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ISOLATION AND CHARACTERIZATION OF MOLECULAR MARKERS FOR *BRASSICA NAPUS* MICROSPORE EMBRYOGENESIS

by

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Thesis submitted to the
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University of Ottawa
in partial fulfillment of the requirements for the
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University of Ottawa,
Ottawa, Ontario
1994

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ABSTRACT

Brassica napus microspores can be diverted from pollen development toward haploid embryo formation in culture by subjecting them to a heat stress treatment. This switch in developmental pathways has been shown to be accompanied by the induction of high levels of napin seed storage protein gene expression (DeMoor, 1992). Specific members of the napin multigene family that were expressed at this time were cloned from a cDNA library prepared from microspores that had been induced to undergo embryogenesis. The majority of napin clones represented three members (BnmNAP2, BnmNAP3 and BnmNAP4) that, along with a previously isolated napin genomic clone (BngNAP1), are members of the highly conserved BnmNAP subfamily of napin genes. DNA gel blot analysis, using a subfamily-specific probe, suggested that this subfamily may consist of up to 5 members. RNA gel blot analysis, also using the subfamily-specific probe, indicated that the BnmNAP subfamily was also expressed during embryo development. BnmNAP mRNA was detected as early as the globular stage of development in microsporic embryos, but not until the late torpedo/early cotyledon stage of development in zygotic embryos.

A BngNAP1 promoter-ß-glucuronidase (GUS) gene fusion was introduced into *B. napus* and *Nicotiana tabacum* (tobacco) plants in order to examine the spatial and temporal pattern of expression of one member of the BnmNAP subfamily. The BngNAP1-GUS construct was shown to be highly expressed in microspores that had been induced to undergo embryogenesis, but was not expressed in microspores continuing pollen development in culture. Furthermore, BngNAP1-directed GUS activity appeared to be predominantly localized in those microspores that have been shown to have the greatest potential to form embryos in culture.

Fluorogenic and histochemical analysis of developing microsporic and zygotic embryos of *B. napus* indicated that the BngNAP1-GUS fusion was expressed as early as the globular stage of development. GUS activity was first detected in the micropylar region of the future embryonic axis and continued to spread upward during subsequent stages of development.

In tobacco, GUS activity was first detected in the endosperm of seeds containing globular stage embryos. GUS activity did not begin to accumulate in tobacco embryos until the early heart stage of development, where it appeared as a band in the middle of the embryo, just under the lobes of the emerging cotyledons. This activity continued to spread outward in both directions as development proceeded. Thus the timing, but not the spatial localization, of BngNAP1-directed GUS expression was maintained in transgenic tobacco.

This work represents the first detailed analysis of the expression of a molecular marker for the induction of microspore embryogenesis.

To my family, for believing in me long before I believed in myself.

ACKNOWLEDGEMENTS

Throughout my Ph.D program the one constant source of guidance and encouragement has been my supervisor, Brian Miki. I thank him for allowing me the freedom to learn from my mistakes and for instilling in me a sense of pride and self-confidence in my abilities. Sylvia McHugh has been my good friend and teacher. I thank her for sharing with me her incredible wealth of knowledge and for giving me a very caring shoulder to cry on. Jiro Hattori has also been a good friend. I am indebted to him for helping me to improve my writing and computer skills, but mostly for helping me to see things in the simplest of terms. Brian Treacy, besides being either the victim or partner in my practical jokes, has always kept me laughing. Thank-you for always being optimistic and looking on the brightest possible side of things. Lisa James went through this whole thesis ordeal at the same time as I did. Thanks for providing a refreshing dose of reality and for being too cool for words. Pierre Fobert, Janice DeMoor and Thérèse Ouellet were all there for me in the early days. I thank them for their patience, their knowledge and their friendship. Finally, I thank my two dearest friends, Jake and Teddy, for always greeting me at the door with a smile and a wagging tail, and for making me feel like the most important person in the world.

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LIST OF ABBREVIATIONS

A	adenine
ABA	abscisic acid
bp	base pairs
C	cytosine
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
cm	centimetre
cpm	counts per minure
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
ddH2O	double-distilled water
dH_2O	distilled water
DNA	deoxyribonucleic acid
DPA	days post anthesis
EDTA	ethylene diamine tetraacetic acid
g	grams
G	guanine
GUS	β-glucuronidase and gene encoding β-glucuronidase
gusA	gene encoding β-glucuronidase
hr	hour(s)
kb	kilobases
kD	kilodaltons
Km	kanamycin
Km^r	kamamycim resistant

Km^S	kanamycin sensitive
l	litre
LB	T-DNA left border repeat
M	molar
μCi	microCurie(s)
μE	microEinsteins
μg	microgram(s)
mg	milligram
min	minute(s)
μJ	microJoules(s)
μm	micrometre(s)
mM	millimolar
MOPS	morpholino propanesulphonic acid
mRNA	messenger RNA
MU	4-methylumbelliferone
MUG	4-methylumbelliferyl-β-D-glucuronide
NAA	1-napthaleneacetic acid
ng	nanogram
nos	gene encoding nopaline synthase
npt II	gene encoding neomycin phosphotransferase
OD_{600}	optical density at 600 nm
PCR	polymerase chain reaction
pmol	picomoles
RB	T-DNA right border repeat
RNA	ribonucleic acid
rpm	revolutions per minute

RT-PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulfate
SSC (20X)	3 M NaCl, 0.3M Na citrate, pH 7
T	thymine
T-DNA	transferred DNA
TE	10 mM Tris, 1 mM EDTA
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
X-gluc	5- bromo-4-chloro-3-indolyl-β-D-glucuronide
2,4-D	2,4-dichlorophenoxy acetic acid

INTRODUCTION

The plant embryo represents an interesting system with which to study the developmental and tissue-specific regulation of plant gene expression. During seed development embryos undergo profound morphological changes, accumulate large amounts of seed-specific storage compounds and eventually enter into a state of developmental arrest. All of these stages are influenced by environmental and hormonal signals and all are characterized by the expression of different groups of genes.

The ability of isolated somatic or gametophytic plant cells to develop into embryos in culture has proven extremely useful in the study of embryo development. One of the main advantages of embryo culture systems is that they allow one to collect, analyze and experimentally manipulate large amounts of embryos that would normally be inaccessible for experimentation *in planta*. For example, embryo culture systems have been used to define the role of plant hormones during embryo development and to identify specific genes whose products are required during the early stages of embryo formation (Schiavone and Cooke, 1987; de Vries *et al.*, 1988; Sterk *et al.*, 1991; De Jong *et al.*, 1992).

One of the most fascinating but poorly understood aspects of *in vitro* embryogenesis is the mechanism whereby a previously differentiated cell can be induced to develop into an embryo in culture. One approach that can be used to gain insight this process involves the isolation of genes whose expression marks this switch in developmental pathways. While a small number of genes have been characterized that mark the induction of embryogenesis from somatic cells (Sterk and de Vries, 1993), similar studies characterizing genes that mark the induction of embryogenesis from gametophytic cells are lacking.

The goal of this thesis was to isolate and characterize molecular markers for the transition from pollen to embryo development during *B. napus* microspore embryogenesis. Previous studies revealed that expression of napin seed storage protein genes is a molecular marker for the induction of *B. napus* microspore embryogenesis (DeMoor, 1992). The results of the study provided the background for my thesis and are briefly summarized below. A more detailed account of these results can be found in Appendices I and II.

The original intent of this earlier study was to determine if the patterns of gene expression during *Brassica napus* microsporic embryo development paralleled those that occur during zygotic embryo development. As part of this effort, the expression patterns of genes coding for the *B. napus* embryo seed storage proteins, napin and cruciferin, was examined in microspores and in developing microsporic and zygotic embryos. The results indicated that the temporal pattern of storage protein mRNA accumulation was similar in both microsporic and zygotic embryos, however, a high level of napin gene expression was also detected in preparations of microspores immediately after the treatment used to induce embryogenesis (4 days at 32.5 °C), but not in microspores that were cultured to develop as pollen (4 days at 25 °C; Appendix I, Figure 1). No cruciferin gene expression was observed in any of the samples (Appendix I, Figure 1). The induction of napin gene expression in embryogenic microspores was unexpected as other studies have shown that high level seed storage protein gene expression generally occurs during the maturation phase of seed development (Crouch, 1988; Goldberg *et al.*, 1989).

Since napin gene expression was restricted to embryogenically-induced microspores, it was hypothesized that napin gene expression may be an early marker for the induction of *Brassica napus* microspore embryogenesis. This possibility was further explored by examining the relationship between the induction of napin gene expression and the developmental stage of donor microspores, the plant growth conditions and the microspore culture conditions. In brief, the results indicated the following: (1) the induction of high levels of napin gene expression is developmentally regulated in microspores and coincides with the stages of microspore development that are optimal for embryogenesis i.e. late uninucleate/early binucleate microspores (Appendix II, Figure 1A); .

(2) the induction of napin gene expression is not a direct response to the plant growth or microspore culture conditions alone. However, napin gene expression may be induced by elevated temperatures, but only in embryos and microspores with embryogenic potential (Appendix II, Figure 1B).

These results indicate that napin gene expression is an early marker for the induction of *B. napus* microspore embryogenesis. The napin proteins of *Brassica napus* are encoded by a highly conserved gene family consisting of at least 10 members (Josefsson *et al.*, 1987; Scofield and Crouch, 1987), some of which have been cloned and sequenced (Crouch *et al.*, 1983; Ericson *et al.*, 1986; Scofield and Crouch, 1987; Baszczynski and Fallis, 1990; Josefsson *et al.*, 1987; Ericson *et al.*, 1991). The primary objectives of this thesis were to clone and identify the specific members of the napin multigene family expressed in embryogenic microspores and to characterize their expression during subsequent microspore and zygotic embryo development. In addition, a study was initiated to identify other genes expressed in embryogenic microspores that may also serve as markers for the induction of *B. napus* microspore embryogenesis, or that may provide insight into the processes underlying microspore embryogenesis. The results of the latter study are incomplete and have therefore been presented in the form of an appendix (Appendix IV).

CHAPTER ONE

LITERATURE REVIEW

This chapter provides an overview of a number of aspects of plant reproduction including gametogenesis and seed formation. Particular emphasis has been placed on embryo development and the gene expression programs that accompany it. When available, literature pertaining to *B. napus* has been used as examples.

1.1 Overview Of Plant Reproduction

The life cycle of higher plants involves the alternation between haploid and diploid generations. The haploid phase, known as the gametophyte, produces the male and female gametes. These gametes come together during fertilization to produce the young diploid sporophyte or embryo. The mature diploid sporophyte comprises the dominant portion of the life cycle in higher plants and will ultimately produce spores that undergo meiosis to generate, once again, the gamete-producing haploid phase of the plant life cycle.

1.1.1 Development of the Male Gametophyte

In angiosperms, formation of the haploid male gametophyte, or pollen, takes place within the stamen and requires the interaction of both diploid sporophytic and haploid gametophytic tissues. The stamen arises from the floral meristem and, as shown in Figure 1A, is composed of the filament and the anther. The filament is not directly involved in pollen formation but rather serves as a vascular connection to the plant, supplying both water and nutrients to the anther. The anther consists of both reproductive and nonreproductive tissues. Four wall-layers, the epidermis, endothecium, middle layer and tapetum, surround a fluid filled locule of connective tissue (Figure 1B). The tapetum plays a crucial role in pollen development, providing important nutrients, degradative enzymes and cell wall precursors. Within the locule are four chambers or pollen sacs. These pollen sacs contain the sporogenous diploid cells, termed microspore mother cells or microsporocytes, that will ultimately give rise to the male gametes (Figure 1B).

The progression of events leading to the formation of a mature pollen grain are largely conserved from species to species. Pollen development occurs within the sporogenous tissue of the anther and begins with two meiotic divisions of the microspore mother cell. These divisions give rise to an enclosed tetrad of haploid uninucleate microspores (Figure 1C). Each tetrad, and each microspore within a tetrad, is enclosed by a callose (β-1,3-glucan) wall.

Following meiosis the microspores are released from the tetrad by action of the enzyme callase (β-1,3-glucanase) which is secreted by the tapetum. Once released the microspores undergo a rapid expansion, increasing two to three times in volume (Figure 1C, D). Cell wall synthesis, which began during meiosis, is also accelerated. The microspore cell wall consists of an outer exine composed of sporopollenin and an inner intine composed of pectic and cellulosic material. The tapetum contributes to the development of the exine by synthesizing precursors which become incorporated into the outer cell wall layer. After an extensive interphase the microspores undergo an asymmetric mitotic division (microspore mitosis) to produce a binucleate pollen grain consisting of a large vegetative cell and a smaller generative cell that lies within the cytoplasm of the vegetative cell (Figure 1E).

Pollen development in angiosperms differs among species with respect to the timing and location of a second mitotic division (pollen mitosis). In some species, such as *B. napus*, the generative cell divides mitotically to form two sperm cells prior to release from the anther (Figure 1F). However, in the majority of species, this division takes place during subsequent pollen germination.

Figure 1 Schematic Representation of Pollen and Ovule Development

A Structure of the flower and its reproductive organs.
p, petal; s, sepal; st, stigma; sty, style; ov, ovule; a, anther; f, filament

B cross section of the anther
e, epidermis; en, endothecium; ps, pollen sac; t, tapetum; pg, pollen grain; v, vascular bundle

C tetrad of uninucleate microspores
m, microspore; cw, callose wall

D uninucleate microspore
vn, vegetative nucleus; pw, pollen wall

E binucleate pollen grain
vn, vegetative nucleus; gn, generative nucleus

F trinucleate pollen grain
sn, sperm nuclei

G cross section of the pistil
st, stigma; sty, style; trt, transmitting tissue; ovu, ovule

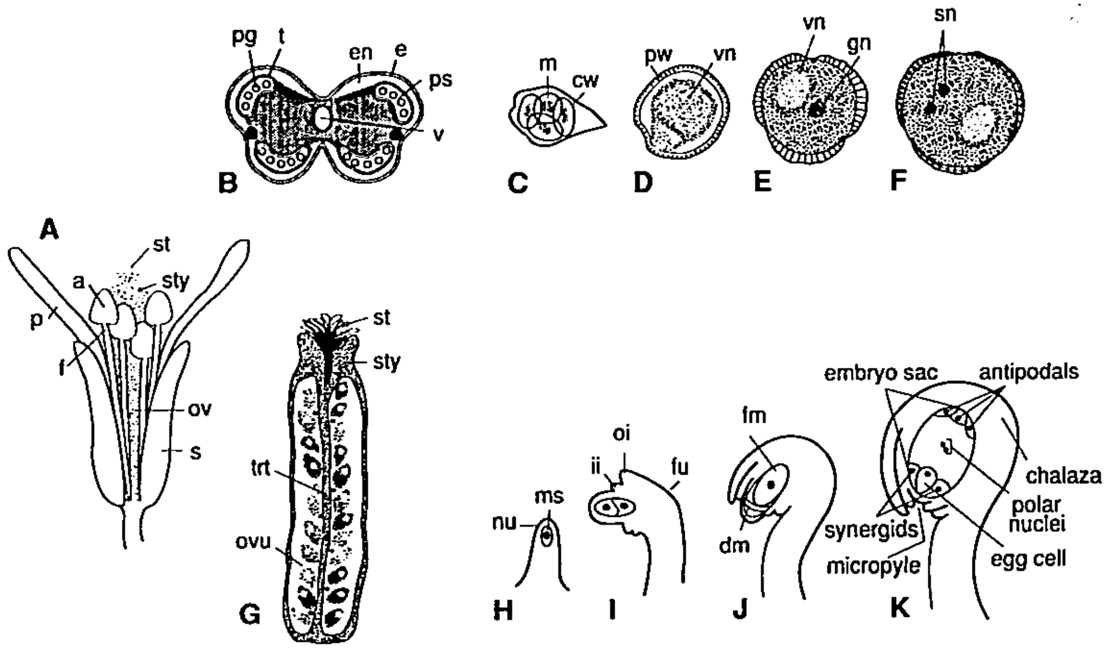
H initiation of ovule development
nu, nucellus; ms, megasporocyte

I ovule after first meiotic division and initiation of integuments
ii, inner integument; oi, outer integument; fu, funiculus

J ovule after meiosis
fm, functional megaspore; dm, degenerate megaspores

K ovule after megagametogenesis

Figure adapted from Coventry *et al.*, 1988, Gasser and Robinson-Beers, 1993, Goldberg *et al.*, 1993 and Reiser and Fischer, 1993. Drawings are not to scale.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H
nu
ms
I
ii
oi
fu
J
fm
dm
K
embryo sac
antipodals
chalaza
polar nuclei
synergids
micropyle
egg cell

During pollen germination the vegetative cell forms the pollen tube that directs the two sperm nuclei to the female gametophyte. Prior to their release from the anther the pollen grains, whether binucleate or trinucleate, must first undergo a desiccation period.

Estimates of the number of genes expressed in the male gametophyte of *Tradescantia paludosa* (Willing and Mascarenhas, 1984), corn (Willing *et al.*, 1988) and tobacco (Kamalay and Goldberg, 1980) are similar; on average approximately 20,000 to 26, 000 genes are expressed in mature pollen. These genes can be divided into different classes based on their relative mRNA abundance. In all three species the majority of the pollen-expressed genes encode the least abundant mRNAs. Estimates of the number of genes expressed in earlier stages of male gemetophyte development have not been carried out.

A considerable overlap of gene expression has been estimated to exist between the sporophyte and the male gametophyte. For example, only 10 to 20% of the genes expressed in the pollen of corn and *Tradescantia* are considered to be pollen specific (Stinson *et al.*, 1980). However, this is likely to be an overestimate as not all sporophytic tissues have been evaluated. There is evidence that at least some of the genes expressed in both the sporophyte and male gametophyte exist as multigene families with pollen specific members (Bryce and Nelson, 1979; Frova *et al.*, 1987; Hussey *et al.*, 1988; Ludwig *et al.*, 1988; Roberts *et al.*, 1993).

Both the level and the nature of gene expression have been shown to change during the course of male gametophyte development. In particular, there is a striking increase in RNA and protein synthesis as well as the onset of expression of a new set of genes after microspore mitosis (Bedinger and Edgerton, 1989; Mandaron *et al.*, 1990). It has been suggested that microspore mitosis marks the initiation of a new developmental program and is a critical point in the commitment to the gametophytic pathway (Bedinger, 1992).

The isolation and characterization of a large number of pollen-specific genes has supported these observations. Pollen-expressed genes have been organized into early and late genes (Mascarenhas, 1989). Early genes are those that are expressed in microspores soon after meiosis and are repressed or expressed at very low levels in pollen. Early genes appear to encode proteins that are involved in the synthesis of the cell wall and cytoskeleton and stored products such as starch. Late genes are defined as those that are expressed from microspore mitosis onward and encode products that are involved in pollen maturation, pollen germination and pollen tube growth. The majority of pollen-expressed genes that have been isolated have been classified as late genes.

1.1.2 Development of the Female Gametophyte

Formation of the haploid female gametophyte takes place within the pistil and, like pollen development, relies considerably on the interaction with sporophytic tissues. The pistil is composed of the stigma, the style and one or more ovaries (Figure 1A). The ovary, which will ultimately form a fruit, contains one or more ovules that give rise to seeds (Figure 1G). The ovule itself is derived from the placental tissue on the inner surface of the ovary wall and consists of a nucellus, one or two integuments and a funiculus. The nucellus is derived from the ovular tissue and differentiates to produce the diploid megasporocyte or megaspore mother cell that gives rise to the megaspores. The integuments, which later become the seed coat, are derived from one or two of the outer cell layers of the ovule and are formed during megasporogenesis. As the integuments develop they leave a pore, or gap, called the micropyle. The micropyle marks the point at which the pollen tube enters the ovule during fertilization. The funiculus, like the stamen, provides both a physical and a vascular connection to the plant.

