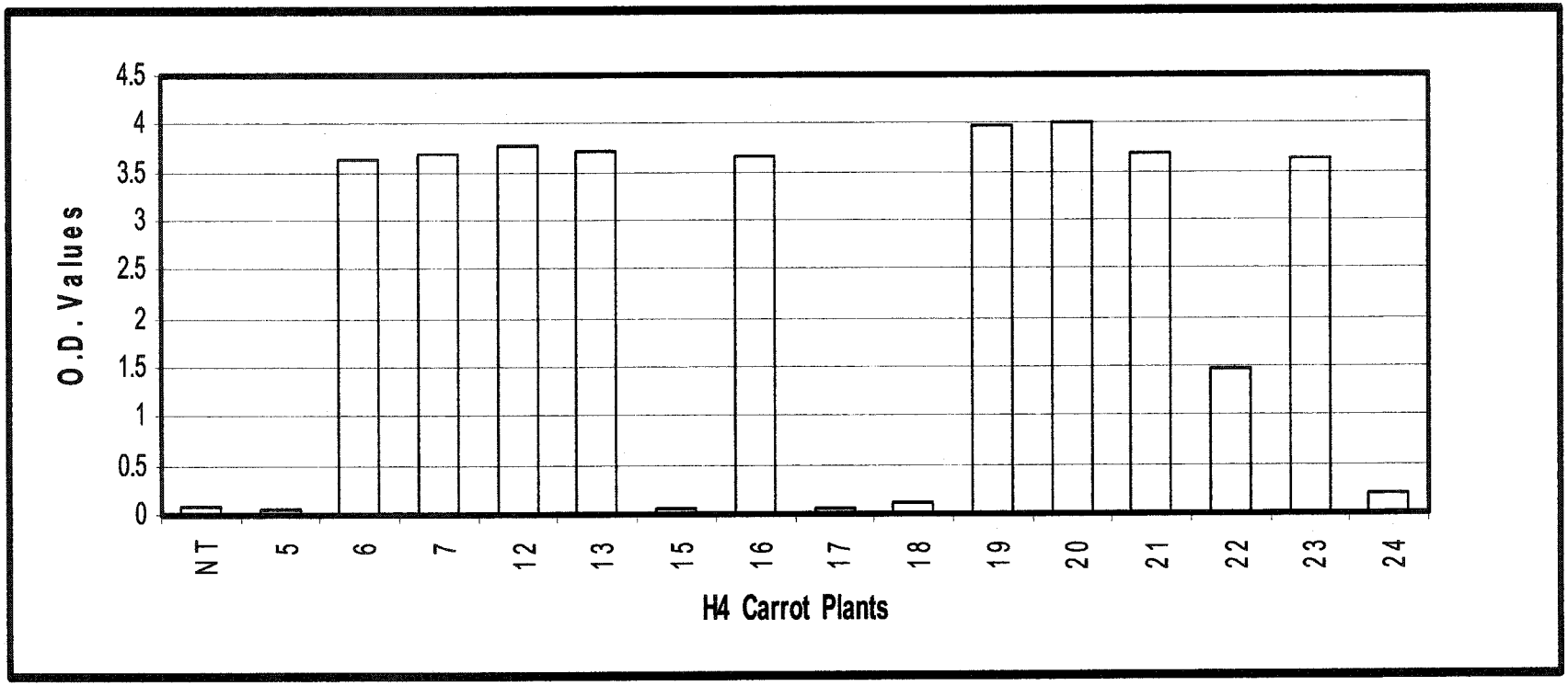


Figure A17. Immunoblot detection of the HBV core particle subunit in transgenic H1 carrot roots. Sample lanes M represent the molecular weight protein standards; lane NT represents protein extracts derived from a non-transformed carrot plant root; lanes 1-15 represent protein extracts derived from the roots of transformed carrot plants. All transgenic carrot root extracts showed a 16.5 kDa band on Western blots but the extracts from an NT carrot plant and plant #13 did not show any bands. Panels A and B are part of the same experiment.