During megasporogenesis, the megasporocyte undergoes meiosis, producing first a dyad then a tetrad of haploid megaspores. In the majority of species, three of these megaspores degenerate, leaving the remaining single cell to function as the megagametophyte (Figure 1H, I, J). The megagametophyte enlarges and subsequently undergoes three free nuclear mitotic divisions. After the first division, the two nuclei migrate to opposite ends of the cell. The two haploid nuclei divide again and the resulting four nuclei divide once more to produce a total of eight nuclei in two polarized groups of four.

The eight nuclei produced by the mitotic divisions of the megasporocyte become rearranged to form, at maturity, an embryo sac, or female gametophyte, consisting of three antipodal cells, a large central cell with two polar nuclei, two synergid cells and a single gamete, the egg cell. One nucleus from either end of the embryo sac migrates to the middle of the cell. These two polar nuclei may fuse to form a diploid nucleus or may remain separate until fertilization. Cell walls begin to form around the remaining six nuclei. The three antipodal cells form at the end farthest from the micropyle (chalazal end). The function of antipodal cells is not known but it is thought that they are involved in nutrient transfer to the embryo (Diboll, 1968). At the micropylar end one cell functions as the female gamete or egg cell, while the other two cells, called synergids, function during fertilization (reviewed in Russell, 1993). The mature embryo sac is shown in Figure 1K

Unlike the male gametophyte, very little is known about the genetics and molecular biology of the female gametophyte. A number of female-sterile mutants have been characterized (see Reiser and Fischer, 1993; Gasser and

Robinson-Beers, 1993). However, no female gametophyte specific gene products have been identified. Several genes that are expressed in other tissues have also been shown to be expressed in the female gametophyte (Gasser *et al.*, 1989; Pennel and Roberts, 1990; Pennel *et al.*, 1991; Bowman *et al.*, 1991; Ma *et al.*, 1991; Jack *et al.*, 1992). This lack of knowledge is due, in part, to the small size and inaccessibility of the developing embryo sac. However, methods that allow for the amplification of mRNA from small amounts of tissue, as well the availability of a number of T-DNA tagged mutants should allow for the isolation of female-gametophyte-specific gene products in the near future (Gasser and Robinson-Beers, 1993; Reiser and Fischer, 1993; Russell, 1993)

1.1.3 Fertilization

Pollination, or the transfer of pollen grains from the anther to the stigma, is brought about primarily by insects and wind. Pollen grains landing on the stigma absorb substances which stimulate the pollen grain to germinate. During germination the vegetative cell bulges out through the pollen grain to form the pollen tube. The vegetative nucleus enters the pollen tube and is followed by the two sperm cells. The pollen tube travels down through the transmitting tissue of the style (Figure 1G) until it reaches the micropyle of the ovule. The contents of the pollen tube are discharged into one of the two synergids, which degenerate either before or during entry of the first sperm cell. The synergid cells are rich in calcium which is thought to direct the pollen tube to enter the embryo sac through the micropyle and to release its two sperm cells (Jensen *et al.*, 1985).

Once released the sperm cells participate in double fertilization, a process which is unique to angiosperms. During double fertilization, the nucleus of one sperm cell fuses with the nucleus of the egg cell to form the diploid zygote, while the nucleus of the other sperm cell fuses with the two polar nuclei to form the triploid endosperm.

1.1.4 Seed Development

The development of the embryo, endosperm and seed coat are triggered by fertilization and culminate in the formation of a seed. During seed development the embryo undergoes morphogenetic events that establish the primordia of the basic organ systems of the sporophyte. The embryo also accumulates storage reserves that will sustain it during germination and early seedling development. Finally the embryo becomes desiccated and enters into a period of developmental arrest. All of these events take place while the seed remains attached to the maternal plant.

1.1.4.1 The Seed Coat

Shortly after fertilization, the integuments undergo cell division, expansion and vascularization to form the seed coat (Bouman, 1984). Unlike the embryo and endosperm, the seed coat is solely maternal in origin. One of the first functions of the seed coat is to act as a conduit for nutrient transfer from the parent plant to the developing seed. In addition, the seed coat acts as a protective covering for the embryo and may also control germination by acting as an impermeable barrier to water, by synthesizing compounds that inhibit germination or by controlling the amount of light reaching the embryo (Bewley and Black, 1978; Crouch, 1988).

1.1.4.2 The Endosperm

In most species, early endosperm development involves a series of free nuclear divisions followed by cellularization (Vijayaraghavan and Prabhakar, 1984). During the initial stages of seed development the endosperm develops more rapidly than the embryo and, as a result, the embryo is almost completely surrounded by endosperm tissue at an early stage in its development. The endosperm is thought to act as a source of nutrients and hormones during the early stages of embryo growth (Crouch, 1988; Lopes and Larkins, 1993). However, it is not clear whether the transfer of hormones and nutrients from the endosperm to the embryo occurs passively, or whether it is orchestrated by the embryo alone or by both the embryo and the endosperm (Lopes and Larkins, 1993).

The degree to which the endosperm persists during seed development varies from species to species. In monocots, the endosperm persists throughout seed development and functions as a storage reserve for carbohydrate, lipid and protein compounds which are catabolized following germination. Pigments and defense compounds, as well as the enzymes needed to catabolize storage products, also accumulate in persistent endosperms. In contrast, many dicotyledonous species, including *B. napus*, have a transient endosperm. In these species the majority of storage reserves accumulate in the cotyledons of the developing embryo. In seeds with transient endosperms, it is thought that the endosperm is consumed by the developing embryo (Raghavan, 1986). However, it is not known whether the endosperm is actively digested by the embryo or whether the process is controlled by the endosperm.

1.1.4.3 The Embryo

In the majority of flowering plants embryo development proceeds through three major phases (Steeves and Sussex, 1989; Walbot, 1978; Crouch, 1988). The first phase is one of rapid cell division and morphogenesis during which the embryo body plan and the major tissue types and organ systems are established. The second phase encompasses a period of rapid cell expansion and is characterized by the synthesis of storage reserves. In the final phase of embryo development the embryo becomes desiccated and enters into a period of quiescence.

The stages of embryo development for a typical dicot such as *B. napus* are shown in Figure 2. After fertilization the diploid zygote (Figure 2A) is located at the micropylar end of the ovule. The first division of the zygote is asymmetric and generates a two-celled structure consisting of a small apical cell (nearest the chalazal end) and a larger elongated basal cell (nearest the micropylar end) (Figure 2B). The apical cell will ultimately divide to form the cotyledons, shoot apex and hypocotyl of the embryo proper, whereas the basal cell will contribute to the development of both the embryo and the suspensor. The first division of the basal cell generates two equally-sized cells (Figure 2B). The upper cell generates the precursors to the root cortex initials and the central region of the root cap, while the lower cell contributes solely to the development of the suspensor. The suspensor is a transient structure, usually consisting of a single file of cells, that attaches the embryo to the wall of the ovule (Figure 2C). As the suspensor grows it pushes the embryo deeper into the endosperm. The suspensor also promotes embryo growth through

Figure 2 Schematic Representation of Embryo Development in a Typical Dicotyledonous Plant

A zygote

B one celled embryo showing the apical (a) and basal (b) cells

C four celled embryo (e) with a file of suspensor cells (s)

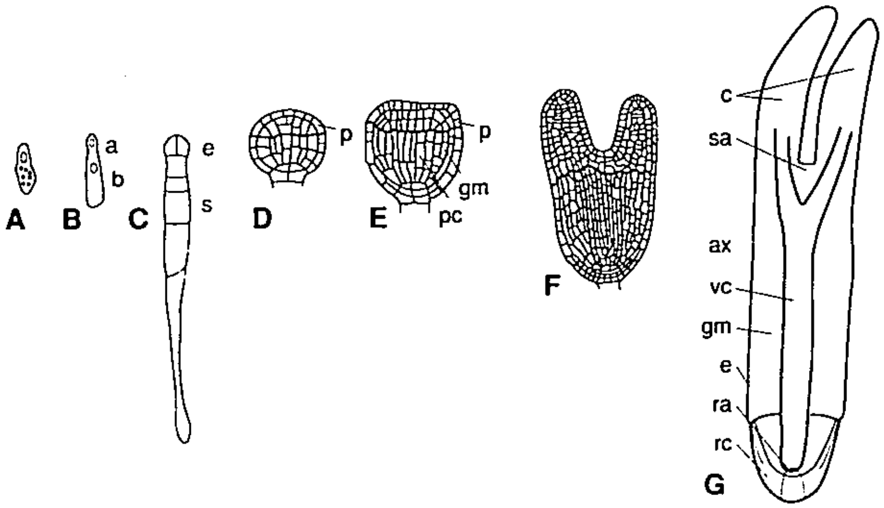
D mid-globular stage embryo with differentiating protoderm (p)

E transition stage embryo with developing protoderm (p), procambium (pc) and ground meristem (gm)

F heart stage embryo

G cotyledon stage embryo showing the elements of the apical basal-axis (c, cotyledons; sa, shoot apical meristem; ax, axis; ra, root apical meristem: rc, root cap) and the radial axis (e, epidermis; vc, vascular cylinder; gm, ground meristem

Adapted from West and Harada, 1993. Drawings are not to scale.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G

nutrient and growth hormone transfer (reviewed in Yeung and Meinke, 1993).

The apical cell of the zygote undergoes a number of longitudinal and transverse divisions to form a radially symmetric, globular-shaped embryo. The first differentiated tissue, the protoderm, is visible at this stage (Figure 2D). In dicots, localized cell divisions in the apical region of the globular embryo result in the emergence of two lobes, which will form the future cotyledons, while cell divisions in the basal region of the embryo result in the emergence of a central axis (Fig 2E). This transition stage marks the switch from a radial to a bilateral type of symmetry. The remaining two tissue systems of the plant, the procambium and the ground meristem, as well as the shoot and root apical meristems also become established during the transition stage. In monocots, only one cotyledon develops and the embryo assumes a cylindrical shape.

In dicots, continued enlargement of both the cotyledon and axis cells results in the successive formation of heart, torpedo and cotyledon stage embryos (Figure 2F, G). The body plan of these embryos can be organized into two superimposed patterns consisting of an apical-basal pattern along the main body axis of polarity and a radial pattern perpendicular to the main axis (Jurgens *et al.*, 1991, Mayer *et al.*, 1991). Each pattern consists of distinct elements; shoot meristem, cotyledons, hypocotyl, root and root meristem in the apical-basal pattern and epidermis, ground tissue and vascular cylinder in the radial pattern (Figure 2G).

Once the embryo has stopped dividing it enters into the maturation phase. In dicot embryos, the first half of the maturation stage is characterized by high levels of the plant growth hormone abscisic acid (ABA) and a decline in the water potential of the seed (Galau *et al.*, 1987). During the maturation stage any increases in size are due solely to the deposition of storage products. Most dicot embryos synthesize and store large amounts of reserves that function after germination to sustain the seedling until it becomes established. The major storage reserves include lipid, carbohydrate and protein, all of which are sequestered into intracellular bodies. In addition, seeds often contain stores of phosphate and various other inorganic compounds. In monocots, and in dicots with a persistent endosperm, the storage function of the embryo is largely taken over by the endosperm. The relative proportion and types of storage reserves varies among different species (Bewley and Black, 1978). For example, the oilseeds of *B. napus* contain approximately 45-55% (w/w) oil and 25-30% (w/w) protein (Murphy and Cummins, 1989; Murphy *et al.*, 1989). Approximately 96% of the oil in *B. napus* seeds is composed of triacylglycerols. 70% of the seed protein is composed of the two storage proteins, napin and cruciferin, and approximately 20 % of the seed protein is composed of an oil body associated protein termed oleosin (Murphy *et al.*, 1989).

The final stage of embryo development for most seed plants involves preparation for quiescence. This period of developmental arrest ensures that subsequent seed germination occurs under environmental conditions that will favour seedling growth. During this phase of embryo development the vascular connection to the maternal plant is severed, the embryo rapidly loses water and eventually becomes desiccated (Galau *et al.*, 1991). The period between ovule abscission and rapid water loss is marked by the synthesis of a new class of proteins termed late embryogenesis abundant or LEA proteins (Hughes and Galau, 1989). Many LEA proteins contain hydrophilic peptide domains that are thought to function to protect cellular components, such as proteins, from subsequent desiccation (Dure *et al.*, 1989).

In *B. napus*, this entire process, from fertilization to maturation through to desiccation, takes place over a period of up to 12 weeks (Figure 3; Murphy and Cummins 1989).

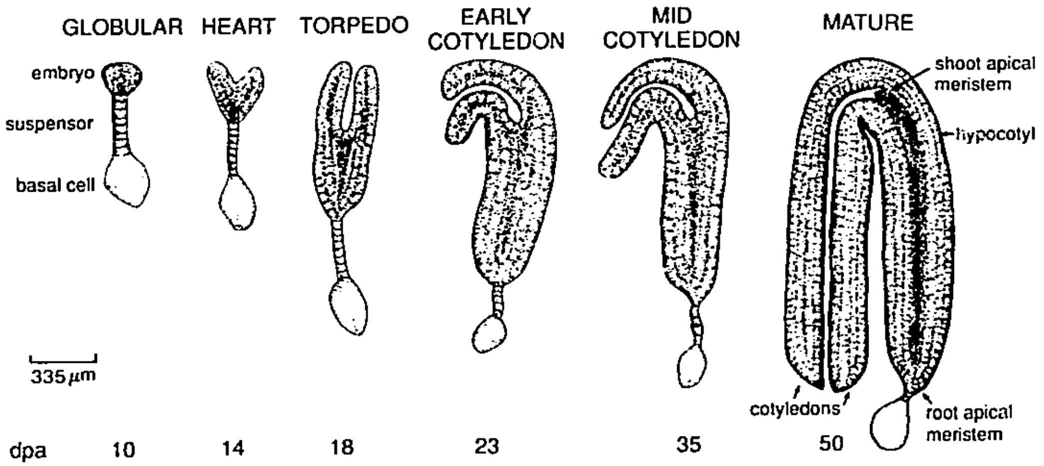
1.2 Asexual Embryo Development

Sexual reproduction in plants involves the fusion of the male and female gametes and results in the formation of an embryo. However, plants are also capable of producing

Figure 3 *B. napus* Embryo Development

dpa, days post anthesis

adapted from DeMoor, 1992


GLOBULAR
HEART
TORPEDO
EARLY COTYLEDON
MID COTYLEDON
MATURE
embryo
suspensor
basal cell
shoot apical meristem
hypocotyl
cotyledons
root apical meristem
335 µm
dpa
10
14
18
23
35
50

functional embryos in the absence of fertilization. This process occurs naturally in many plant species and can also be induced in culture in an even wider range of species.

1.2.1 Apomixis

In a number of species, seeds containing an embryo, and in some cases endosperm, may be generated from ovular tissue in the absence of fertilization. This process is referred to as apomixis and results in the production of a seed that is entirely maternal in its genetic constitution.

Three mechanisms of apomixis have been observed and are termed diplospory, apospory and adventitious embryony (reviewed in Koltunow, 1993). Each mechanism differs with respect to the source of the cell that gives rise to the embryo and with respect to the time during ovule development at which the apomictic process is initiated. However, regardless of the mechanism, the resulting apomictic embryos are virtually indistinguishable from those that are derived from a sexual process.

In diplospory, the megaspore mother cell does not undergo the usual meiotic reduction division, but rather divides mitotically to produce an eight-celled embryo sac that contains diploid rather than haploid cells. One of these cells functions as the egg cell and divides to form the embryo. In a number of species the unreduced polar nuclei may fuse to form the endosperm (Nogler, 1984). In aposporous apomicts embryos are produced from additional cells, the aposporous initials, that differentiate from the nucellus. As with the megagametophyte of diplosporous species, the aposporous initial undergoes mitotic divisions to produce an 8-celled, unreduced embryo sac. Aposporous embryos are not derived from the megagametophyte and can therefore co-exist within a single ovule with sexually produced embryos. Autonomous production of endosperm is rare in aposporous species. The third mechanism of apomixis, termed adventitious embryony, does not involve the production of an embryo sac. Rather, embryos are formed directly from either the integuments or nucellar tissue of the ovule (Lakshmanan and Ambegaokar, 1984). Seeds derived from species exhibiting adventitious embryony are generally polyembryonic and may also contain a single sexually-derived embryo.

A number of studies suggest that apomixis is controlled by a few dominant genes (Mogie, 1988; Asker and Jerling, 1990). A recent model (Koltunow, 1993) suggests that these apomictic genes encode products that act as developmental regulators which normally function to initiate cascades of developmental reactions in sexually reproducing plants. Ectopic expression of these genes in the nucellus at different times during ovule development could lead to the initiation of the observed apomictic processes, while still allowing normal sexual reproduction to occur. Once apomixis is initiated the normal process of embryo development would be free to proceed.

1.2.2 Embryo Culture Systems

Asexual embryos can also be induced to form in culture from a wide variety of gametophytic and sporophytic plant tissues (reviewed in Raghavan, 1986). These embryos may be induced to form either directly, from single cells, or indirectly, through an intermediary callus phase. Cultured embryos, although not identical to sexually-derived embryos, do develop through similar morphological stages and, given the appropriate culture conditions, express many of the same genes at the same developmental time as their zygotic counterparts.

Embryo culture systems have proven to be a valuable tool for the study of embryo development. The primary advantage of these systems resides in the ability to rapidly generate large numbers of embryos at specific developmental stages. This allows one access to the early stages of embryo development that would normally be inaccessible in zygotic embryos. Furthermore, embryos grown in culture are amenable to a variety of experimental manipulations which either cannot be carried out or would be difficult to perform *in planta*. Finally, embryo culture systems provide an opportunity to understand the mechanism whereby a terminally-differentiated cell is induced to switch developmental pathways and develop into an embryo.

1.2.2.1 Somatic Embryogenesis

Somatic embryogenesis was first observed in carrot (*Daucus carota*) by Steward *et al.* (1958) and Reinert (1959) and has since been achieved in a wide variety monocot and dicot species (Ammirato, 1983). However, the somatic embryo culture system established with carrot remains the most fully characterized. In carrot, somatic embryos can be obtained from cultures of leaf petioles, root parenchyma, flower peduncles and epidermal strips from the hypocotyls of germinating seedlings. When these tissues are cultured on synthetic medium containing high concentrations of plant growth hormones, usually auxins, cell division is reinitiated and leads to the formation of a dedifferentiated callus. These dediffereniated callus tissues can be propagated in liquid culture or on a solid agar surface. When callus suspension cultures are diluted and transferred to hormone free growth medium they give rise to somatic embryos. Highly purified populations of somatic embryos at specific developmental stages can be obtained by sieving the embryo cultures through different sizes of nylon filters (Giuliano *et al.*, 1983).

The most effective inducer of somatic embryogenesis is the synthetic auxin 2,4-D (Ammirato 1983; Sung *et al.*, 1984; Dudits *et al.*, 1991). In carrot, prolonged exposure to high levels of 2,4-D is necessary to initiate embryogenic cell divisions in the explanted tissue . However, if tissue explants from some plant species, such as alfalfa (*Medicago sativa*), are first grown in the presence of a weak auxin, such as napthalene acteic acid (NAA), only a very short pulse of 2,4-D (a few minutes to a few hours) is needed to initiate organized growth in the callus tissue and to induce subsequent embryo formation in hormone free culture medium (Bögre *et al.*, 1990; Dudits *et al.*, 1991).

Auxin, in addition to its role in initiating embryogenic cell divisions, also appears to promote early embryo development. Somatic embryos generally originate from the surface cells of small cell clusters termed proembryogenic masses (PEMs; Halperin, 1966). PEMs are established early, during culture in auxin containing media. A variety of evidence suggests that PEMs are, in fact, somatic embryos that have become blocked at or before the globular stage of development (Bokird *et al.*, 1986; de Vries *et al.*, 1988). The block to further embryo development is removed when the cultures are transferred to a medium lacking auxin. Thus auxin may either promote or inhibit embryo development depending on the stage at which it is applied.

The carrot somatic embryo system offers a number of experimental advantages for the study of embryogenesis. Among these are the availability of a number of chemical inhibitors of somatic embryogenesis and a variety of well characterized mutant cell lines (Lo Schiavo *et al.*, 1986; Sung et al., 1984). Both have proven useful in the isolation of proteins that are required during the early stages of somatic embryo development (Cordewener *et al.*, 1991; de Jong *et al.*, 1992). An added advantage of the carrot somatic embryo system is the ability to stably transform PEMs and to subsequently regenerate transgenic plants through somatic embryogenesis (Scott and Draper, 1987).

There are, however, a number of inherent disadvantages associated with the use of the carrot somatic embryo system. For example, the prolonged culture periods employed during carrot somatic embryogenesis often result in the accumulation of mutations due to somaclonal variation. This variation can potentially result in the appearance of new phenotypes or, alternatively, in the loss of important mutant cell phenotypes, both of which can confound the interpretation of experimental results.

An added disadvantage of the carrot somatic embryo system is that it is unsuitable for studying the earliest events associated with the induction of the embryogenic pathway. Because the explants are cultured for extended periods in auxin-containing medium the precise timing of the auxin induced initiation of embryogenesis is ill defined. Furthermore, carrot somatic embryos are derived from multicellular structures, making it difficult to identify the cell types that will ultimately give rise to embryos. Both of these factors make this system unsuitable for studying the very early events associated with the induction of embryogenesis.

The alfalfa somatic embryo system offers potential solutions to both these problems. In alfalfa, single cells, such as leaf protoplasts, can be induced to form embryos directly, without developing through an intermediate callus stage (Kao and Michayluk, 1980; Song *et al.*, 1990). Furthermore, the timing of embryogenic induction in alfalfa cultures can be precisely controlled. Alfalfa somatic embryo culture is therefore better suited for studying the very early events involved in the initiation of the embryogenic pathway, while the carrot system seems to be better suited for studying events that occur after transfer to hormone free medium.

1.2.2.2 Gametophytic Embryogenesis

Embryos can also be induced to form in culture from the gametophytic tissues of ovaries and anthers. In ovary culture, embryos may form from both the synergid cells (Zhou *et al.*, 1986) and the egg cell (Yang *et al.*, 1986) of the ovule. The major limitation of this system is that there is only one embryo sac per ovule and embryo yields are therefore restrictively low. In contrast, anther culture, which was first reported by Guha and Maheswari (1964) in *Daturia innoxia*, has been used to generate high yields of embryos in over 200 species of plants (reviewed in Dunwell, 1986). The major disadvantage associated with the use of this system is that embryos can develop from both the sporophytic and gametophytic tissues of the anther, generating a population of embryos with mixed ploidy levels. Furthermore, the early stages of embryo development are inaccessible since they occur within the confines of the anther.

Culture of isolated microspores or pollen avoids the problems that occur in anther culture such as low embryo yields and formation of embryos with mixed ploidy levels. Furthermore, microspore or pollen culture systems are well suited to the study of the early events that accompany the induction of embryogenesis in culture and, in fact, appear to more closely approximate *in vivo* embryo sac embryogenesis than somatic embryogenesis systems.

In general, pollen or microspore embryogenesis is induced by a single stress treatment of defined duration, allowing one to define the events that occur immediately before, during, and after the treatment used to induce embryogenesis. Embryos are derived from single cells allowing one to define and characterize the precursor cells that give rise to embryos in culture. An added advantage of pollen culture systems is that pollen development is fairly synchronous and one can easily collect large amounts of donor pollen at specific developmental stages.

Microspore or pollen embryogenesis has been observed in a wide range of species (reviewed in Raghavan, 1986), but is particularly well described for tobacco (Heberle-Bors, 1985; Heberle-Bors, 1989) and *B. napus* (Chuong and Beversdorf, 1985; Keller *et al.*, 1987; Fan *et al.*, 1988; Kott *et al.*, 1988; Pechan and Keller, 1988; Telmer *et al.*, 1992, 1993).

A highly efficient culture system has been developed for isolated pollen grains of tobacco. This system allows for strict control of pollen maturation and pollen embryogenesis (Benito Moreno *et al.*, 1988; Garrido *et al.*, 1991) and thus is suitable for study of both gametogenesis and embryogenesis. Culture of mid-binucleate stage tobacco pollen grains for 6 to 8 days in a medium lacking nutrients, followed by transfer to a nutrient-rich medium, induces the vegetative nucleus of the pollen grains to divide and form embryos. In contrast, the same stage pollen, when cultured in nutrient rich medium, continues to develop as pollen. Therefore, in tobacco, the switch between embryogenesis and pollen development can be controlled by the nutritional status of the plant.

The induction of tobacco pollen embryogenesis appears to occur in three phases: (1) degradation of the cytoplasm of mid-binucleate pollen grains; (2) derepression of the cell cycle in the vegetative cell and; (3) activation of gene expression.

During culture in medium lacking nutrients, embryogenic pollen grains, referred to as P-grains, develop cytological characteristics that distinguish them from normal gametophytic pollen. P grains exhibit characteristics that are typical of a starving cell that is converting stored nutrients into energy (reviewed by Heberle-Bors, 1989). These features include numerous lysosomes and structurally empty spaces, the regression of plastids and starch grains, a decrease in the number of ribosomes and the maintenance of mitochondrial integrity. This pollen dimorphism occurs naturally *in vivo,* but the proportion of P-grains can be increased by manipulating the growth conditions of the donor plants (Heberle-Bors and Reinert, 1981) or by the application of growth substances or gametocides that are known to increase male sterility (Heberle-Bors, 1983). In all instances, the increase in P grain formation results in an increase in the frequency of pollen embryo formation *in vitro*. Pollen starvation, whether it occurs *in vivo* or *in vitro,* may be required to eliminate the products of gametophytic gene expression and thereby facilitate the transition from pollen to embryo development.

The induction of tobacco pollen embryogenesis also appears to involve the derepression of the cell cycle. During normal pollen development the vegetative nucleus is arrested in G1. However, in mid-binucleate pollen cultured in starvation medium, the vegetative nucleus is induced to enter S phase and replicate its DNA (Zarsky *et al.*, 1992). Furthermore, changes in protein kinase activity (Garrido *et al.*, 1993) and protein phosphorylation patterns (Kyo and Harada, 1990) during the starvation treatment also suggest that the starvation treatment acts to reactivate the cell cycle in the vegetative nucleus.

In *B. napus*, as in tobacco, the switch from pollen to embryo development can be experimentally controlled by manipulating the plant growth and cell-culture conditions.

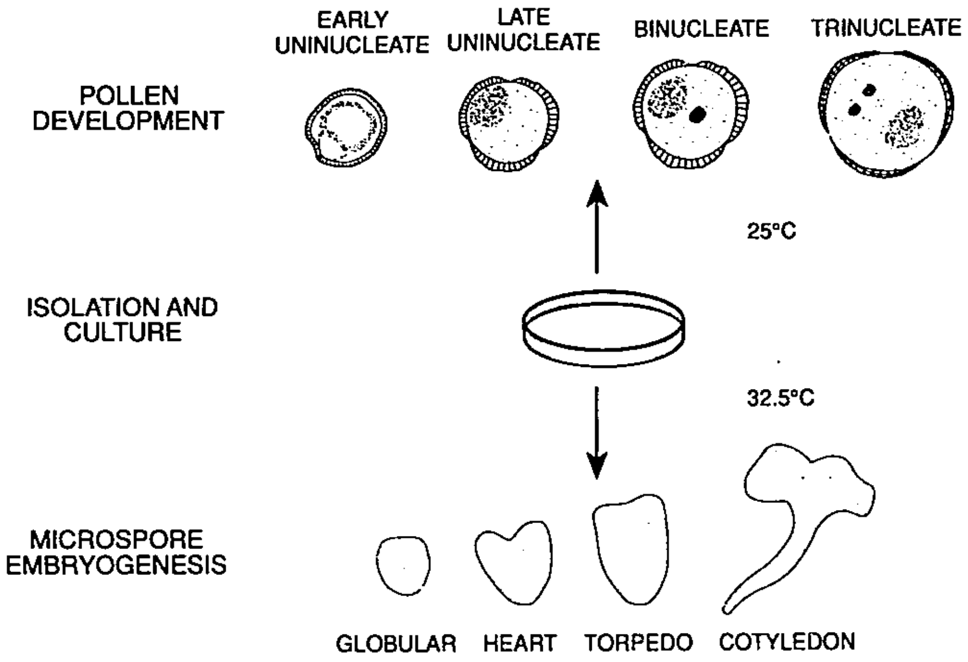
Heat treatment of late uninucleate/early binucleate microspores, isolated from plants grown under low temperatures, followed by transfer to normal growth temperatures, is sufficient to simultaneously block pollen development and induce microspore embryogenesis in a large proportion of the population (Chuong and Beversdorf, 1985; Fan *et al.*, 1988; Keller *et al.*, 1987; Gland *et al.*, 1988; Pechan and Keller, 1988; Telmer *et al.*, 1992, 1993). The same microspores, when cultured in the absence of the heat treatment, continue to develop as pollen. Thus a single stress treatment is sufficient to induce and sustain embryogenesis in *Brassica* microspores. The process of *Brassica* microspore embryogenesis is illustrated in Figure 4.

Changes in microspore morphology and ultrastructure can be seen soon after the microspores have been heat stressed to induce embryogenesis (Chuong *et al.*, 1988; Kott *et al.*, 1988; Fan *et al.*, 1988; Zaki and Dickinson, 1990; Telmer *et al.*, 1993). Heat-treated microspores, isolated just prior to or immediately after microspore mitosis, appear spherical and slightly swollen. Cultures with a high proportion of this type of microspore generally give rise to the highest embryo yields. In contrast, heat-treated microspores or pollen from either earlier or later stages of development do

Figure 4 Schematic Representation of Pollen Development and Microspore Embryogenesis in *B. napus*

B. napus microspores isolated at the late uninucleate to early binucleate stage of development can be induced to form haploid embryos *in vitro* by culturing them at 32.5 ^{o}C. In contrast, microspores from the same developmental stages continue to develop as pollen *in vitro* when cultured at 25 ^{o}C.

Adapted from Coventry *et al.*, 1988.


POLLEN DEVELOPMENT
EARLY UNINUCLEATE
LATE UNINUCLEATE
BINUCLEATE
TRINUCLEATE
25°C
ISOLATION AND CULTURE
32.5°C
MICROSPORE EMBRYOGENESIS
GLOBULAR
HEART
TORPEDO
COTYLEDON

not exhibit this response. Microspores from earlier stages swell only slightly, whereas those isolated well past microspore mitosis appear oval, tend to swell enormously and usually burst within a few days in culture. Neither of these stages is embryogenic.

Interestingly, embryogenic microspores often appear starch-free and contain distinct organelle-free regions (Kott *et al.*, 1988; Telmer *et al.*, 1993). This is similar to the ultrastructural profile observed in tobacco pollen after the starvation treatment.

In heat-treated embryogenic microspores the first mitotic division toward microsporic embryo development takes place in vegetative nucleus and, in contrast to the first pollen mitosis, is symmetric in nature (Fan *et al.*, 1988; Zaki and Dickinson, 1990, 1991; Hause *et al.*, 1993). Continued mitotic divisions give rise successively to globular, heart, torpedo and cotyledon stage embryos which are morphologically similar to and accumulate the same seed-specific storage products as zygotic embryos (Crouch, 1982; Pomeroy *et al.*, 1991; Taylor *et al.*, 1990; Wiberg *et al.*, 1991).

1.3 Gene Expression During Embryo Development

1.3.1 Zygotic Embryos

Using RNA-excess and DNA/RNA hybridization experiments Goldberg *et al.*, (1981) and Galau and Dure (1981) have shown that approximately 30,000 genes are expressed during soybean and cotton embryogenesis. Most of the mRNAs are rare sequences that are present in a few copies per cell, a few thousand mRNAs are low to moderately abundant, while only a few hundred mRNAs are considered to be abundant. The majority of these seed mRNAs are expressed during the maturation stage, are stored in mature embryos and are also present in germinated seedlings and in the mature plant.

Genes expressed during embryo development can be organized into a number of groups based both on the timing of their expression and the nature of the products they encode (Dure, 1985; Goldberg *et al.*, 1989; Hughes and Galau, 1989). The first group includes constitutively expressed genes such as actin and tubulin. These genes are not embryo specific, but rather are expressed throughout the life cycle of the plant. The second group of genes is embryo specific. The expression of these genes is restricted to the embryo proper and ceases prior to germination. The third group of genes is expressed early in embryo development, during the period of rapid cell division and morphogenesis. The fourth group contains genes coding for the seed storage proteins. These genes are highly expressed during the maturation phase but are also expressed to a lesser extent during earlier and later stages. The fifth group contains genes coding for LEA proteins and is generally expressed late in embryogenesis, at the time of ovule abscission.

The majority of embryo expressed genes have been isolated from developmental stages that are accessible in large amounts. This has led to the preferential isolation of genes that are highly expressed during the mid to late stages of embryo development, such as those encoding seed storage and LEA proteins. As a result, very little is known about genes that are expressed during early zygotic embryo development.

Jurgens and coworkers performed a saturating genetic screen for putative embryo pattern mutants in *Arabidopsis*. These mutants exhibited disturbances in both the apical-basal and radial patterns. The phenotypes of at least two of these mutants, *gnom* and *monopteros* have been shown to occur very early in embryo development (Berleth and Jurgens, 1993; Mayer *et al*., 1993). The *gnom* gene appears to be required for the first asymmetric division of the zygote, while the action of the *monopteros* gene can be traced back to the octant-stage embryo. These embryo-pattern mutants represent the earliest acting genes known for zygotic embryogenesis; however, none of the genes responsible for these mutations has been cloned.

Of all the genes that are expressed during embryo development perhaps the best characterized are those that code for the seed storage proteins. Seed storage proteins differ from other seed proteins in that they are generally synthesized and accumulate to very high levels only within the developing seed. In dicots, storage proteins are deposited in protein bodies derived from the central vacuole of the storage parenchyma cells (Lott, 1980). Protein bodies are generally located in the endosperm of monocots and throughout the embryo in dicots. After germination the seed storage proteins are broken down within the protein bodies by a variety of hydrolytic enzymes including both endo- and exo-proteases (Müntz *et al.*, 1985; Shutov and Vaintraub, 1987; Ericson, 1988).

Seed storage proteins generally lack enzymatic activity and have therefore been classified on the basis of both their sedimentation coefficient values and their solubility characteristics (Osborne, 1924). The four solubility groups for seed storage proteins are the albumins (water soluble), the globulins (soluble in mild salt solutions), the prolamins (soluble in aqueous alcohol) and the glutelins (soluble in weakly acidic or alkali solutions). In general, dicotyledonous plants mostly contain albumin and globulin type seed storage protein, whereas monocotyledonous plants contain the prolamins and glutelins.

Seed storage protein genes are expressed to high levels in a developmentally-regulated, tissue-specific manner, and therefore provide a model system with which to study the regulation of plant gene expression. A great deal of variability exists with respect to the expression of seed storage protein genes; however, a number of common characteristics are evident.

Seed storage protein genes are generally expressed exclusively within the developing embryo and/or endosperm, although in a few cases, seed storage mRNAs or proteins have been detected in nonseed tissues (Finkelstein & Crouch, 1984; Ueng *et al.*, 1988; Quattrocchio *et al.*, 1990). Neither the significance of this phenomenon nor the extent of its occurrence has been investigated.

Seed storage proteins are generally encoded by multigene families that vary in size, organization, complexity and chromosomal location. Individual members within a multigene family may show different temporal and spatial patterns of expression. For example, in *Arabidopsis thaliana*, the 2S albumins are encoded by a small multigene family consisting of 4 members, At2S1 to At2S4 (Krebbers *et al.*, 1988). Although the At2S sequences are all temporally regulated in a similar fashion, they appear to have different tissue specificities; At2S2, At2S3 and At2S4 are expressed throughout the entire embryo whereas At2S1 expression is restricted to the embryonic axis (Guerche *et al.*, 1990). Similar observations have been made with the 12S globulins of *B. napus* (Sjödahl *et al.*, 1993).

Seed storage protein mRNA usually begins to accumulate at an early stage, while cell division is still occurring. The mRNA levels reach a maximum during the period when cell division ceases, remain high for many weeks, and decline to low or negligible levels in quiescent seeds (for reviews see

Crouch, 1988, Goldberg *et al.*, 1989; Shirsat, 1991; Thomas, 1993).

Seed storage protein genes have been shown to be regulated at both the transcriptional and posttranscriptional level, although transcriptional control appears to be the major regulatory process. Most seed storage protein genes are transcriptionally activated at very low levels during early embryo development, transcribed at much higher rates during the maturation stage and are transcriptionally repressed as the seed becomes quiescent (Bostan *et al.*, 1986; Gatehouse *et al.*, 1986; Walling *et al.*, 1986; Harada *et al.*, 1989).

Posttranscriptional processes also play an important role in the accumulation of seed storage protein mRNAs (Goldberg *et al.*, 1989). These processes have been shown to affect both mRNA prevalence and temporal patterns of mRNA accumulation during development. In soybean, mRNAs corresponding to four glycinin genes accumulate and decay at the same time during development. Their transcription is also co-ordinately activated and repressed at the same times (Neilson *et al.*, 1989). In contrast, two different soybean ß-conglycinin mRNAs accumulate and decay at different times during development, whereas their corresponding genes are transcriptionally activated and repressed in a co-ordinate manner (Goldberg *et al.*, 1989). Post-transcriptional regulation may take the form of differential processing of the primary transcript, selective nuclear transport of specific mRNAs or differential mRNA stability.

Gene expression is regulated, in part, by a number of sequences lying in the promoter region of the gene. These *cis*-acting sequences are believed to bind specific *trans*-acting nuclear protein factors which activate or repress transcription of the gene. It is generally assumed that the promoter region of a developmentally-regulated gene or set of genes contains specific segments of *cis*-acting DNA which are responsible for directing the developmental or tissue specificity of the gene.

One method for determining a role for putative *cis* elements is to mutate the promoter of the gene of interest, usually by deletion analysis, reintroduce the altered gene into the plant and study its pattern of expression *in vivo*. Due to the absence of suitable transformation and regeneration systems for most plants, the altered genes are usually introduced into dicots such as tobacco by *Agrobacterium*-mediated transformation. In these experiments the detection of gene activity is often facilitated by replacing the coding region of the gene of interest with the coding region of a reporter gene such as the neomycin phosphotransferase II gene (*nptII*) from Tn5 (Fraley *et al.*, 1983), the chloramphenicol acetyl transferase gene (*cat*) from Tn9 (Herrera-Estralla *et al.*, 1983), the firefly or *Vibrio harveyi* luciferase genes (*luc* and *lux*, respectively; (Ow *et al.*, 1986; Koncz *et al.*, 1987), or the ß-glucuronidase (*gusA*) gene from *E. coli* (Jefferson *et al.*, 1986). These enzymatic activities are generally present in low or negligible amounts in non-transformed plants.

Deletion analysis of the promoters of a number of seed storage protein genes has shown that in most cases only a few hundred base pairs of upstream sequence are required for the correct tissue specificity and temporal regulation of the genes; however, the addition of further upstream sequences often enhances the level of expression. These observations have lead to the development of a bipartite model for the regulation of seed protein gene expression (Thomas *et al.*, 1991; Thomas, 1993; Nunberg *et al.*, 1994). In this model, proximal promoter regions confer seed specific expression while more distal promoter regions function to refine and enhance the basic expression patterns conferred by the proximal regions.