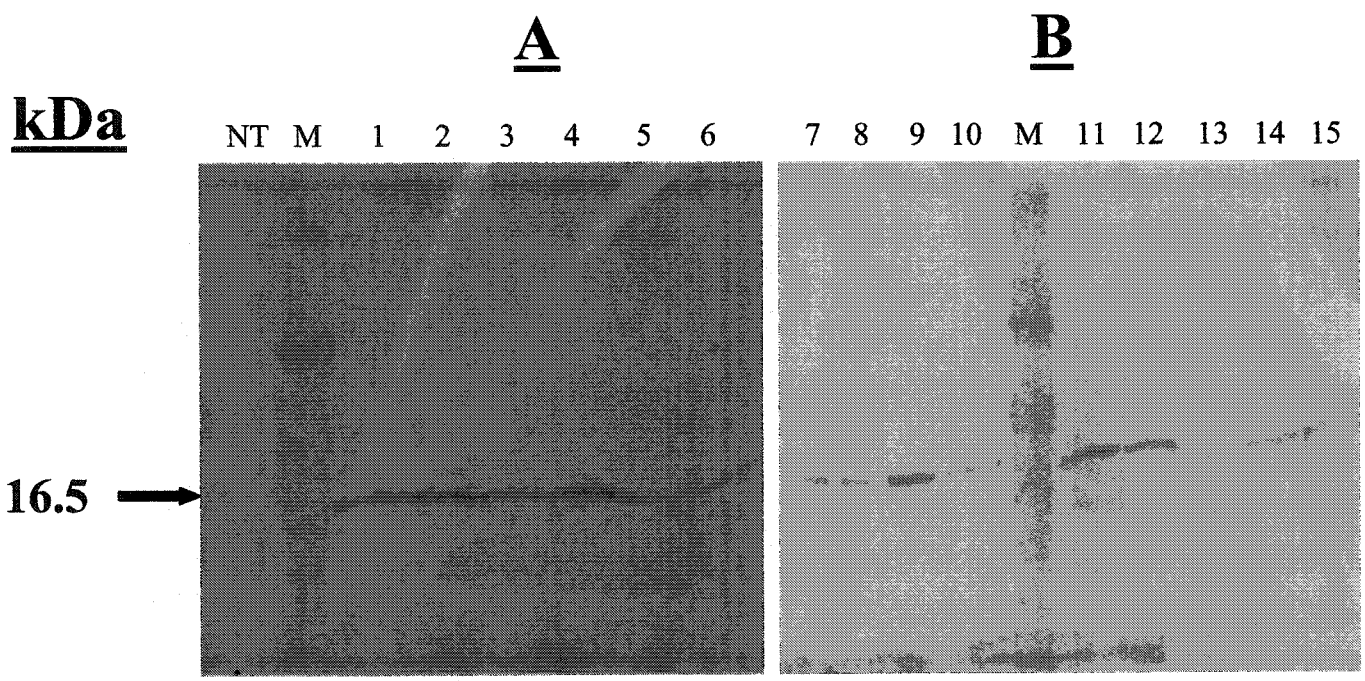


Figure A17

Figure A18. Immunoblot detection of the HBV core particle subunits in transgenic H1 carrot roots and leaves. Sample lanes M represent the molecular weight protein standards; lanes NT represent protein extracts derived from a non-transformed carrot plant root; lanes 16 and 17 represent protein extracts derived from the roots and leaves of transformed H1 carrot plants. All transgenic carrot root extracts showed a 16.5 kDa band on Western blots but the extracts from the leaves of transformed carrot plants or from an NT carrot plant did not show any bands. Panels A and B are part of the same experiment that used root extracts or leaf extracts respectively.