A number of putative proximal control elements can be identified within the genes coding for similar types of storage proteins. These include the endosperm box, a 20 bp conserved element found in almost all of the promoters of the cereal prolamine seed storage proteins (Forde *et al.*, 1985), as well as other endosperm-specific genes such as the maize sucrose synthetase gene (Werr *et al.*, 1985), and the vicilin box, a 42 bp motif found in the promoters of various 7S globulin genes (Gatehouse *et al.*, 1986; Newbigin *et al.*, 1990).

A variety of potential *cis*-acting distal elements have also been identified in the promoter regions of seed storage protein genes. These elements are thought to modulate level, timing and spatial boundaries of seed protein gene expression. Among these *cis*-acting elements are the legumin box, a 28 bp concensus sequence found in the promoter region of 11S seed storage protein genes of legumes (Bäumlein *et al.*, 1986); the RY motif, an 8 bp motif that is prevalent in the promoters of seed storage protein genes (Bäumlein *et al.*, 1992; Chamberland et al., 1992; Leleivre et al., 1992); and a variety of A/T rich enhancer motifs (Allen *et al.*, 1989; Bustos *et al.*, 1989; Jordano *et al.*, 1989). The G-box motif, a common sequence element of plant promoters (Williams *et al.*, 1992), is thought to mediate ABA-induced expression in several embryo-abundant genes (Gomez et al., 1988).

Binding of *trans*-acting nuclear proteins to potential *cis*-acting sequences of seed storage protein genes has been widely observed (Jordano *et al.*, 1989; Allen *et al.*, 1989; Bustos *et al.*, 1989); however, for the most part there is no direct evidence for the involvement of these sequences in the regulation of gene expression. One clear example of a *trans*-acting protein that is involved in the regulation of seed storage protein gene expression is that of the O2 protein encoded by the *opaque 2* locus of maize. Mutations in this locus result in a drastic reduction of one class of seed storage proteins, the 22 kD zeins, but not the 19 kD zeins (Tsai *et al.*, 1978). The *opaque-2* allele (*o2*) encodes a protein which contains a leucine zipper DNA binding domain. This domain is characteristic of a class of mammalian and fungal transcription factors (Schmidt *et al.*, 1987; Motto *et al.*, 1988). An O2 binding site is present in a region of the endosperm box that is highly conserved in the 22 kD zeins, but which is divergent in the 19 kD zeins. O2 may also interact with a second leucine zipper protein, OHP1, to activate zein gene expression (Pysh *et al.*, 1993).

1.3.1.1 *B. napus* Seed Storage Proteins

Mature *B. napus* seeds contain 20-25% protein, 60 to 70 % of which consists of both the neutral 12S globulins (cruciferin) and the basic 1.7S albumins (napin). The ratio (w/w) of cruciferin to napin in mature seeds varies from cultivar to cultivar but is approximately 2.5:1. Napin is a 13 kD protein consisting of two subunits (9 and 4 kD) held together by two disulphide bonds (Crouch *et al.*, 1983). This protein is initially synthesized as a 20 kD polypeptide product which subsequently undergoes a number of processing steps. Processing of the 20 kD precursor involves the cotranslational removal of the endoplasmic reticulum signal peptide as well as the posttranslational cleavage of two acidic peptides and a carboxy terminal tyrosine (Ericson *et al.*, 1986). These post-translational modifications are believed to occur in the protein bodies (Ericson *et al.*, 1986).

Napin-like seed storage proteins have been characterized in a number of plants other than *B. napus* and are collectively referred to as 2S albumins. 2S albumins have been described in other members of the Cruciferae, including other *Brassica* species (Finlayson, 1976), *Raphanus sativus* (LaRoche *et al.*, 1984), *Sinapis alba* (Kirk & Pylotis, 1976) and *A. thaliana* (Krebbers *et al.*, 1988), as well as more distantly related species such as castor bean (Sharief & Li, 1982) and Brazil nut (Ampe *et al.*, 1986). Napin-like proteins have even been characterized in the fern *Matteuccia struthiopteris* (Templeman *et al.*, 1988). The similarity among these napin-like proteins lies more in the placement of cysteine residues than in nucleotide sequence similarity (Krebbers *et al.*, 1988).

In *B. napus*, napin is encoded by a small gene family that is estimated to contain 10 to 16 members (Joseffsen *et al.*, 1987; Scofield & Crouch, 1987). Of the possible 16 members, five genomic and three cDNA clones have been isolated (Table I). All of the genomic clones lack introns. These napin sequences were isolated from a number of different cultivars and, in the case of the cDNAs, under a variety of growth conditions.

All of the isolated napin clones are highly conserved at the nucleotide level (90-97%); however, there are minor differences in both the length and the deduced amino acid content of the proteins. The differences at the amino acid level among the various napin clones points to the existence

TABLE I Cloned 2S Albumin Sequences of the Cruciferae

GENE	SPECIES	TYPE OF CLONE	ACCESSION NUMBER	REFERENCE
At2S1	*A. thaliana*	genomic	M22032	Krebbers *et al.*, 1988
At2S2	*A. thaliana*	genomic	M22034	Krebbers *et al.*, 1988
At2S3	*A. thaliana*	genomic	M22035	Krebbers *et al.*, 1988
At2S4	*A. thaliana*	genomic	M22033	Krebbers *et al.*, 1988
napA	*B. napus*	genomic	J02798	Josefsson *et al.*, 1987
Na	*B. napus*	genomic	J02782	Scofield and Crouch, 1987
napB	*B. napus*	genomic	X58142	Baszczynski and Fallis, 1990
napB'	*B. napus*	genomic	X14492	unpublished
N1	*B. napus*	cDNA	K01544	Crouch *et al.*, 1983
N2	*B. napus*	cDNA	K01545	Crouch *et al.*, 1983
Bc2Sc	*B. rapa*	PCR amplified protein coding region	X65037	unpublished
Bo2SC	*B. oleracea*	PCR amplified protein coding region	X65038	unpublished
Bj2SC	*B. juncea*	PCR amplified protein coding region	X65040	unpublished
radnapA	*R. sativus*	cDNA	M36630	Raynal *et al.*, 1991
radnapB	*R. sativus*	cDNA	M36629	Raynal *et al.*, 1991
radnapC	*R. sativus*	cDNA	M36630	Raynal *et al.*, 1991

of a number of napin isoforms. The presence of napin isoforms is supported by the work of Lonnerdal and Janson (1972) who were able to isolate four discrete protein species from mature seeds of *B. napus*.

The pattern of napin protein and mRNA accumulation has been studied in zygotic embryos of *B. napus*. Although the timing of the induction of napin gene expression varies from study to study depending on the plant growth conditions and the cultivars used, in general napin protein can first be detected in zygotic embryos at approximately 20 days after pollination (DAP). Protein accumulation peaks by 30 to 35 DAP, and ceases approximately 20 days later, at the beginning of the desiccation phase (Ericson *et al.*, 1989; Höglund *et al.*, 1991). Napin protein accumulation begins in the embryo axis and then spreads toward the cotyledons, where it accumulates first in the outer and then in the inner faces of the cotyledons (Höglund *et al.*, 1991). Low levels of napin can also be detected in the endosperm of cotyledon stage embryos (Höglund *et al.*, 1991).

Although napin mRNAs begin to accumulate slightly earlier than the protein, their spatial distribution throughout embryo development is almost identical to that of the protein (Fernandez *et al.*, 1991). Expression of one member, Na (Scofield and Crouch, 1987), has been shown by RNAse protection assays to peak and decline earlier than other members of the napin gene family (Blundy *et al.*, 1991).

Run-on transcription experiments have shown that, depending on the developmental stage, napin gene expression can be regulated either transcriptionally or post-transcriptionally (DeLisle & Crouch, 1989). For example, during the maturation stage of embryo development both napin mRNA levels and transcription rates remain high; however, as the embryos begin to dehisce the mRNA levels fall while the transcription rate remains approximately the same.

Common sequence motifs, that may be involved in the regulation of expression of napin genes, have been identified in the 5' flanking regions of a number of *B. napus* napin genes (Ericson *et al.*, 1991; Gustavsson *et al.*, 1991; Stälberg *et al.*, 1993). Chief among these are a G-box like motif, an Opaque-2-like binding site, and an RY-like repeat that is also part of a longer repeated motif. A number of these motifs have been shown to bind nuclear proteins *in vitro* (Ericson *et al.*, 1991; Gustavsson *et al.*, 1991).

Studies on deletion derivatives of *B. napus* promoters (Radke, *et al.*, 1988; Stälberg *et al.*, 1993) support the bipartite model of seed-protein gene regulation in which proximal promoter regions confer seed specific expression while the more distal promoter regions control the level of expression. These results indicated that all of the information necessary to direct correct expression of at least two napin genes is located within a 200 to 300 bp region contiguous to the transcription start site.

Interestingly, the common sequence motifs identified above are also found within this region.

1.3.2 Somatic Embryos

A study by de Vries *et al.* (1988) using 2-D gel electrophoresis of *in vitro* translated carrot mRNAs revealed that only a small number of abundant proteins differ between hypocotyl explants, nonembryogenic starting cell cultures, embryogenic cultures containing PEMs and somatic embryos. These results suggest that the process of dedifferentiation, induction and initiation of somatic embryogenesis does not involve large changes in the pattern of gene expression. Nonetheless, it has been possible to isolate a number of genes that are differentially expressed in carrot somatic embryos as compared to callus cell cultures (reviewed in Sterk and de Vries, 1993; Zimmerman, 1993). Many of the genes expressed during the early stages of carrot somatic embryo development have proven useful in characterizing similar gene expression programs that occur in their zygotic counterparts.

The expression of at least three carrot genes, Dc3, ECP31 and ECP40, is correlated with the acquisition of embryogenic potential (Wilde *et al.*, 1988; Kiyosue *et al.*, 1992, 1993). These genes are highly expressed in PEMs but are not expressed in non-embryogenic cell cultures. These genes are expressed up to the torpedo stage in somatic embryos. The Dc3, ECP31 and ECP40 genes all encode LEA proteins. The expression profiles of LEA proteins in somatic embryos does not appear to mimic those seen in zygotic embryos. Furthermore, it is not known how these genes function during somatic embryogenesis since, in zygotic embryos, LEA proteins are thought to act as hydration agents to protect cellular components of the embryo from desiccation. Expression of at least one LEA gene, EMB-1, has been shown by *in situ* hybridization to begin during the globular stage in both somatic and zygotic embryos (Wurtele *et al.*, 1993). It is possible then, that other LEA proteins may normally be expressed during the early stages of embryo development, but that more sensitive techniques are required to detect this expression.

A number of other genes have been isolated that are expressed during the early stages of carrot somatic embryo development. Aleith and Richter (1990) used a differential screening approach to isolate genes that were transiently expressed upon removal of 2,4-D from the culture medium. Expression of these genes in somatic embryos began at the globular or heart stages of development and, for a few of the genes, continued through to the torpedo stage. Three genes encoding potential cell wall proteins rich in either glycine or proline were among those identified. The role that they play in embryo development has not been determined.

Carrot somatic embryo cultures have been shown to secrete a wide variety of compounds into the culture medium. The resulting "conditioned medium" harbours polysaccharides, proteoglycans and proteins that have been shown to be associated with both embryogenic and nonembryogenic cells (for reviews see Van Engelen and de Vries, 1992; Sterk and de Vries, 1993). A number of genes encoding proteins secreted into the conditioned medium have recently been characterized. The EP1 gene, which encodes a protein with regions similar to the *Brassica* S locus glycoprotein gene, is expressed in nonembryogenic suspension cells, in somatic embryos up to the torpedo stage and in germinated seedlings, but not in PEMs or zygotic embryos (Van Engelen *et al.*, 1991). The EP2 gene encodes a lipid transfer protein that is secreted by PEMS as well as both somatic and zygotic embryos. *In situ* hybridization demonstrated that EP2 expression was restricted to the peripheral cells of PEMS and to the protoderm cells of somatic and zygotic embryos. The EP2 protein may be involved in the export of cutin monomers at the epidermal cell surface (Sterk *et al.*, 1991). EP2 expression is not specific to embryos as it is also expressed in epidermal cells of the shoot apical meristem, leaf primordia and floral organs. Nonetheless, EP2 expression is significant in that it marks the appearance of the first differentiated tissue of the embryo i.e. the protoderm.

1.3.3 Gametophytic Embryos

In contrast to carrot somatic embryo culture, the molecular events underlying the transition from pollen to embryo development have not been examined in detail; however, preliminary investigations have been carried out in a few species. In tobacco, *de novo* transcription of specific genes is thought to occur during the starvation treatment of pollen. Although no newly induced proteins were detected by 2-D gel electrophoresis of protein extracts from mid-binucleate pollen cultured in starvation medium (Kyo and Harada, 1990; Garrido *et al.*, 1993), at least 2 new acidic proteins could be detected by 2-D gel electrophoresis of their *in vitro* translated mRNAs (Garrido *et al.*, 1993). The fact that these proteins were not detected using protein extracts from the same samples suggest that their mRNAs are not translated *in vivo*. This observation is analogous to the storage of preformed maternal mRNAs as ribonucleoproteins in unfertilized animal eggs and suggests that the starvation induced mRNAs are translated only after transfer to nutrient rich medium.

Pechan *et al.* (1991) have shown that a number of mRNAs and proteins are also differentially expressed within the first 8 hours of heat treatment of *B. napus* microspores (Pechan *et al.*, 1991). It was suggested, on the basis of molecular weight comparisons, that some of these proteins may correspond to known heat shock proteins. Although the evidence for this specific conclusion is weak, it does suggest, as in tobacco, that the switch from pollen to embryo development in culture is accompanied by early changes in the pattern of gene expression. Whether these changes occur in response to the conditions used to induce embryogenesis or whether they are directly involved in the process of embryo induction remains to be elucidated.

Reynolds and Kitto (1992) used differential screening of a cDNA library to isolate sequences with enhanced expression in embryogenically induced pollen and pollen embryos of wheat anther cultures. While the majority of cloned mRNAs were also expressed in vegetative tissues, a number of embryo-specific clones were obtained. Each of the pollen embryo genes was expressed at different times during development. Two of the clones, pEMB4 and pEMB94, were shown to be expressed during the very early stages of culture as well as during subsequent stages of pollen embryo development, while expression of the other three clones was restricted to developing embryos. The identity of these cDNA clones was not determined.

Gene expression studies during the later stages of pollen/microsporic embryo development have focused primarily on genes encoding embryo-specific storage products, such as seed storage proteins. During *B. napus* microspore embryogenesis the pattern of storage protein gene expression in microsporic embryos appears to parallel that seen in zygotic embryos, although differences in the extent of protein accumulation have been observed (Crouch, 1982; Taylor *et al.*, 1990; Wilen *et al.*, 1990). For example, Taylor *et al.* (1990) observed that in microsporic embryos the level of napin mRNA was approximately 40 times lower than that in zygotic embryos of the same stage; however, the addition of 10 µm ABA increased the level of napin message to that in zygotic embryos. Despite the effect of ABA on napin gene transcription, no concomitant increase in napin protein was observed. It is not known whether these differences are due to the culture conditions or whether they are due to some inherent difference between microsporic and zygotic embryos.

1.4 Thesis Objectives

In vitro embryo culture systems have been used extensively in the selection of variants and clonal production of crop plants and as tools for studying numerous aspects of embryo-specific processes (Morrison and Evans, 1988; Taylor *et al.*, 1990; Wiberg *et al.*, 1991). Despite the widespread use of embryo culture sytems, relatively little is known about the molecular and physiological mechanisms underlying the ability of differentiated cells to form embryos in culture. This lack of understanding is especially true for gametophytic embryo culture systems.

The goal of this thesis is to isolate a number of genes whose expression is associated with induction of *B. napus* microspore embryogenesis. The *B. napus* microspore embryogenesis system is one of the most efficient embryo culture systems and has been reasonably well characterized at the cytological and ultrastructural levels. An added advantage of working with *B. napus* is that both pollen and embryo development have also been well described in this organism.

The isolation and characterization of molecular markers for the very early stages of *B. napus* microspore embryogenesis is of importance for a number of reasons. Firstly, these markers may provide insight into the mechanism whereby a previously differentiated cell is induced to switch developmental pathways and form an embryo *in vitro*. This insight may be gained either through analysis of the nature and deduced role of protein products encoded by the marker genes or through the use of these genes as "traps" for the isolation of important *cis*-acting elements and transcription factors that function during the induction period. From a more applied point of view, expression of these markers could also be used to enhance the various tissue culture manipulations and hence the frequency of embryogenesis well before the appearance of actual embryos. This becomes particularly feasible when the marker gene is used in conjunction with a reporter gene such as GUS. Finally, the isolation and characterization of molecular markers for the induction of *B. napus* microspore embryogenesis may also provide insight into the mechanism underlying the induction of embryogenesis in a variety of *in vitro* embryo systems.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Plant Growth Conditions and Collection of Plant Tissues

2.1.1 Greenhouse Grown Plants

Plants were grown in a 16- to 18-h photoperiod under sunlight supplemented with high pressure sodium lamps (600 $\mu E/m^2/s$) in a soil:peat moss:sand:perlite (1:2:1:1) mixture containing small amounts of lime and superphosphate. Plants were watered twice daily and fertilized weekly with Hoaglands solution. The greenhouse temperature ranged from approximately 18 to 32 ^{o}C.

The flowering stalks of *B. napus* and tobacco plants were covered with bags to prevent cross pollination. Flowers were hand pollinated and tagged on the day of anthesis. Seed samples were collected on various days after pollination (DAP). Whole seeds were collected (for RNA extraction and GUS fluorogenic analysis) from developing tobacco and *B. napus* fruits using either a scalpel (tobacco) or watchmakers forceps (*Brassica*). The samples were immediately frozen in liquid nitrogen and stored at -80 ^{o}C. Individual tobacco and *B. napus* embryos used for GUS histochemical analysis were excised by hand under a dissecting microscope, using watchmakers forceps and a scalpel, and then assayed immediately.

Somatic tissues (for RNA extraction and GUS fluorogenic analysis) were collected from greenhouse-grown plants and immediately frozen in liquid nitrogen.

2.1.2 Microspore Isolation and Culture

2.1.2.1 Growth Conditions of Donor Plants

Donor plants for microspore culture (*Brassica napus* cvs. Topas and Westar) were grown in a growth cabinet at 20°/15°C (day/night) with a 16 h photoperiod (400 $\mu E/m^2/s$) provided by VHO cool white fluorescent lamps (165W, Sylvania) and incandescent bulbs (40W, Duro-test). Four weeks after germination the plants were transferred to either embryogenic (10°/5°C day/night) or non-embryogenic (25°/20°C day/night) conditions.

2.1.2.2 Microspore Isolation

Brassica napus cvs. Topas and Westar microspores were isolated and cultured essentially as described in Keller *et al*. (1987). Flower buds, 3.0 to 5.0 mm in length, were harvested and surface sterilized for 10 to 15 min by immersion in 7% (w/v) calcium hypochlorite. Buds were then rinsed 3 times, for 10 min each time, in sterile dH_2O. Sterile buds were then transferred to a 50 ml beaker containing 5 ml B5 medium (Gamborg *et al*., 1968) without hormones and 13% (w/v) sucrose (= B5-13 medium). Microspores were released from the anther by macerating the buds against the sides and bottom of the beaker with a sterile flat-bottomed ground-glass rod. The macerate was filtered through a 44 µm nylon screen into a 50 ml Erlenmeyer flask and rinsed with a few ml of B5-13 medium. The microspores were transferred from the flask to 16 x 100 mm glass-capped tubes and centrifuged at 1000 rpm for 3 min. The microspores were washed and centrifuged (100g, 3 min) 3 more times in 10 ml B5-13 and then resuspended in 1/2 NLN-13 (1/2X NLN salts plus 13% sucrose (w/v); Keller *et al.*, 1987) at a density of 50,000 to 100,000 microspores/ml. Ten ml of microspore suspension was added per Petrie dish (100 x 15 mm) and the plates sealed with Parafilm (American Can Co., Greenwich, CT).