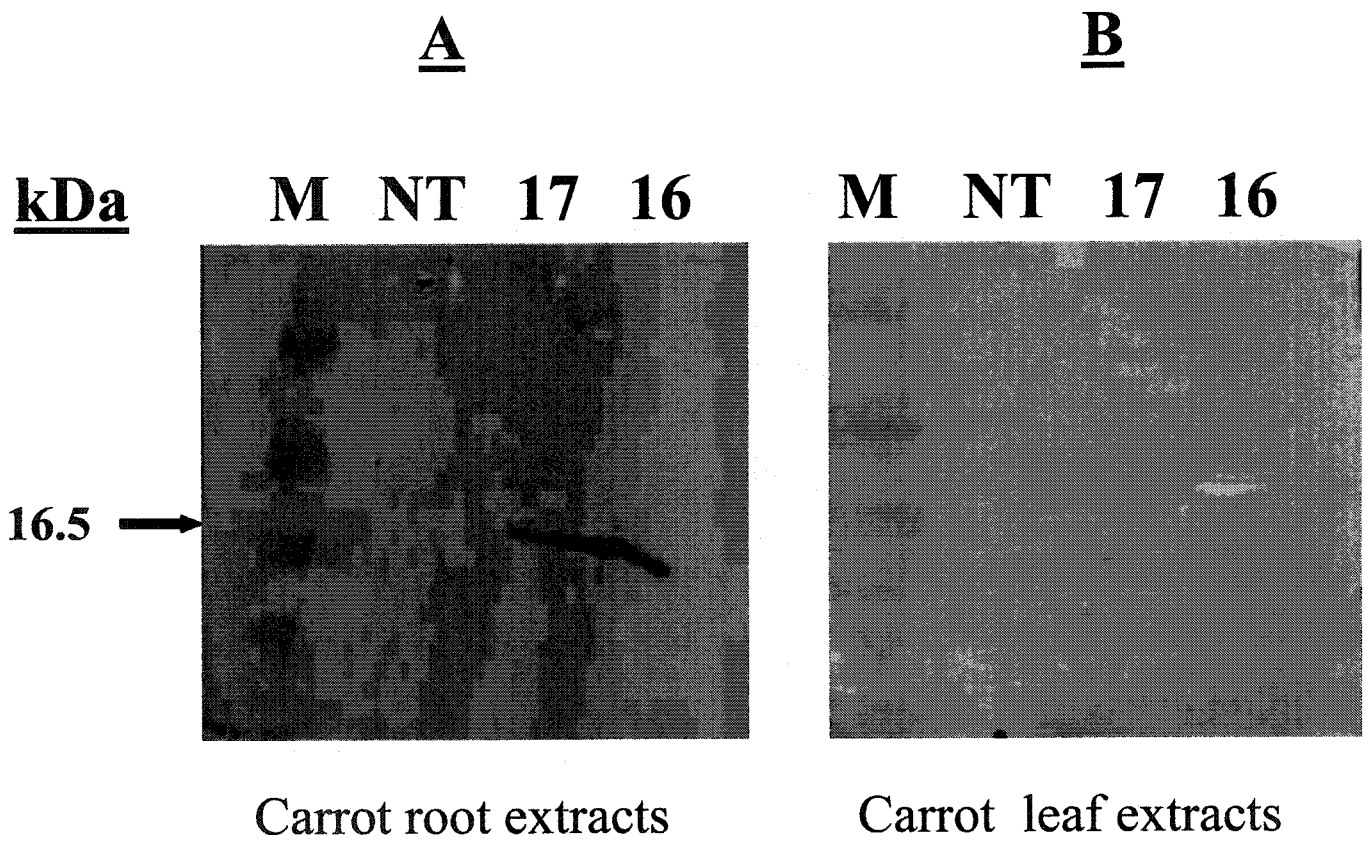

A
B
kDa
M NT 17 16
M NT 17 16
16.5
Carrot root extracts
Carrot leaf extracts

Figure A18

Figure A19. Immunoblot detection of HBV core particle subunits in H4 carrot leaf extracts. Lane M: molecular weight protein standards; Lanes +: positive control derived from *E. coli*, quantity loaded from M lane left to right are 200 ng and 100 ng respectively; lanes 5-20 were loaded with 10 µL each of total leaf protein extract from H4 carrot plants; the far right "+" lane in Panel A was loaded with 50 ng of *E. coli* derived HBV core antigen subunits. All transgenic carrot leaf extracts showed bands on Western blots but the extracts from the leaves of an NT carrot plant did not show any bands. Panels A and B are part of the same experiment that used leaf extracts of the H4 carrot line.

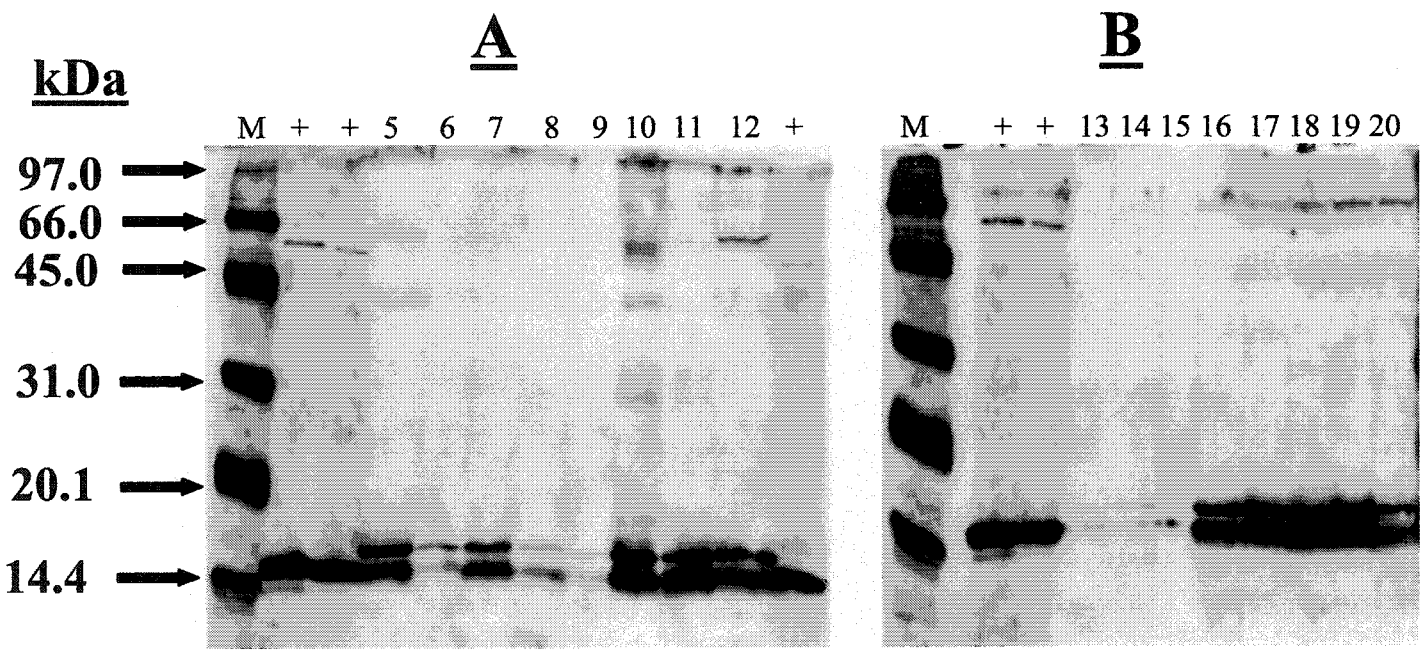

A
B
kDa
M + + 5 6 7 8 9 10 11 12 +
M + + 13 14 15 16 17 18 19 20
97.0
66.0
45.0
31.0
20.1
14.4

Figure A19

Figure A20. Immunoblot detection of HBV core particle subunits in H4 carrot root extracts. Lane M: molecular weight protein standards; lanes +: positive control derived from *E. coli*; lanes 6, 7 and 13 represent the same plants from the H4 line but lanes 6 and 7 were loaded with 20 μL of protein extracts from roots. Lane 13 contained 10 μL of total root protein extract from H4 carrot plants. This experiment is further discussed in the text.