2.1.2.3 Microspore Culture

Microspores were induced to undergo embryogenesis by culturing them for 4 days at 32.5 °C in the dark and then transferred to 25 °C for the remainder of the culture period. Uninduced cultures, i.e. microspores continuing pollen development, were cultured for four days at 25 °C.

Plates containing microsporic embryos at the globular stage of development (approximately 10 days after the start of culture) were transferred to a rotary shaker set at 50 rpm. Heart, torpedo and cotyledon stages embryos were visible at approximately 12, 14 and 21 days after the start of the culture period. After 21 days in culture, cotyledon-stage embryos were transferred to a maturation medium consisting of 1/2X NLN salts, 1% sucrose, 0.35 M mannitol and 5 µM ABA.

2.1.2.4 Microsporic Embryo Collection

Microspores and microsporic embryos were collected for RNA extraction and GUS histochemical analysis. Microspores were isolated as described above and either frozen in liquid nitrogen and stored at -80 °C (RNA extraction) or assayed immediately (GUS histochemical analysis). Microsporic embryos used for RNA extraction were collected at the globular, heart, torpedo and cotyledon stages of development by filtration through nylon membranes of various pore sizes as described in Giulano *et al.*, (1983). Globular, heart, torpedo and developing cotyledon stage embryos were collected on 62 to 82 µm meshes, 125 to 200 µm meshes, 250 µm meshes and 500 µm meshes respectively. The populations were from 75 to 95% pure. Samples were immediately frozen in liquid nitrogen and stored at -80 °C. Microsporic embryos used for GUS histochemical analysis were collected by hand using either a Pasteur pipet or a small paintbrush and assayed immediately.

2.2 Growth and Storage of Bacterial Strains

All *Escherichia coli* bacterial strains were grown in LB (10g Bacto-Tryptone, 5g Bacto-Yeast extract, 5g NaCl, pH

7.5) with the exception of host cultures for packaging and plating of lambda DNA which were grown in LB supplemented with 0.2% maltose. Bacterial strains to be stored were grown overnight in 50 ml LB, pelleted, resuspended in 40% glycerol, 5 mM $MgSO_4$ and stored at -20 °C and -80 °C.

E. coli strains DH5α and DH5αF' were used as hosts for the propagation of plasmid DNAs. *E. coli* strains WA802 (Raleigh *et al.*, 1988)and LE392 (Promega, Madison, WI) were used as hosts for Lambda-GEM4.

The plasmid cloning vector was pGEM4Z (Promega Madison, WI)

2.3 Preparation of Competent Cells and Bacterial Transformation

Competent cells were prepared using the "Frozen Storage of Competent Cells, Protocol 2" method, exactly as outlined by Hanahan (1985).

Approximately 5 to 25 ng of plasmid DNA was used to transform competent cells. DNA was added to 50 to 100 µl of frozen cells that had been thawed on ice. The mixture was incubated for 30 min on ice and then heated at 37 °C for 5 min. The cells were placed on ice for 2 min and then added to 900 µl SOC (2 g/l Bacto-Tryptone, 0.5 g/l Bacto-Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). The culture was incubated at 37 °C for 45 to 60 min with moderate aeration. Aliquots were plated on selective media and incubated overnight at 37 °C. Control samples were included to monitor the efficiency of transformation as well as the efficiency of restriction digests and ligations.

2.4 DNA Isolation Procedures

2.4.1 Isolation of Plant Genomic DNA

Genomic DNA was isolated from *B. napus* and tobacco leaf tissue using a modified version of the method described in Sanders *et al.* (1987). Approximately 2.5 g of leaf tissue was frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle with 0.2 g aluminum oxide per gram fresh weight of tissue. Ten ml of extraction buffer (50 mM Tris-HCl pH 8, 50 mM EDTA pH 8, 50 mM NaCl, 1% (w/v) sarcosyl, 400 µg ethidium bromide) was added. The tissue was ground gently for another 5 min and then centrifuged for 5 min at 5000 rpm. Cesium chloride was added to the supernatant at approximately 1 mg/ml. The samples were transferred to a Quick seal tube (Beckman Instruments Inc., Toronto, ON) and centrifuged 18 to 20 h at 60,000 rpm. The DNA band was collected under UV light and extracted several times with 10 mM Tris-HCl (pH 7.5) saturated iso-propanol or n-butanol. Two volumes of ddH_2O and two volumes of 95% ethanol were used to precipitate the DNA out of solution. The DNA was collected immediately or after overnight precipitation at

-20 °C. The DNA was removed using a bent Pasteur pipette or by centrifugation (30 min, 7000 rpm), then rinsed in 70% ethanol, air dried briefly, and resuspended in TE (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8) at a concentration of 250 ng/µl.

2.4.2 Isolation of Plasmid DNA

Large scale preparations of plasmid DNA were isolated from *E. coli* using the alkaline lysis procedure of Birnboim and Doly as described in Maniatis *et al.* (1982). Small scale plasmid preparations were performed using the Magic Miniprep or Wizard Miniprep kits from Promega (Madison, WI).

2.4.3 Isolation of Lambda DNA

Lambda DNA was isolated using a modified version of the plate lysate method of Sambrook *et al.* (1989). A single plaque was picked using a Pasteur pipette and placed in 1 ml of phage buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$) with a drop of chloroform. The mixture was vortexed and left at room temperature for 30 min to allow for diffusion of the phage particles. The solid debris was removed by centrifugation (7000 rpm, 15 min). 100 µl of the supernatant was mixed with 100 µl of *E. coli* strain WA802 and incubated at 37 °C for 20 min. Molten top agarose (2.5 mls; 10 g/l Bactotryptone, 5 g/l NaCl, 6 g/l agarose, 10 mM

$MgSO_4$) was added to the mixture and the contents spread over the surface of an LB agarose plate (LB with 0.6% agarose). The plates were incubated at 37 °C for 9-11 hr. Six ml of lambda diluent (10 mM Tris-HCl, pH7.5, 10 mM $MgSO_4$) was added and the plates gently shaken for 2 hr at room temperature. The supernatant was centrifuged (8000 rpm, 10 min, 4 °C) to remove the bacterial debris and then treated with RNaseA (10 μg) and DNaseI (10 μg) for 45 min at 37 °C. An equal volume of PEG/NaCl solution (20% w/v polyethelene glycol 8,000, 2 M NaCl in lambda diluent) was added, the mixture vortexed and then incubated for 1 hr in an ice water bath. The precipitate was recovered by centrifugation (10,000 g, 10 min, 4 °C), briefly air dried and then resuspended in 500 μl TE. Five μl of 10% SDS was added and the mixture incubated for 5 min at 68 °C. The mixture was extracted once with an equal volume of phenol, once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and then once with an equal volume of chloroform:isoamyl alcohol (24:1). The phage DNA was precipitated with 1 volume of isopropanol for 15 min at -80 °C and then centrifuged for 30 min at room temperature. The pellet was washed with ice cold 70% ethanol, air dried and then redissolved in 100 μl TE.

2.4.4 Isolation of DNA Fragments

DNA restriction fragments were isolated from TAE agarose gels using the GENECLEAN kit as recommended by the manufacturer (BIO 101 Inc., LaJolla, CA).

2.5 RNA Isolation Procedures

2.5.1 Isolation of Total RNA

Total RNA was isolated from *B. napus* tissues using a guanidinium isothiocyanate procedure adapted from Chirgwin *et al.* (1979) and Glisin *et al.* (1974). Approximately 1 g of frozen tissue was ground in a frozen mortar and pestle with a small piece of dry ice. The ground powder was resuspended in at least 3 volumes of ice cold homogenization buffer (4 M guanidinium isothiocyanate, 25 mM Na citrate, 1 mM EDTA, pH 8, containing 150 µl antifoam A and 100 µl β-mercaptoethanol per 10 ml of buffer). The sample was homogenized on ice for 1 min at high speed using a Polytron Omnimixer (Brinkman Instruments, Waterbury, CT). 20% sarcosyl was added to the homogenate at 1 ml/10 ml of sample volume and the tissue homogenized for another minute. The homogenized sample was centrifuged at 7000rpm for 15 min at 4 °C until the supernatant was clear. The supernatant was loaded onto 2 ml of CsCl solution (5.7 M CsCl dissolved in 0.1 M EDTA, pH 7.0) in polyallomer tubes and centrifuged at 30,000 rpm for 14 to 42 h at 18 °C in a swinging bucket rotor. Following centrifugation the RNA pellet was resuspended in 500 µl of a solution containing 7.5 M guanidine hydrochloride, 0.025 M sodium citrate in 5 mM dithiothreotol, adjusted to pH 7. The RNA was precipitated at -20 °C with 0.025 volumes of 1 M acetic acid and 0.5 volumes ethanol. The precipitate was centrifuged for 30 min, rinsed with 70% ethanol, dried slightly under vacuum and then resuspended in 200 µl ddH$_2$O by alternately vortexing and heating (60 °C) the pellet over 1 min intervals. The mixture was centrifuged for 5 min at room temperature and the supernatant combined with 250 µl ddH$_2$O. This procedure was repeated until no RNA remained in the pellet. The resuspended RNA was precipitated overnight at -20 °C with 0.3 volumes 10 M sodium acetate and 2 volumes of ethanol. The mixture was centrifuged for 30 min at 4°C, washed with 70% ethanol, dried under vacuum and then resuspended in ddH$_2$O at a concentration of 1 µg/ml.

2.5.2 Isolation of Poly(A)$^+$ RNA

Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972) as described in Maniatis *et al.*(1982).

2.6 Restriction Digests

Plasmid and genomic DNA digests were carried out in buffers supplied by the restriction enzyme manufacturers. Lambda DNA digests were carried out in 1X TA buffer (O'Farrell *et al.*, 1980; 33 mM Tris-acetate, pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DTT, 100 μg/ml gelatin). Approximately 2, 3 and 5 units of restriction enzyme/μg DNA were used to digest lambda, plasmid and genomic DNAs respectively. Restriction digests were carried out at 37 °C for 1.5 to 2 hrs (lambda and plasmid DNA) or 3 to 6 hrs (plant genomic DNA). Restriction enzymes were inactivated by heating the samples to 65 °C for 15 min.

2.7 Probe Synthesis

2.7.1 Random Primers Labeling of Long DNA Probes

Restriction fragments isolated from agarose gels were radiolabelled with [α-^{32}P]dCTP (3,000 Ci/mmol) by the random primer method of Feinberg and Vogelstein (1983) using kits purchased from either BRL (Burlington, ON) or Pharmacia (Montreal, PQ). Unincorporated nucleotides were removed using Sephadex G-50 Nick Columns (Pharmacia, Montreal, PQ).

Probes were denatured prior to hybridization by heating them in a 95 °C water bath for 10 min and then cooling them on ice for 5 min.

2.7.2 End Labeling of Oligonucleotides

The sequence of the redundant oligonucleotide probe used to detect the MiNAP sequences is as follows: 5' AGC CTC AAA [C/T]AT CCT AGA TAA GAC CTT ACA TAT TAC G[A/C]G 3'. Twenty-four pmol of the oligonucleotide was end-labeled for 30 min at 37 °C in a 30 µl reaction mixture containing 10 units of T_4 polynucleotide kinase (Pharmacia, Montreal, PQ), 1X One-Phor-All Buffer PLUS (Pharmacia, Montreal, PQ) and 130 µCi of [γ-^{32}P]ATP (specific activity 5000 Ci/mmol; Amersham, Oakville, ON). The reaction was stopped by adding 1 µl of 500 mM EDTA, pH 7.8. Unincorporated nucleotides were removed using Sephadex G-50 Nick Columns (Pharmacia, Montreal, PQ) as per the manufacturers' recommendations.

The oligonucleotide probe was denatured prior to hybridization by heating it in a 95 °C water bath for 5 min and then cooling it in an ice-water bath for 5 min.

2.8 Agarose Gel Electrophoresis

2.8.1 DNA Electrophoresis

DNA restriction fragments were resuspended in 5X loading buffer (15% (w/v) Ficoll, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 0.2M EDTA) and separated by electrophoresis through agarose gels in TAE buffer. The concentration of the gels ranged from 0.7 to 1% depending on the size of the restriction fragments. DNA molecular weight markers were lambda *Hind* III restriction fragments. DNA was generally visualized by staining the gels in a 1 µg/ml ethidium bromide solution. Gels were destained by rinsing them in water (2X, 10 min each). When the fragment of interest was less than 500 bp ethidium bromide was added directly to the gel before pouring.

2.8.2 RNA Electrophoresis

RNA samples (5 µg) were prepared for electrophoresis as in Maniatis *et al.* (1982). RNA was separated by electrophoresis through 1% agarose gels containing formaldehyde (Lehrach *et al.*, 1977) and 1X MOPS buffer, pH 7(20 mM morpholinopropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA). The running buffer contained 1X MOPS buffer and 1.1 M formaldehyde. Ethidium bromide (5 µg/ml) was added to the RNA samples prior to electrophoresis. Following electrophoresis RNA gels were rinsed for 1 to 2 hrs in numerous changes of ddH_2O.

2.9 Gel Blot Analysis

2.9.1 DNA Gel Blots

DNA restriction fragments were transferred to BIOTRANS+ nylon membranes (ICN Biomedicals Canada Ltd.) by either capillary or vacuum blotting. Capillary blots were carried out using the "ICN Improved Procedure" according to the manufacturer's recommendations. Vacuum blots were performed with a VacuGene XL Vacuum Blotting System (Pharmacia, LKB Biotechnology) using the "VacuGene XL Protocol No. 1"

(Hydrochloric Acid Depurination and Vacuum Transfer of High Molecular Weight DNA) procedure.

Following transfer the DNA was fixed to the membrane by UV crosslinking using either a UV transilluminator (3 min) or a Hoeffer UVC-500 crosslinker (Hoeffer Scientific Instruments, San Francisco, CA) set to deliver 120,000 $\mu J/cm^2$.

2.9.1.2 Hybridization of Long DNA Probes to DNA Gel Blots

DNA blots were prehybridized and hybridized in heat sealable bags following the manufacturers recommendations. DNA blots were hybridized overnight at either 50 ^oC (PN2 probe) or 65 ^oC (*gusA* probe) to 5 X 10^5 to 1 X 10^6 cpm of radioactive probe/ml of hybridization solution. Following hybridization, blots were washed twice at room temperature (15 min each) in 2X SSC, 0.1% SDS, once at room temperature (15 min) in 0.1X SSC, 0.1% SDS, and then once (30 min) at either 50 ^oC (PN2 probe) or 65 ^oC (*gusA* probe) in prewarmed 0.1X SSC, 0.1% SDS.

Membranes were exposed to Kodak X-Omatic AR film at -80 ^oC in Kodak X-Omatic Cassettes with regular or Dupont Cronex Lightning Plus FF intensifying screens.

2.9.1.3 Dried Agarose Gel Hybridization

2.9.1.3.1 Preparation of Dried Gels

Dried gels were prepared according to Tsao *et al.*, (1983). Melted agarose for dried gels was filtered through a 0.45 µm disposable filter unit prior to pouring. After electrophoresis agarose gels were denatured in 0.15 M NaCl, 0.5 M NaOH (2 x 30 min) and then neutralized in 0.15 M NaCl, 0.5 M Tris-HCl, pH 7.5 (2 x 30 min). The gels were transferred to 2 sheets of Whatman 3MM filter paper cut to size of gel, covered with Saran wrap and then dried under vacuum (without heat, until flat and then at 60°C for an additional hour). Prior to hybridization the gels were removed from the saran wrap and paper backing by floating them in ddH_2O.

2.9.1.3.2 Hybridization of Oligonucleotide Probes to Dried Gels

Dried agarose gels were hybridized at 60 °C in a solution containing 5X SSPE (0.9 M NaCl, 50 mM $NaPO_4$, 5mM EDTA), 0.5% SDS, 100-200 µg/ml tRNA, which had been heated to 65 °C and filtered through a 0.45 µm disposable filter prior to use. 1 X 10^6 cpm/ml of radioactive oligonucleotide probe was added/ml hybridization solution. Following hybridization the dried gels were washed twice at room temperature (30 min each) in 6X SSC, 0.1% SDS, twice at room temperature (30 min each) in 0.1X SSC, 0.1% SDS, and then once for 30 to 60 min at 65 °C in prewarmed 0.1X SSC, 0.1% SDS. The melting temperature (T_d) of the probe was calculated to be 78 °C using a modified version (J. Hattori, Plant Research Centre, Agriculture Canada) of the computer program of Rychlik and Rhoads (1989); however, empirical observation indicated that the T_d was actually closer to 70 ^{o}C.

Dried gels were exposed to Kodak X-Omatic AR film at -80 ^{o}C in Kodak X-Omatic Cassettes with regular or Dupont Cronex Lightning Plus FF intensifying screens.

2.9.2 RNA Gel Blot Analysis

2.9.2.1 Preparation of RNA Gel Blots

RNA was transferred to BIOTRANS nylon membranes (ICN Biomedicals Canada Ltd.) either by capillary or vacuum blotting. Capillary blots were carried out using the protocol supplied by the manufacturer. Vacuum blots were performed with a VacuGene XL Vacuum Blotting System (Pharmacia LKB Biotechnology) using the "VacuGene XL Protocol No. 4" (Vacuum Transfer of RNA) procedure.

Following transfer the RNA was fixed to the membrane by UV crosslinking using either a UV transilluminator (3 min) or a Hoeffer UVC-500 crosslinker (Hoeffer Scientific Instruments, San Francisco, CA) set to deliver 120,000 µJ/cm^2.

2.9.2.2 Hybridization of Oligonucleotide Probes to RNA Gel Blots

RNA gel blots were prehybridized at 60 ^{o}C for at least 1 hr in a solution containing 5X SSPE, 5X Denhardt's, 0.5% SDS and 200 µg/ml tRNA. The blots were hybridized overnight at the same temperature to 1 X 10^6 cpm/ml of end-labeled oligonucleotide probe. Following hybridization the blots were washed in 6X SSC, 0.1% SDS, two times for 30 min each time at room temperature, then in 0.1X SSC, 0.1% SDS for 30 min at room temperature, followed by a 30 min stringent wash at 65°C in prewarmed 0.1X SSC, 0.1% SDS.

Membranes were exposed to Kodak X-Omatic AR film at -80 °C in Kodak X-Omatic Cassettes with regular or Dupont Cronex Lightning Plus FF intensifying screens.

2.10 Construction and Screening of cDNA Library

2.10.1 Construction of Library

Approximately 2 µg poly[A]$^+$ mRNA from late uninucleate and early binucleate *B. napus* cv. Topas microspores that had been cultured for four days at 32.5°C in order to induce embryogenesis was used for cDNA synthesis. First and second strand cDNAs were synthesized using the RiboClone cDNA synthesis kit (Promega, Madison, WI) and an *Xba* I primer-adaptor. The cDNA was size fractionated as in Sambrook *et al.*, (1989). *Eco* RI adaptors were ligated to the size-fractionated cDNA and the resulting products simultaneously phosphorylated with T_4 polynucleotide kinase and digested with *Xba* I as outlined in the Promega instruction manual. The size fractionated cDNA was purified on a Sephacryl S-400 spun column. The resultant cDNA was directionally cloned into *Eco* RI, *Xba* I double digested LambdaGEM-4 arms as described by the manufacturer (Promega, Madison, WI). Phage DNA was packaged using the Packagene extract system (Promega, Madison, WI).