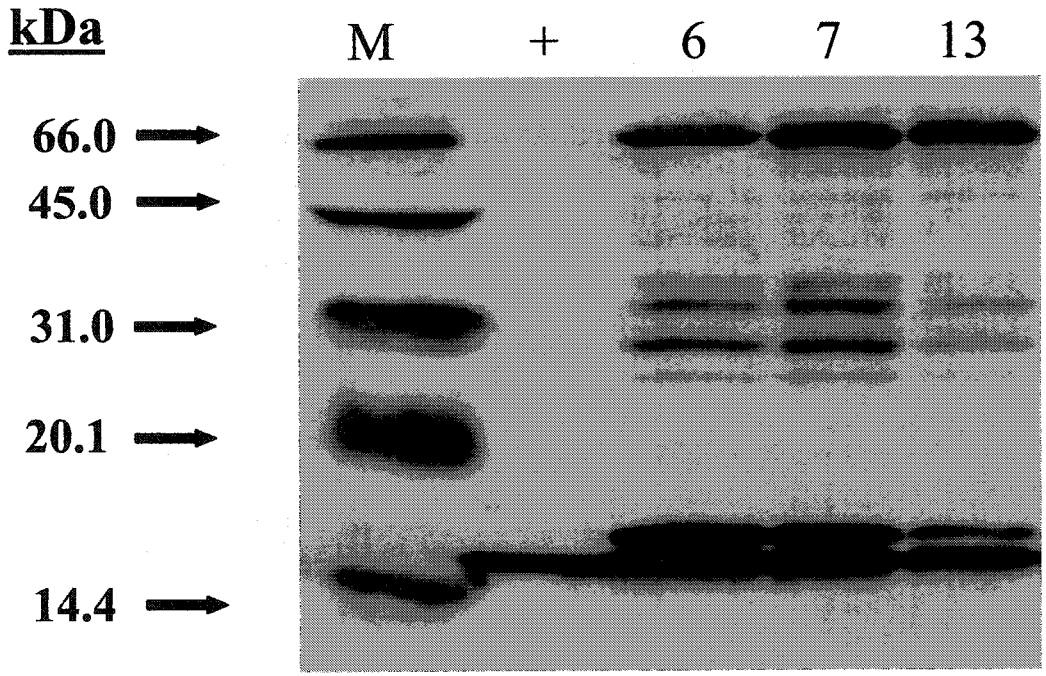


Figure A20

Figure A21. Electron micrograph of assembled HBV core particle from H1 carrot leaf extract. Total protein extracts from H1 carrot plant #2 was purified via sucrose density gradient ultracentrifugation for electron microscopy. The assembled HBV core particle was detected on negatively stained grids by electron microscopy. No antibodies were used in this experiment.

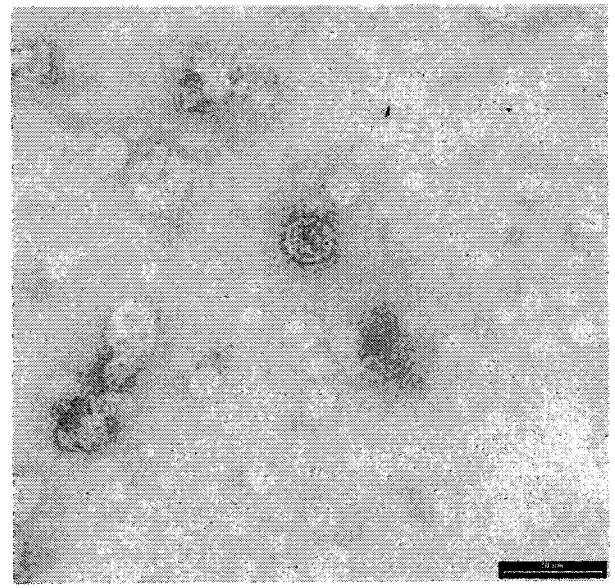


Figure A21

Figure A22. Immunoblot detection of the HBV core particle subunit in transgenic H1 rice plants. Sample lane M represents the molecular weight protein standards; lane + represents 50 ng of *E. coli* derived positive control; lane NT represents protein extracts derived from a non-transformed rice plant; lanes 1-3 represent protein extracts derived from the leaves of transformed rice plants #1, #3 and #5 respectively. All transgenic rice extracts showed a 16.5 kDa band and sometimes a 33 kDa band (e.g. rice plant #3) on Western blots. The extracts from an NT rice plant did not show any bands.

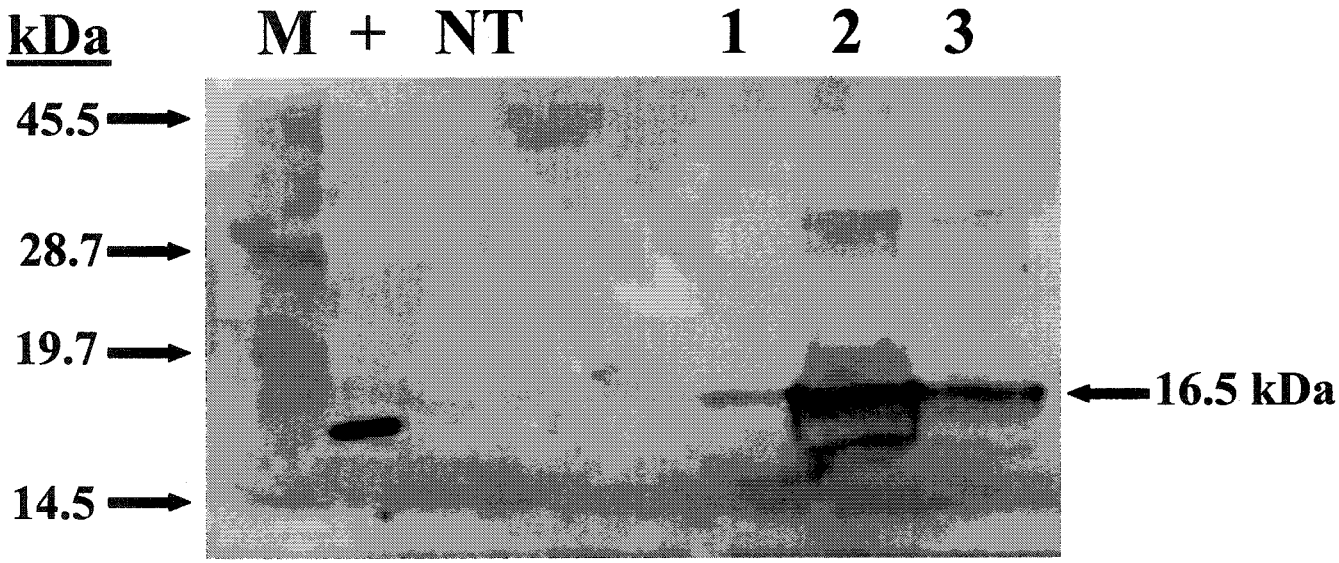


Figure A22

Figure A23. Immunoblot detection of the HBV core particle subunits in successive seed protein extracts of transgenic H1 rice plant #3. Sample lane + represents the *E. coli* derived positive control while lanes A to D were loaded with 10 µL each of rice seed extract where the same sample was subjected to successive protein extractions. Lane A represents 10 µL of protein extract from the first extract while Lane B represents the second extract etc. Lane E was loaded with 10 µL of an equal mixture of the four extracts from A-D. The "+" lanes from left to right were loaded with 1 µg, 500 ng, 250 ng and 125 ng of *E. coli* derived positive control respectively. All transgenic rice extracts showed a 16.5 kDa band, however, the *E. coli* derived positive control showed a 16.5 kDa and sometimes a 33 kDa band on Western blots. The extracts from an NT rice plant did not show any bands.