2.10.2 Screening of Library

Aproximately 33,000 plaque-forming units were mixed with 500 µl of *E. coli* LE392 cells (OD_{600} = 0.6) and incubated for 30 min at 37 °C. The mixture was added to 25 ml of molten top agar (47 °C) and spread onto a 20 X 30 cm Pyrex dish containing 300 ml LB agar. The top agar was allowed to harden and the plate incubated overnight at 37 °C. Following incubation the plate was placed at 4 °C for at least one hour prior to making duplicate nylon lifts (Hybond-N; Amersham, Oakville, ON). The membranes were transferred, phage side up, to filter paper (Whatman 3MM) soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 10 min, in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl) for 2 X 5 min and then in 5X SSC for 5 min. The DNA was immobilized on the membrane by UV irradiation for 3 min on a transilluminator. Agarose particles were washed off the membrane by gently rubbing it in 5X SSC.

Lifts were hybridized overnight at 50°C to 1 x 10^6 cpm/ml of random primed PN2 cDNA insert, as per the manufacturers instructions. Following hybridization the lifts were washed twice at room temperature (15 min each) in 2X SSC, 0.1%, once at room temperature (15 min) in 0.1X SSC, 0.1% SDS, and then once for 30 min at 50 °C in prewarmed 0.1X SSC, 0.1% SDS.

Twenty positive plaques were picked, using the large end of a Pasteur pipette, transferred to 500 µl of modified SM buffer (10mM Tris-HCl, pH 7.4, 10mM $MgSO_4$, 0.5% (w/v) NaCl, 0.1% gelatin) and left to elute for 30 min. The sample was spun (3 min, 3000g, 4 °C) to remove the debris and the supernatant mixed with 100 µl of an overnight culture of *E. coli* WA802. 2.5 ml of top agar (at 45 °C) was added, the mixture poured onto small plates containing LB agar and the plates incubated overnight at 37 °C. Plates containing well spaced plaques were lifted and hybridized to the PN2 cDNA probe as described above. A single positive plaque was picked from each of the plates , using the narrow end of a Pasteur pipette, and then subjected to a third round of screening (as above).

Lambda DNA was isolated from each of the twenty plaques as described in section 2.4.3. DNA from each of the lambda clones was digested with *Spe* I (*Spe* I digestion removes the pGEM1 vector containing the cDNA insert), religated (Maniatis *et al.*, 1982) and used transform *E. coli* DH5α competent cells.

2.11 Sequencing

Cesium chloride purified double stranded DNA was sequenced using the dideoxy chain termination method of Sanger *et al.* (1977) with kits purchased from either USB (Cleveland, OH) or Promega (Madison, WI). [α-^{35}S]dATP (specific activity, 1,000 Ci/mmol) was used as the radioactive label. DNA inserted into pGEM4Z was sequenced from either end using the vector based SP6 and T7 primers, and internally with synthetic primers. All primers were synthesized by J. Hattori (Plant Research Centre, Agriculture Canada, Ottawa).

Sequencing reactions were resolved on 6% denaturing polyacrylamide gels using 0.5 to 1X TBE (1X TBE: 89 mM Trizma base, 2.5 mM EDTA, pH 8, 89 mM boric acid) as the running buffer. Gels were dried under vacuum (80 °C, 1.5 hr) on Whatman 3MM filter paper and exposed to Kodak X-Omatic AR film at room temperature.

DNA sequence information was manipulated using IBI Pustell sequence analysis software (New Haven, CT).

2.12 Plant Transformation

2.12.1 Vector Construction

Vector construction was carried out by C. Baszczynski, Pioneer Hi-Bred International Inc. Based on the sequence of the genomic clone, BngNAP1 (Baszczynski and Fallis, 1990), two synthetic oligonucleotides were made and used to amplify by PCR, 796 nucleotides of 5' sequence directly upstream of the BngNAP1 ATG polypeptide translation start site. These oligonucleotides also contained *Xba* I and *Bam* HI restriction sites near the 5' and 3' ends, respectively, of the amplified promoter region. A single PCR product of the correct size was obtained, digested with both *Xba* I and *Bam* HI, purified using GeneClean (BIO101 Inc. LaJolla, CA) and ligated to purified *Xba*`I and *Bam* HI digested pBI101.1 (Jefferson *et al.*, 1987; Clonetech Laboratories, Inc., Palo Alto, CA) directly in front of the β-glucuronidase (*gusA*) coding region, generating the vector pALLNAPG. The *Hind* III-*Sst*I insert, containing the BngNAP1 promoter and *gusA* coding sequences from pALLNAPG was digested, purified and ligated to *Hind* III and *Sst* I digested pALLTKRep vector DNA (C. Baszczynski, unpublished data) to generate pALLTKNAPG. pALLTKRep differs from pBI101.1 by having the CaMV 35S promoter, rather than the nos promoter, driving the *npt*II selectable marker gene. The 35S promoter yielded higher *npt*II expression and allowed selection at higher levels of kanamycin. pALLTKNAPG was transferred from *E. coli* to *Agrobacterium tumefaciens* strain GV3101(pMP90; Van Larebeke, 1974).

2.12.2 *B. napus* Transformation

The BngNAP1-GUS construct was transferred to *Brassica napus* cv. Westar by *Agrobacterium tumefaciens*-mediated transformation. Transformation was carried out by Bin Huang (Pioneer Hi-Bred International) according to the method of

Moloney *et al.*, 1989. A number of independent primary transformants, exhibiting a similar histochemical pattern of BngNAP1 directed GUS expression, were obtained (C. Baszczynski, personal communication); however, only the results obtained from the selfed progeny of one transformant, designated C18D, are presented here.

2.12.3 Tobacco Transformation

Agrobacterium-mediated transformation of *N. tabacum* cv. Petit Havana SR1 was carried out by Hélène Labbé (Plant Research Centre, Agriculture Canada) according to the method outlined by Horsch *et al* (1985). Three independant primary tobacco transformants were made available for this study.

2.12.3.1 Tobacco Segregation Analysis

Seeds from individual capsules were placed in small envelopes made from Whatman 3MM filter paper and sterilized by dipping the packets in 70 % ethanol (1 min) and then in undiluted Javex (6% sodium hypochlorite) containing a few drops of Tween 20 (25 min). The packets were rinsed with sterile dH_2O (3 x 30 min) and dried overnight in a laminar flow hood.

Seeds from individual packets were germinated on MS salts (Murashige and Skoog, 1962) containing B5 media, 1% sucrose, 6 g/l agarose and 100 µg/ml kanamycin. The ratio of bleached (kanamycin sensitive) to green (kanamycin resistant) seedlings was determined approximately 6 weeks after germination. Seeds from transgenic and untransformed plants were also germinated on control plates containing no kanamycin.

2.13 β-Glucuronidase Assays

Fluorogenic and histochemical GUS assays were performed based on methods outlined by Jefferson (1987).

2.13.1 Fluorogenic Assays

Samples were removed from -80 ^{o}C and placed in liquid nitrogen. Samples were placed on ice one at a time and ground until homogeneous in 3 volumes of ice-cold extraction buffer (50 mM $NaPO_4$ buffer, pH 7, 10 mM β-mercaptoethanol, 10 mM EDTA, pH 8, 0.1% (w/v) N-laurosarcosine, 0.1% (v/v) Triton-X-100) using an OMNI 1000 hand-held homogenizer (OMNI International, Waterbury, CT). The cellular debris was removed by centrifugation (4 ^{o}C, 10 min). The supernatant was transferred to a fresh tube, frozen in liquid nitrogen and stored at -80 ^{o}C until use.

Frozen tissue extracts to be assayed were thawed on ice and then vortexed. Five to 50 µl of tissue extract was added to 500 µl of prewarmed assay buffer (extraction buffer containing 1 mM 4-methyl umbelliferyl-β-D-glucuronide [MUG; Sigma, St. Lois, MO]) and incubated in a 37 ^{o}C water bath. 100 µl aliquots were removed at various time points, including time "0", and added to 1.9 ml stop buffer (0.2 mM Na_2CO_3). Fluorescence was measured using an Hitachi F2000 fluorescence spectrophotometer calibrated with known methylumbelliferone (MU) standards. The excitation and emission wavelengths were set to 365 and 455 nm respectively. Activity is expressed in nmol MU produced per min per mg protein (nmol/min/mg protein) or log pmol/min/mg protein. The protein content of the extracts was determined with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as a standard.

2.13.2 Histochemical Assays

All microspore and embryo samples were rinsed in 0.1 M $NaPO_4$ buffer, pH 7-8 prior to staining. Samples were incubated in histochemical assay buffer containing 100 mM $NaPO_4$ buffer, pH 7-8, 10 mM EDTA, pH 8, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]:H_2O$, 20% methanol and 0.5 to 1.0 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucoronide (X-gluc: Clonetech Laboratories Inc., Palo Alto, CA) at 37 ^oC until no further development in colour was detected (a few hours to overnight). Some samples were cleared of chlorophyll using several washes in 70% ethanol.

CHAPTER THREE

RESULTS

3.1 Isolation and Characterization of Napin Clones Expressed in Embryogenic Microspores

B. napus cv. Topas microspores at the late uninucleate and early binucleate stages of development can be induced to undergo embryogenesis by culturing them at 32.5 °C for 4 days. Previously, we had shown that this switch in developmental pathways was accompanied by the induction of high level napin seed storage protein gene expression. Napin gene expression was shown to be a marker for the induction of microspore embryogenesis as changes in the plant growth or microspore culture conditions were not, by themselves, sufficient to induce napin expression.

The napin proteins of *B. napus* are encoded by a small multigene family that is estimated to contain 10 to 16 members (Joseffsen *et al.*, 1987; Scofield & Crouch, 1987). Sequence analysis of eight published napin clones indicates that the coding regions of these genes are all very highly conserved, exhibiting 90 to 97% similarity at the nucleotide level. Probes derived from these sequences should detect, under moderately stringent conditions, mRNAs corresponding to any expressed member of the napin multigene family. Thus the moderately stringent hybridization conditions used to detect napin mRNA in embryogenically-induced microspores reflects the sum of all the napin genes expressed at that time.

3.1.1 Screening of cDNA Library for Napin Clones

To identify the specific members of the napin multigene family expressed in embryogenically-induced microspores, a cDNA library was constructed with poly $(A)^+$ mRNA from a population of *B. napus* cv. Topas microspores at the late uninucleate and early binucleate stage of development that had been induced to undergo embryogenesis by culturing them for 4 days at 32.5°C.

The library was screened, under low stringency, using the napin cDNA N2 (Crouch *et al.*, 1983) as a probe. Twenty hybridizing plaques were obtained from approximately 33,000 lambda clones after three rounds of plaque purification. DNA gel-blot analysis was performed under higher stringency on *Eco* RI/*Xba* I digests of lambda DNA isolated from each of the plaques to confirm that the clones contained a napin insert. This analysis indicated that each plaque contained a single napin insert ranging in size from approximately 450 to 2000 bp in length (data not shown).

The LambdaGEM-4 vector used in this study contains a complete copy of the pGEM 1 plasmid. During the cloning procedure, the cDNA is inserted into the multiple cloning site of the plasmid. The napin cDNAs were subcloned by *Spe* I digestion and religation of the pGEM 1 plasmid contained within LambdaGEM-4.

3.1.2 Characterization of Napin Clones

Due to the large number of napin clones obtained from the library a sequencing strategy was devised that would allow for an initial characterization of these clones. This strategy involved partial sequencing of the 5' and 3' ends of the napin clones using the SP6 and T7 promoter primer sites present within the pGEM1 vector. Extensive stretches of poly(A) residues in the 3' ends of a number of the clones prohibited sequencing through this region. Therefore, in order to obtain 3'-end sequence data for these clones a 17-nt primer, complementary to a region approximately 200 nt upstream of the site of poly(A) addition was synthesized (see Figure 5). The primer was designed to contain sequence information that was common to all the napin clones from the induced microspore library and to other previously published napin clones. At least 150 nt of sequence data were obtained from the 5' and 3' ends of each of the 20 clones using these two approaches.

Based on this information the napin cDNA clones were organized into groups of similar sequences (Table II). Two

TABLE II Classification of Napin cDNA Clones Expressed in Embryogenically Induced *B. napus* Microspores

member[a]	**no. clones**	**clone identity**[b]
BnmNAP2	6	3, 4, 5, 8, 9, 12
BnmNAP3	8	11, 13, 14, 16, 17, 18, 20, 21
BnmNAP4	4	1, 6, 7, 10,
gNa	1	2
napA/nap1	1	19

[a] napin cDNAs were organized into groups of similar sequences based sequence data obtained from the 5' and 3' ends of the cDNA clones.

[b] each cDNA clone was given a numerical designation prior to being organized into groups of similar sequences

of the isolated napin clones showed significant nucleotide similarity to previously published napin clones (data not shown). Clone 2 was 96% similar over the sequenced region to the napin cDNA clone pNAP1 (and its genomic counterpart, NapA) (Ericson *et al.*, 1986). Clone 19 showed similarity to the 3' end of the napin genomic clone Na but the 5' end of clone 19 appears to have been replaced by another unidentified, non-napin sequence. The napl/napA- and Na-like clones isolated in this study were not sequenced in full so it is not known how similar they are to their homologues in the other *B. napus* cultivars.

The remaining 18 napin cDNA clones were organized into a single subfamily consisting of three, highly similar members or subgroups of napin clones. This subfamily has been designated BnmNAP and the representative members within this group BnmNAP2, BnmNAP3 and BnmNAP4.

Two representative cDNAs from each of the BnmNAP2, BnmNAP3 and BnmNAP4 subgroups were fully sequenced in both directions using synthetic primers based on sequence information obtained from the previous round of sequencing (Figure 5). The complete nucleotide and predicted amino acid sequences of these representative cDNA inserts are shown in Figures 5 and 6 respectively.

The BnmNAP2, 3 and 4 nucleotide sequences are 96 to 99% similar at the nucleotide level and have no insertions or deletions relative to each other. The majority of the representative sequences are approximately 715 nt in length

Figure 5 Sequencing Strategy and Nucleotide Sequence Alignment for the *B. napus* BnmNAP Napin cDNAs.

DNA sequences are numbered at the right. Identical bases shared between BnmNAP2 and the two other cDNAs are shown by dashes. The translational start and stop codons are shown in bold and the putative polyadenylation signal is boxed. The location and direction (5' to 3') of synthetic primers used to obtain overlapping BnmNAP sequence information are depicted by arrows below the corresponding sequence. *represents the synthetic primer complementary to all cloned *B. napus* genes that was used to sequence BnmNAP genes with long poly(A) tails.

```
                                                        50
BnmNAP2 AAAAAACATACACACAAATAGCAAATGGCCAACAAGCTCTTTCTCGTCTC
BnmNAP3 --------------------------------------------------
BnmNAP4 -----------------------------------------C--------

                                                       100
BnmNAP2 GGCGACTCTCGCCCTCTTCTTCCTTCTCACCAATGCCTCCATCTACCGAA
BnmNAP3 --------------------------------------------------
BnmNAP4 ---A-----T---T-------------------C--------------C-

                                                       150
BnmNAP2 CGGTCGTAGAAGTCGAGGAAGATGATGCCACAAACCCAGCCGGTCCATTT
BnmNAP3 -------------------------------------------C------
BnmNAP4 -CA----G--------C--------------------------C------

                                                       200
BnmNAP2 AGGATTCCGAAATGTAGGAAGGAGTTTCAGCAAGCACAACACCTAAGAGC
BnmNAP3 C-------------------------------------------------
BnmNAP4 --------A-----------------------------------G-A---

                                                       250
BnmNAP2 TTGCCAGCAATGGCTCCACAAGCAGGCAATGCAGTCCGGTAGTGGTCCAA
BnmNAP3 --------------------------------------------------
BnmNAP4 ------A--------------------------------------C----

                                                       300
BnmNAP2 GCTGGACCCTCGATGGTGAGTTTGATTTTGAAGACGACATGGAGAACCCT
BnmNAP3 -------------C------------------------------------
BnmNAP4 -------------C--------------------T--------------C

                                                       350
BnmNAP2 CAGAGCCCACAGCAGAGGCCACCGCTACTCCAGCAGTGCTGCAACGAGCT
BnmNAP3 --------------------------------------------------
BnmNAP4 ---G-------A--------G--A--------------------------

                                                       400
BnmNAP2 CCACCAGGAAGAGCCACTTTGTGTTTGCCCAACCTTGAAAGGAGCATCCA
BnmNAP3 --------------------------------------------------
BnmNAP4 ---------------------C----------------------------

                                                       450
BnmNAP2 AAGCCGTTAAACAACAGGTTCGACAACAGCAAGGACAGCAGGGACAGCAG
BnmNAP3 --------------------------------------------------
BnmNAP4 --------------------------------------------------

                                                       500
BnmNAP2 CTGCAGCAAGTAATTAGCCGTATCTACCAGACTGCTACGCACTTACCTAA
BnmNAP3 -----------------T--------------------------------
BnmNAP4 --------------------------------------------------

                                                       550
BnmNAP2 AGTTTGCAACATCCCTCAAGTTAGCGTTTGTCCCTTCCAGAAGACCATGC
BnmNAP3 ---------------G----------------------------------
BnmNAP4 ---------------G----------------------------------
                                *

                                                       600
BnmNAP2 CTGGACCCTCCTACTAGATTCCAAACGAAACCCTCGAGTGTATGAATGTG
BnmNAP3 ----G---------------------------------------------
BnmNAP4 --------------------------------------------------

                                                       650
BnmNAP2 GTTGTCGGTATATGTCAACGCCACACTTCATCGCGTAATATGTAAGGTCT
BnmNAP3 -------A------------------------------------------
BnmNAP4 -------A------------------------T-----------------

                                                       700
BnmNAP2 TATCTAGGATGTTTGAGGCTATGTAATTAGCACTACTCCATAATAAAAGA
BnmNAP3 --------------------------------------------------
BnmNAP4 ---------------------------------------G----------

                                                       750
BnmNAP2 GGTTTTTAATGTTTTATTCGGTGTGCATGCTCTCTAAGTGGTCCAAGATT
BnmNAP3 --A-----G------
BnmNAP4 ----------C----

               760
BnmNAP2 TTAGTCTTCA
```

excluding the poly(A) tail: owever one of the BnmNAP4 group clones contains an extra 45 bp of sequence in the 3' untranslated region of the clone. All three representative BnmNAP sequences contain putative translational start and stop codons as well as the conserved poly(A) addition signal sequence AAUAAA (Proudfoot and Brownlee, 1976). The BnmNAP4 gene presumably contains an alternative polyadenylation signal sequence; however, it does not appear to be favoured as only one of the four BnmNAP4 sequences exhibited this feature.

Each of the BnmNAP2, 3 and 4 inserts can potentially encode a 180 amino-acid-protein (Figure 6). The amino acid sequence of the BnmNAP2 and BnmNAP3 proteins is identical, while that encoded by BnmNAP4 differs from BnmNAP2 and BnmNAP3 by only 4 amino acids.

3.1.3 Comparison of the BnmNAP Subfamily with Other Napin Genes

Comparison of the BnmNAP sequences to published napin sequences from *B. napus* (Figure 7) indicates that the BnmNAP sequences are most similar to the previously described napin genomic clone BngNAP1, which was isolated from *B. napus* cv. Westar (Baszczynski and Fallis, 1990). In fact, BngNAP1 differs from BnmNAP4 by only one nucleotide at position 661 in the 3' untranslated region of the gene (G in BnmNAP4 and

Figure 6 Alignment of the Predicted Amino Acid Sequence of the *B. napus* BnmNAP Napin cDNAs.

The amino acids are numbered at the right. Identical amino acids shared between BnmNAP2 and the two other cDNAs are shown by dashes. NAP2 = BnmNAP2, NAP3 = BnmNAP3, NAP4 = BnmNAP4.

```
                                                                       60
nap2     MANKLFLVSATLALFFLLTNASIYRTVVEVEEDDATNPAGPFRIPKCRKEFQQAQHLRAC
nap3     ------------------------------------------------------------
nap4     --------------------------I---D--------------------------K--

                                                                      120
nap2     QQWLHKQAMQSGSGPSWTLDGEFDFEDDMENPQSPQQRPPLLQQCCNELHQEEPLCVCPT
nap3     ------------------------------------------------------------
nap4     ---------------------------------G--------------------------

                                                                      180
nap2     LKGASKAVKQQVRQQQGQQGQQLQQVISRIYQTATHLPKVCNIPQVSVCPFQKTMPGPSY
nap3     ------------------------------------------------------------
nap4     ------------------------------------------------------------
```

A in BngNAP1). BnmNAP4 and BngNAP1 may therefore represent the same gene in cultivars Topas and Westar. BngNAP1 represents a fourth member of the BnmNAP subfamily of napin genes and is hereafter be referred to as such.

The nucleotide sequences of BngNAP1, BnmNAP2, BnmNAP3, BnmNAP4 as a group are more similar to each other than they are to the other members of the *B. napus* napin multigene family. Comparison of the coding regions of the BnmNAP sequences with other available napin sequences indicates that while all of the napin sequences share extensive nucleotide similarity, they differ in the length of the primary translation product (Figure 7). For example, the BnmNAP sequences encode a 180 amino acid protein while the other napin genes encode protein products that are either shorter (napA, napB, N1, pN2) or longer (Na) than the BnmNAP sequences. The differences at the amino acid level among the various napin clones support the observations of Lonnerdal and Janson (1972) who were able to isolate four discrete isoforms of napin protein from mature seeds of *B. napus*.

The sequence divergence between the BnmNAP sequences and the other members of the napin multigene family is greatest in the 3' untranslated region of the gene. The BnmNAP sequences exhibit a number of nucleotide substitutions in addition to a number of specific deletions and insertions which distinguish them from other members of the napin gene family (Figure 7).

Figure 7. Comparison of the Nucleotide Sequence of the Protein Coding and 3' Untranslated Regions of the *B. napus* Napin Genes

All sequences were compared with BnmNAP2. A dash was used to indicate where a nucleotide was the same as that in BnmNAP2. The nucleotide is indicated in positions where the sequence differed from BnmNAP2. Positions where gaps were introduced are indicated with an asterisk. The translational start and stop codons are underlined. The position spanning the BnmNAP subfamily oligonucleotide probe is indicated in bold. The numbering is for the BnmNAP2 sequence. Gene nomenclature as in Table I.

```
                                                                              94
BnmNAP2 ATGGCCAACA AGCTCTTCCT CGTCTCGGCG ACTCTCGCCC TCTTCTTCCT TCTCACCAAT GCCTCCATCT
BnmNAP3 ATG--G---- ---------- ---------- ---------- ---------- ---------- ----------
BnmNAP4 ATG--G---- ---------- ---------A -----T---T ---------- ---------C ----------
BngNAP1 ATG--G---- ---------- ---------A -----T---T ---------- ---------C ----------
N1      ********** ********** ********** ********** ********** ********** **********
N2      ATG--G---- ---------- ---------A ---------T ---------- ---------C ----------
Na      ATG--G---- ---------- ---------A ---------T -G-------- ---------- ------G---
napA    ATG--G---- ---------- ---------A ---------T ---------- ---------- ----------
napB    ATG--G---- ---------- ---------A ---------T ---------- ---------- ----------
napB'   ATG--G---- ---------- ---------A ---------T ---------- ---------- ----------

                                                                             164
BnmNAP2 ACCGAACGGT CGTAGAAGTC GAGGAAGATG ATGCCACAAA CCCAGCCGGT CCATTTAGGA TTCCGAAATG
BnmNAP3 ----- ---- ---A------ ---------- ---------- ---------C ------C--- ----------
BnmNAP4 ----C- CA- ---G------ --C------- ---------- ---------C ---------- ----------
BngNAP1 ----C--CA- ---G------ --C------- ---------- ---------C ---------- ----------
N1      ********** ********** ********** ********** ********** ********** ----------
N2      ----G----- ---C--GT-- --C------- ---------- -T-------C ---------- ----------
Na      --A-G----- T--G------ --C-----C- ---------- T--------C ---------- ----------
napA    ----G----- ---C--GT-- --C------- --------G- -T-------C ---------- ----------
napB    ----G----- G--C--GT-- --C------- ---------- ---------C ---------- ----------
napB'   ----G----- C--C--GT-- --C------- ---------- -T-------C ---------- ----------

                                                                             234
BnmNAP2 TAGGAAGGAG TTTCAGCAAG CACAACACCT AAGAGCTTGC CAGCAATGGC TCCACAAGCA GGCAATGCAG
BnmNAP3 ---------- ---------- ---------- ---------- ---------- ---------- ----------
BnmNAP4 ---------- ---------- ---------- G-A*------ --A------- ---------- ----------
BngNAP1 ---------- ---------- ---------- G-A------- --A------- ---------- ----------
N1      ---------- --C------- ---------- G--------- --A------- ---------- A---------
N2      ---------- ---------- ---------- A--------- -----G---- ---------- A---------
Na      ---------- ---------- ---------- A--------- --A------- ---------- ----------
napA    ---------- ---------- ---------- A--------- -----G---- ---------- A---------
napB    ---------- ---------- ---------- --A------- -----G---- ---------- A---------
napB'   ---------- ---------- ---------- --G------- -----G---- ---------- A---------

                                                                             298
BnmNAP2 TCCGGT**** **AGTGGTCC AAGCTGGACC CTCGATGGTG AGTTTGATTT TGAAGACGAC ATGGAGAACC
BnmNAP3 ------**** **-------- ---------- -----C---- ---------- ---------- ----------
BnmNAP4 ------**** **-----C-- ---------- -----C---- ---------- ------T--------------
BngNAP1 ------**** **-----C-- ---------- -----C---- ---------- ------T--- ----------
N1      --******TG GCG------- T--------- -----C---- -------C-- ---------- --------**
N2      --******TG GCG------- T-A------- -----C---- -------C-- ---------- ----------
Na      C-----GGTG GT-------- ---------T -----C---- ---------- ---------- G---------
napA    --******TG GCG------- T--------- -----C---- -------C-- ---------- ----------
napB    --******TG GC-------- T--------- -----C---- -------C-- ---------- ----------
napB'   --******TG GCG------- T--------- -----C---- -------C-- ---------- ----------

                                                                             365
BnmNAP2 CTCA***GAG CCCACAGCAG AGGCCACCGC TACTCCAGCA GTGCTGCAAC GAGCTCCACC AGGAAGAGCC
BnmNAP3 ----***--- ---------- ---------- ---------- ---------- ---------- ----------
BnmNAP4 -C--***-G- ------A--- -----G--A- ---------- ---------- ---------- ----------
BngNAP1 -C--***-G- ------A--- -----G--A- ---------- ---------- ---------- ----------
N1      ****GCA-G- T--------- --A--G--T- ---------- --A---T--- --------A- ----------
N2      -***GCA-G- T--------- --A--G--T- ---------- ------T--- -----C---- -A--------
Na      AA--ACA-G- ---------- -----A---- C--C------ ------C--- ---------- ----------
napA    C***GCA-G- T--------- --A--G--T- ---------- ------T--- ---------- ----------
napB    C***CCA-G- T--------- --A--G--T- ---------- ------T--- ---------- ----------
napB'   C***GCA-G- T--------- --A--G--T- ---------- ------T--- ---------- ----------

                                                                             435
BnmNAP2 ACTTTGTGTT TGCCCAACCT TGAAAGGAGC ATCCAAAGCC GTTAAACAAC AGGTTCGACA ACAGCAAGGA
BnmNAP3 ---------- ---------- ---------- ---------- ---------- ---------- ----------
BnmNAP4 ------C--- ---------- ---------- ---------- ---------- ---------- ----------
BngNAP1 ------C--- ---------- ---------- ---------- ---------- ---------- ----------
N1      C-----C--- -----G---- --A------- ---------G ---------- -AA---A--- ----***-A-
N2      C-----C--- ---------- ---------- ---------G ---------- -AA---A--- ----***---
Na      ------C--- ---------- ---------- ---------- ----G----- -GG------- ---A-AG---
napA    C-----C--- ---------- ---------- ---------G ---------- -AA---A--- ----***---
napB    C-----C--- ---------- ---------- ---------G ---------- -AA---AG-- ----***---
NapB'   C-----C--- ---------- ---------- ---------G ---------- -AA---A--- ----***---
```

```
                                                                           499
BnmNAP2 CA******GC AGGGACAGCA GCTGCAGCAA GTAATTAGCC GTATCTACCA GACTGCTACG CACTTACCTA
BnmNAP3 --******-- ---------- ---------- ---------T ---------- ---------- ----------
BnmNAP4 --******-- ---------- ---------- ---------- ---------- ---------- ----------
BngNAP1 --******-- ---------- ---------- ---------- ---------- ---------- ----------
N1      --ACA***-- -A---A---- ******---- A-GG-G-A-- ---------- ---C------ ----------
N2      --ACA***-- -A---A---- ******---- A-GG------ ---------- ---C------ ----------
gNa     --ACAAAT-- ---------- -A-------- ---------- --G------- ---------- ----------
napA    --ACA***-- -A---A---- ******---- A-GG-G---- ---------- --CA------ ----------
napB    --ACA***-- -A---A---T ******---- A-GG-G---- ---------- ---A-----T ----------
NapB'   --ACA***-- -A---A---- ******---- A-GG-G---- ---------- ---CCG---- A---------

                                                                            567
BnmNAP2 AAGTTTGCAA CATCCCTCAA GTTAGCGTTT GTCCCTTCCA GAAGACCATG CCTGGACCC* **TCCTACTAG
BnmNAP3 ---------- ------G--- ---------- ---------- ---------- -----G---* **------TAG
BnmNAP4 ---------- ------G--- ---------- ---------- ---------- ---------* **------TAG
BngNAP1 ---------- ------G--- ---------- ---------- ---------- ---------* **------TAG
N1      ---------- ------G--- ---------- ---------- ---------- -----G---* **------TAG
N2      ---------- ------G--- ---------- ---------- ---------- -----G---* **------TAG
Na      G--------- -----GG--- ------A--- ---------- ---------- -----G---G GC-T----TAG
napA    ---------- ------G--- ---------- ---------- ---------- -----G---* **------TAG
napB    ---------- A-----G--- --------A- ---------- ---------- -----G---* **------TAG
NapB'   ---------- ------G--- ---------- ---------- ---------- -----G---* **------TAG

                                                                           621
BnmNAP2 ATTCCAAACG AA**ACCCTC GAGTGTATGA ATGTGGTTGT CGGTATAT** **GTCAACGC **********
BnmNAP3 ---------- --**------ ---------- ---------- --A-----** **-------- **********
BnmNAP4 ---------- --**------ ---------- ---------- --A-----** **-------- **********
BngNAP1 ---------- --**------ ---------- ---------- --A-----** **-------- **********
N1      ---------- GG**G----- ---C---C-- ---------- --A-----** **---GG-A- **********
N2      ---------- --**------ ---------- ---------- --A-----** **------A- **********
Na      ---------- --AT-T---- ---A--G--T --ACCACG-- *-A---GAGT GT-GTTG*** TTGATGTATG
napA    ---------- --**------ ---------- ---------- --A-----** **------A- **********
napB    ---------- --**------ ---------- ---------- --A-----** **------A- **********
napB'   ---------- --**------ ---------- ---------- --A-----** **------A- **********

                                                                           676
BnmNAP2 *CACACTTCA TCGCGT**** ***AATA*** TGTAAGGTCT TATCTAGGAT GTTTGAGGCT A*TGTAA***
BnmNAP3 *--------- ------**** ***----*** ---------- ---------- ---------- -*-----***
BnmNAP4 *--------- --T---**** ***----*** ---------- ---------- ---------- -*-----***
BngNAP1 *--------- --T---**** ***----*** ---------- ---------- A--------- -*-----***
N1      *-----C--- ------GTTT CAT----ATA --------T- ---------- ---------- -A-----*AA
N2      *-----C--- ------GTTT CAT----ATA --------T- ---------- -----C---- -A-----*AA
Na      TT-*-***-- CTA-A-A*** *GTC--GGTG -***GT--TC C--AA-TA-- --ACT-ATG- -C-AATGTAA
napA    *-----C--- ------GTTT CAT----ATA --------T- ---------- ---------- -A-----*AA
napB    *-----C--- ------GTTT CAT----ATA --------T- ---------- ---------- -A-----*AA
napB'   *-----C--- ------GTTT CAT----ATA --------T- ---------- ---------- -A-----*AA

                                          712
BnmNAP2 TTAGCACTAC TCCATA*ATA AA**AGAG** GTTTTTAATGT
BnmNAP3 ---------- ------*--- --**----** -A-----G---
BnmNAP4 ---------- ---G--*--- --**----** ---------C-
BngNAP1 ---------- ---G--*--- --**----** -----------
N1      ---------- ------*--- --**----AG -C-C-------
N2      ---------- ------*--- --**----AG -C-C-------
NA      ----A----- ---G--G-CC GGTA-T-AAA --****G-A--
napA    ---------- ------*--- --**----AG -C-C-------
napB    ---------- ------*--- --**----AG -C-C-------
napB'   ---------- ------*--- --**----AG -C-C-------
```

If the proportion of each of the napin clones isolated is representative of the relative abundance of each transcript in the original mRNA population then approximately 95% of the napin clones expressed in embryogenically induced microspores correspond to a new, highly similar subfamily of napin genes.

A preliminary study was also undertaken to identify additional genes that are co-expressed with napin in embryogenically-induced microspores. The results of this study are presented in Appendix III.

3.1.2. DNA Hybridization

Nucleotide sequence alignment suggests that the BnmNAP sequences constitute a distinct subfamily within the larger napin multigene family. To determine the number of sequences in the BnmNAP subfamily, an oligonucleotide probe was designed that would specifically detect members of this subfamily. A redundant 39-mer oligonucleotide probe was constructed, comprising the reverse complement of the nucleotide sequence spanning positions 632 to 670 of the BnmNAP2, BnmNAP3 and BnmNAP4 sequences and the homologous region of the BngNAP1 sequence (in bold, Figure 7B) . The sequence of this oligonucleotide is given in section 2.7.2 of the Materials and Methods.

To estimate the number of BnmNAP genes in the *B. napus* genome *B. napus* cv Topas genomic DNA was digested with *Eco*

RI, run on agarose gels and the gels either blotted or dried under vacuum. None of the published *B. napus* napin sequences contains *Eco* RI restriction sites within the coding region of the gene. Therefore, digestion with *Eco* RI should give a minimum estimate of the number of napin genes in the *B. napus* genome. The oligonucleotide probe was hybridized *in situ* to dried gels rather than to gel blots to increase the sensitivity of detection.

Figure 8 shows that under conditions of low stringency the full length cDNA probe pN2 detects 14 hybridizing fragments of varying intensities (Figure 8B). Among these 14 fragments are five that are specifically detected by the BnmNAP subfamily oligonucleotide probe (Figure 8A). The same pattern of hybridizing bands was detected for *B. napus* cv. Westar, and suggests that the two cultivars are highly similar (data not shown).

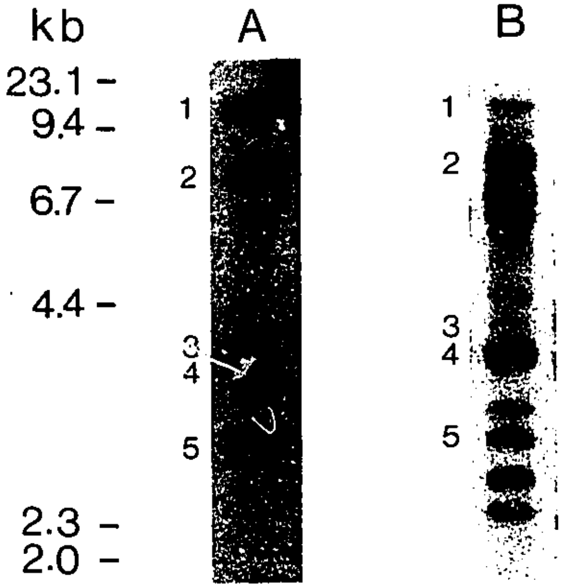
Three of the fragments detected by the oligonucleotide probe are of the same intensity (fragments 1, 2 and 5) while two of the fragments are lighter (fragments 3 and 4). A number of hypotheses can be invoked to explain this observation. The three darker fragments may correspond to comigrating, duplicate alleles, i.e. alleles that are homozygous for the *Eco* RI restriction site, while the two lighter fragments may correspond to individual alleles that are polymorphic for the *Eco* RI restriction site. These alleles would migrate as different-sized restriction fragments and, because they contain only half as much DNA,

Figure 8 Detection of the BnmNAP Subfamily

B. napus cv. Topas genomic DNA (10 µg) was digested with *Eco* RI and separated on agarose gels. The bands hybridizing to the BnmNAP subfamily specific oligonucleotide probe are indicated (1-5). The molecular size markers are lambda *Hind* III restriction fragments.

(A) dried gel hybridized directly with the ^{32}P-labeled oligonucleotide probe

(B) DNA gel blot hybridized with the ^{32}P-labeled pN2 cDNA probe.



kb
A
B
23.1
9.4
6.7
4.4
2.3
2.0
1
2
3
4
5
1
2
3
4
5

would appear less intense on the autoradiograph. An alternative explanation is that the lighter fragments represent napin genes with a lower similarity to the BnmNAP subfamily probe. However when the gel was washed at higher temperatures, all five fragments were removed simultaneously, making this alternative less likely.

The pN2 cDNA probe hybridizes only weakly to fragment 3, but hybridizes to fragment 4 with an intensity that is at least double the intensity of the next strongest hybridizing bands. Fragment 4 therefore, may contain comigrating napin restriction fragments, one of which corresponds to a BnmNAP gene.

Attempts to distinguish the bands corresponding to the individual BnmNAP members using gene-specific probes that differ by a single nucleotide were unsuccessful.

3.2 Developmental Expression of the BnmNAP Subfamily

The BnmNAP subfamily of napin genes was found to be highly and predominately expressed during the induction of microspore embryogenesis; however, it was not known if BnmNAP expression was restricted to embryogenically-induced microspores or whether the BnmNAP genes were also expressed during subsequent stages of embryo development. Therefore, the temporal pattern of BnmNAP gene expression was examined during microsporic and zygotic embryo development. RNA gel blot analysis, using the BnmNAP-subfamily specific oligonucleotide probe, was carried out on total RNA isolated from microspores, developing microsporic embryos, zygotic embryos and somatic tissues.

Figure 9 shows the temporal pattern of BnmNAP expression detected in microspores and developing microsporic embryos. High levels of BnmNAP mRNA were present in microspores that had been induced to undergo embryogenesis (Mi sample), but not in microspores that were cultured to develop as pollen (Mu sample). BnmNAP gene expression in microsporic embryos was very low during the globular, heart and torpedo stages and could usually only be seen in heart and torpedo stage embryo samples after a long exposure of the blots. BnmNAP mRNA was not detected in the Mu sample after the same long exposure. BnmNAP expression increased substantially at the cotyledon stage and remained high for the duration of the culture period.