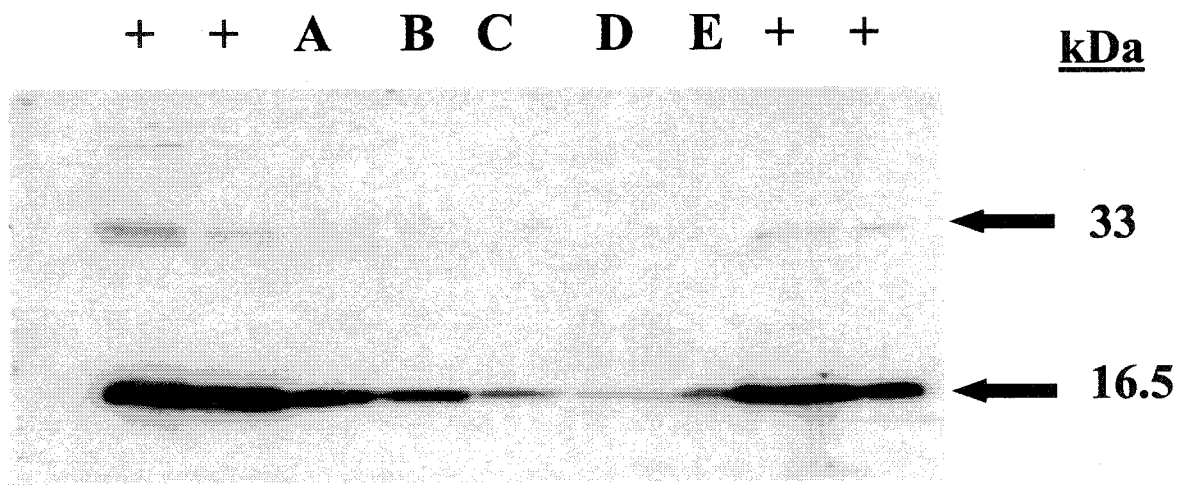


Figure A23

Figure A24. Electron micrograph of the assembled HBV core particles derived from an H1 rice plant leaf extract. Total protein extract from H1 rice plant #3 was purified via sucrose density gradient ultracentrifugation. The assembled HBV core particles were detected by electron microscopy via negatively stained grids. The grids were coated with the purified protein extract. The particles varied in size between 25-30 nm in diameter.

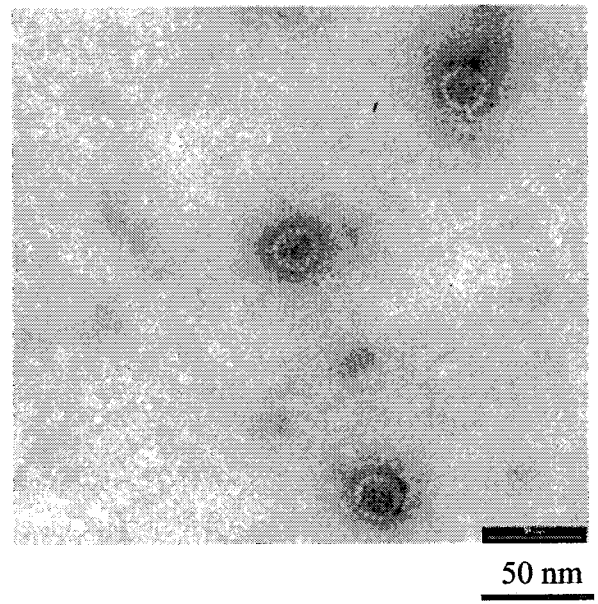


Figure A24

Collaborators

This has been a collaborative project between the University of Ottawa, Health Canada's National Microbiology Laboratory in Winnipeg and Agriculture and Agri-food Canada, Eastern Cereal Oilseed Research Centre (ECORC) in Ottawa. Please see the acknowledgement section for the people involved.

Appendix 3

Figure B1. A standard curve for the estimation of the recombinant HBcAg in transgenic plants. The standard curve was generated by loading known amounts of *E. coli* derived HBcAg on SDS-PAGE gels as standards. The resulting Western blot was analysed via densitometry. The densitometry units and the amounts of *E. coli* derived HBcAg were used to generated the standard curve for estimation of the unknown HBcAg concentrations in transgenic plants.

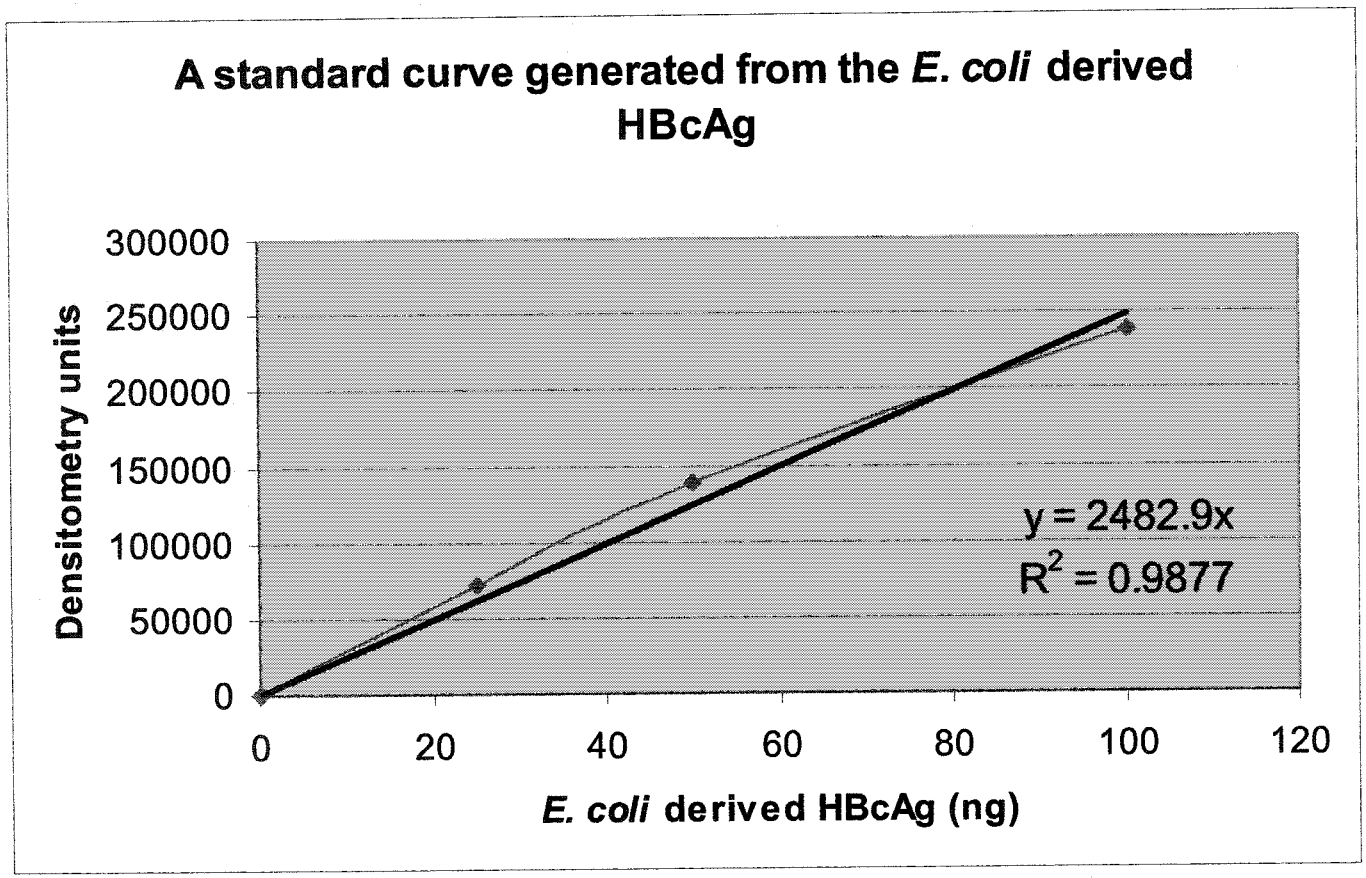

A standard curve generated from the E. coli derived HBcAg
Densitometry units
300000
250000
200000
150000
100000
50000
0
0
20
40
60
80
100
120
E. coli derived HBcAg (ng)
y = 2482.9x
R2 = 0.9877

Figure B1