The BnmNAP subfamily specific oligonucleotide probe detected a similar developmental pattern of napin mRNA accumulation as the non-specific pN2 cDNA probe (Appendix I, Figure 1). However, the level of napin mRNA detected by the BnmNAP oligonucleotide probe appears to be higher in microspores induced to undergo embryogenesis than in developing embryos, whereas the level of napin mRNA detected by the pN2 cDNA probe appears to be higher in embryos as compared to embryogenically induced microspores. This may reflect the specificity of the oligonucleotide probe for the

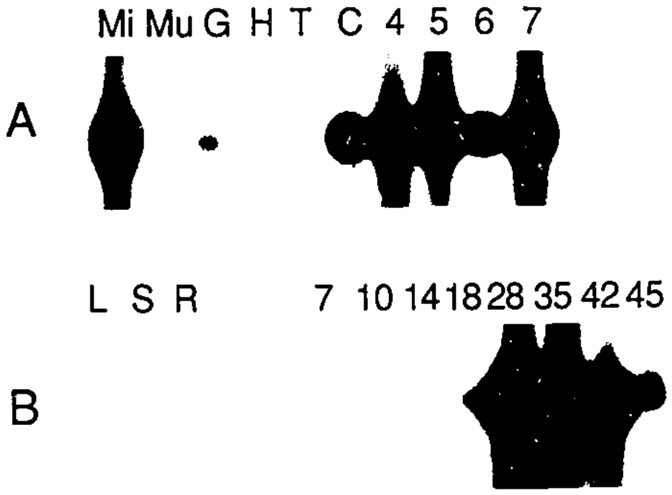
Figure 9 Detection of BnmNAP mRNA in Microspores and Developing Embryos of *B. napus*

(A) RNA gel blots containing 5 µg of total RNA per lane from microspores and developing microsporic embryos probed with the ^{32}P-labeled BnmNAP subfamily-specific oligonucleotide probe

RNA was isolated from late uninucleate and early binucleate microspores, collected from preconditioned donor plants grown at 10°/5°C (day/night), after 4 days in culture at 32.5°C (Mi) or after 4 days in culture at 25°C (Mu) and microsporic embryos at the globular (G), heart (H), torpedo (T), 21-day cotyledon (C), 28-day cotyledon (4), 35-day cotyledon (5), 42-day cotyledon (6) and 49-day cotyledon (7) stage of development

(B) RNA gel blots containing 5 µg of total RNA per lane from somatic tissues and developing zygotic seeds probed with the ^{32}P-labeled BnmNAP subfamily specific oligonucleotide probe

Total RNA was isolated from leaf (L), stem (S) and root (R) tissues as well as from seeds containing developing embryos at the following points in development (DAP): 7, early globular stage; 14, heart stage; 18, torpedo stage; 28, early cotyledon stage; 35, mid-cotyledon stage; 42, late cotyledon stage; 45, late cotyledon stage


Mi Mu G H T C 4 5 6 7
A
L S R 7 10 14 18 28 35 42 45
B

BnmNAP subfamily, which appears to comprise the majority of napin gene expression in embryogenically induced microspores.

The expression of the BnmNAP subfamily was also examined during zygotic embryo development. Whole seeds, rather than excised embryos, were used for RNA isolation as the small size of the developing embryo prior to the early cotyledon stage made it impossible to isolate sufficient numbers of embryos for RNA gel blot analysis.

The temporal pattern of BnmNAP expression during zygotic embryo development is shown in Figure 9B. A low level of BnmNAP mRNA was first detected at the torpedo stage (18 DAP) of development. BnmNAP mRNA levels peaked between 28 and 35 DAP and then dropped off by 45 DAP. BnmNAP mRNA was not detected in leaf, stem or root tissues (Figure 9B).

This temporal pattern of BnmNAP mRNA accumulation in zygotic embryos is similar to that previously reported for the napin subfamily as a whole (DeLisle & Crouch, 1989), but differs from the pattern of BnmNAP gene expression observed in developing microsporic embryos. While it is possible that the expression of the BnmNAP subfamily is regulated differently in microsporic and zygotic embryos it is also possible that the observed differences relate to the manner in which the RNA samples were collected. RNA samples were collected from whole seeds. The developing embryo initially comprises only a very small portion of the seed prior to the cotyledon stage and therefore only a small portion of the total seed RNA. In contrast, RNA samples collected at later stages of development, when the embryo comprises the majority of the total seed tissue, contain significantly more embryo RNA. Thus although the BnmNAP subfamily (and other napin genes) may be expressed prior to 18 DAP, the amount of embryo RNA present in the samples may be too small to detect BnmNAP gene expression. This second possibility is supported by the results presented below.

3.3 Analysis of the BngNAP1 Promoter in Transgenic Plants

To characterize the temporal and spatial regulation of one member of the BnmNAP subfamily in greater detail a 796-bp fragment of the BngNAP1 promoter was fused to the GUS reporter gene and to the 3' nos terminator (C. Basczcynski, Pioneer Hi-Bred International) and introduced into *B. napus* cv. Westar (Bin Huang, Pioneer Hi-Bred International) and *N. tabacum* cv. Petit Havana SR1 (Hélène Labbé, Plant Research Centre, Agriculture Canada) by *Agrobacterium*-mediated transformation. The pattern of GUS expression directed by the BngNAP1 promoter was examined using both histochemical and fluorogenic assays.

3.3.1 Expression of the BngNAP1-GUS Fusion in Transgenic *B. napus*

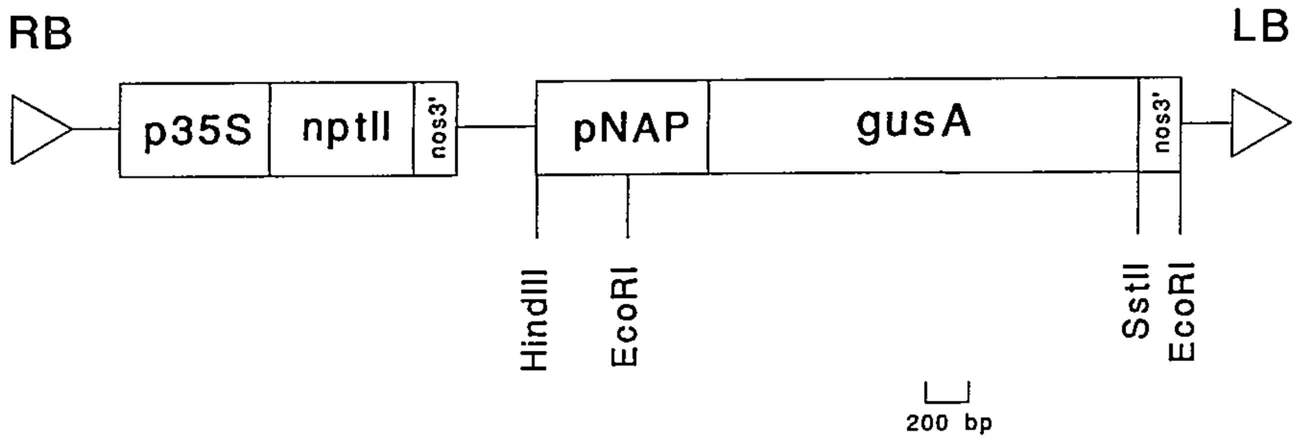
3.3.1.1 Gel Blot Analysis

A fragment containing 796 nucleotides of 5' sequence lying directly upstream of the BngNAP1 ATG polypeptide translation start site was fused to the coding region of the GUS reporter gene and introduced into *B. napus* cv. Westar by *Agrobacterium tumefaciens*-mediated transformation (Moloney *et al.*, 1989). A number of independently transformed lines were provided by B. Huang (Pioneer Hi-Bred International); however, only one line, C18D, was used in this study.

Gel blot analysis, using the GUS coding region as a probe, was carried out on DNA isolated from the selfed progeny of the original transformants. The isolated DNA was digested with either *Hind* III or *Eco* RI. As shown in Figure 10, *Hind* III cuts once within the the T-DNA, adjacent to the GUS gene, and outside the T-DNA insert, within the plant genome. The size of the *Hind* III fragment will vary depending on the proximity of the first *Hind* III site flanking the integrated T-DNA. Digestion with *Hind* III therefore, can be used to determine the number of T-DNA inserts, while digestion with *Eco* RI, which removes the entire GUS coding region of the T-DNA insert, can be used to determine if this region has undergone any gross rearrangements.

Figure 10 pBngNAP1-*gusA*-3'*nos* Vector Construct Used to Transform *B. napus* and *N. tabacum*

796 nucleotides of 5' sequence directly upstream of the BngNAP1 ATG polypeptide translation start site (pNAP) was ligated directly in front of the β-glucuronidase (gusA) coding region and the nopaline synthase terminator (nos3'). The cauliflower mosaic virus promoter (p35S) was used to drive the nopaline synthase (nptII) selectable marker gene. RB, T-DNA right border; LB, T-DNA left border

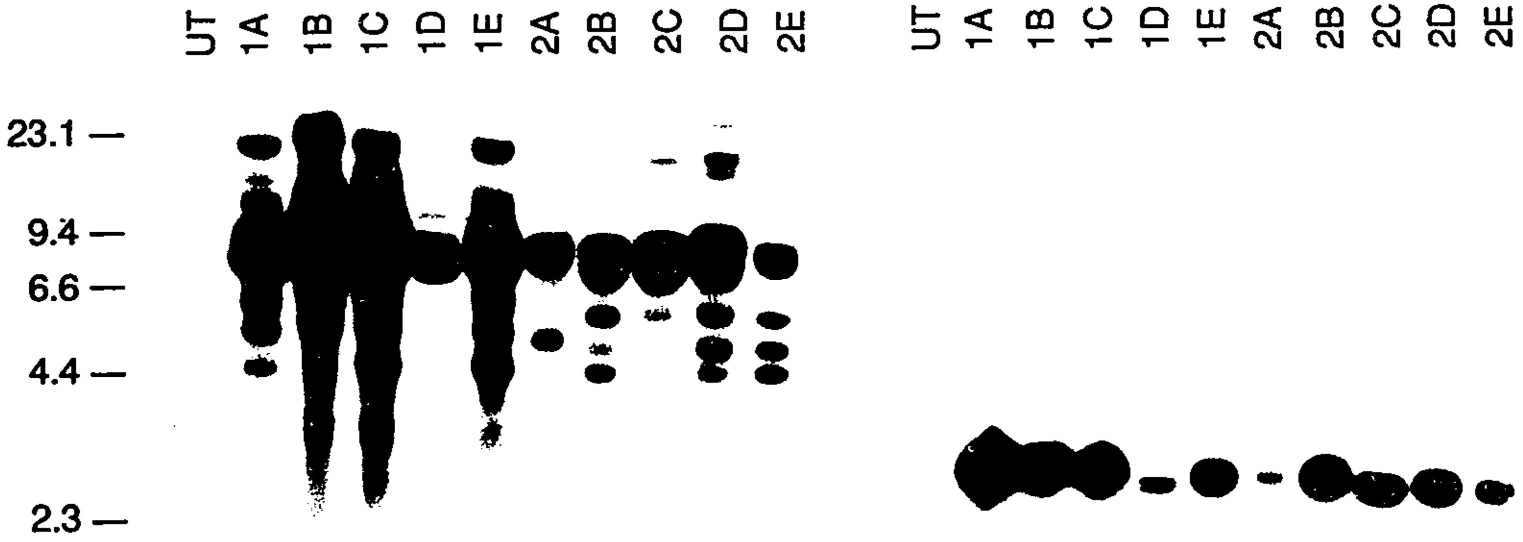

RB
p35S
nptII
nos3'
pNAP
gusA
nos3'
LB
HindIII
EcoRI
SstII
EcoRI
200 bp

The results obtained from 10 selfed progeny of C18D are shown in Figure 11. All 10 plants show multiple hybridizing bands. The observed pattern of bands is very similar to that derived from the parental line C18D (B. Huang, personal communication). A number of the hybridizing bands have been inherited by all of the progeny, suggesting that these fragments are tightly linked and therefore do not segregate in the offspring. None of the sizes of the observed fragments correspond to either direct (predicted size- 4.8 kb) or inverted (predicted size- 5.2 kb) repeats of the integrated T-DNA. However, the intensity and size of the 9.5 and 7.8 kb fragments suggests these fragments contain tandem or inverted repeats of the integrated T-DNA that have also been accompanied by a large plant DNA duplication. Supporting this is the observation that the same 9.5 and 7.8 kb fragments were detected when a probe derived from the *nptII* coding region was hybridized to the blot. The duplication of large regions of plant target DNA can be accounted for by the current model of T-DNA integration (Gheysen *et al.*, 1990).

Digestion of the same DNA with *Eco* RI gave a single hybridizing band of approximately 2.6 kb for each sample (Figure 11), suggesting that the GUS gene has not undergone any major rearrangements.

Figure 11 DNA Gel Blot Analysis of Transgenic *B. napus* Plants Carrying the pBngNAP1-*gusA*-3'*nos* Construct

DNA samples were digested with either HindIII (left) or *Eco* RI (right) and hybridized with a ^{32}P-labeled probe derived from the *gusA* coding region. The individual plants listed across the top are: **UT**, untransformed *B. napus* cv Westar; **1A-E** and **2A-E**, progeny plants derived from transgenic line C18D. The molecular size markers are lambda *Hind* III restriction fragments.


UT
1A
1B
1C
1D
1E
2A
2B
2C
2D
2E
23.1
9.4
6.6
4.4
2.3
UT
1A
1B
1C
1D
1E
2A
2B
2C
2D
2E

3.3.1.2 Choice of Plants for Further Analyses

Each of the 10 plants analysed above was used as a donor for both microspore embryo culture (with assistance from Nancy Long and Keith Pomeroy, Plant Research Centre, Agriculture Canada) and for collection of zygotic embryos. All plants were initially assayed histochemically for GUS activity. While all ten plants exhibited the same temporal and spatial pattern of GUS expression, they differed in their ability to form embryos in culture. Three plants that gave consistently high embryo yields (1D, 2C and 2E) were chosen for further analysis. These plants were selfed and three progeny from each selfed plant were chosen for subsequent studies. Each plant was originally used as a donor for microspore embryogenesis and then the flowering stalks were cut back and the same plants transferred to the greenhouse for seed collection.

3.3.1.3 Fluorogenic Analysis of BngNAP-GUS Activity

Fluorogenic GUS assays were only performed on zygotic seeds due to limited amounts of microspore-derived material.

The temporal pattern of GUS expression directed by a 796-bp fragment of the BngNAP1 promoter was examined during *B. napus* seed development. The time points analysed span the first two phases of seed development, i.e. the period of major morphological changes and the period of reserve accumulation, but do not include fully-mature, dry seeds.

Seeds from untransformed plants were analysed at some, but not all of these timepoints. Somatic tissues including leaf, stem and root from transformed and untransformed plants were also analysed.

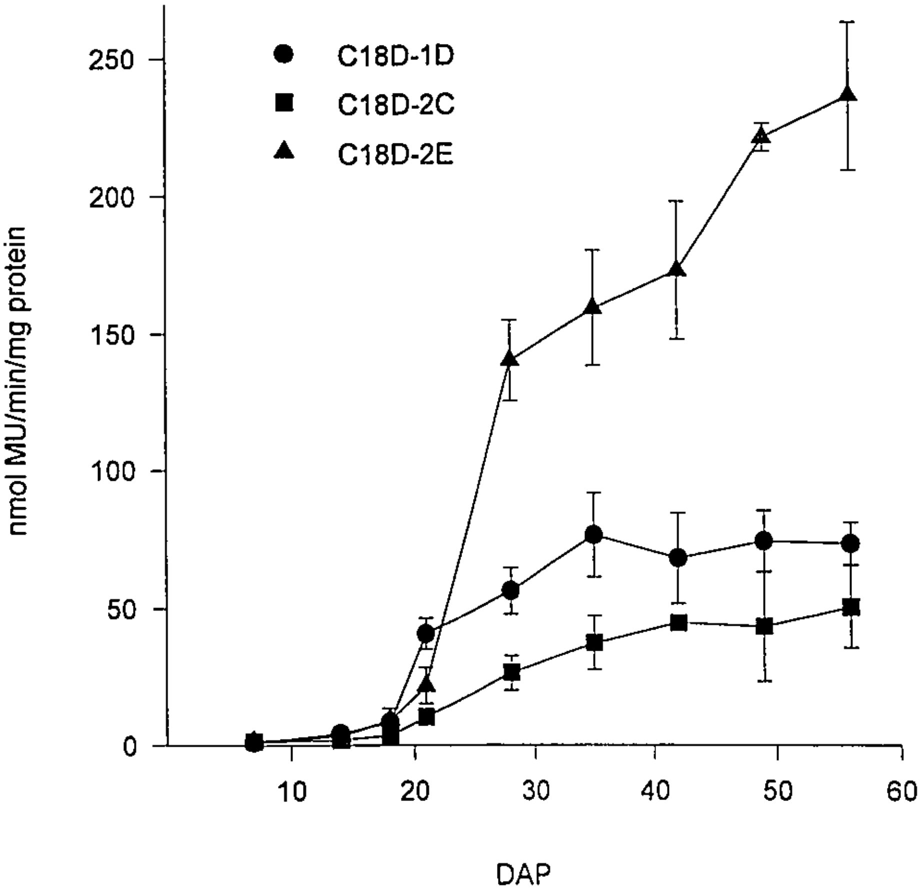
Figure 12 shows the time course of GUS accumulation during *B. napus* seed development. GUS activity was initially low during the globular and heart stages (7-10 DAP), but increased during the torpedo to early cotyledon stages (18 to 21 DAP). GUS activity began to level off by the mid-cotyledon stage (28 to 35 DAP) and remained steady for the duration the time points analysed. GUS activity in untransformed seed tissues and in somatic tissues from transformed plants was less than 0.03 nmol MU/min/mg protein and was considered to be negligible (Appendix II, Table I).

The GUS reporter gene was a sensitive marker for detecting BnmNAP promoter activity. GUS activity was detected in whole seeds as early as the globular stage of development whereas total napin and BnmNAP mRNA were not detected until the late torpedo/early cotyledon stage.

Figure 12 GUS activity in Developing Seeds of *B. napus cv.* Westar Plants Transformed with the pBngNAP1-*gusA*-3'*nos* Construct

Seeds from one or more siliques at various timepoints throughout development were collected and pooled. Three samples were collected for each timepoint from three selfed progeny of lines C18D-1D, C18D-2C and-C18D-2E. Samples were assayed in duplicate and the average value plotted.

MU, 4-methylumbelliferone
DAP, days after pollination



● C18D-1D
■ C18D-2C
▲ C18D-2E
nmol MU/min/mg protein
0
50
100
150
200
250
10
20
30
40
50
60
DAP

3.3.1.4 Histochemical Analysis of BngNAP-GUS Expression

The spatial pattern of GUS activity directed by the BngNAP1 promoter was also examined by histochemical analyses of microspores and developing microsporic and zygotic embryos of *B. napus*.

Figure 13 shows the pattern of GUS accumulation in microspores and developing microsporic embryos. Late uninucleate/early binucleate microspores from preconditioned plants were isolated (Mo sample) and either induced to undergo embryogenesis by culturing them for 4 days at 32.5 ^{o}C (Mi sample) or allowed to continue pollen development by culturing them for 4 days at 25 ^{o}C (Mu). Low levels of GUS activity were often observed in a small percentage of freshly isolated microspores (Mo sample, Figure 13a). In general, the highest yields of microsporic embryos were obtained from microspores that exhibited GUS activity at the time of collection; however the validity of this observation was not rigorously tested.

The BngNAP1 promoter was highly expressed in samples of microspores that had been induced to undergo embryogenesis (Figure 13b) but was not expressed in samples of microspores that were allowed to undergo pollen development in culture (Figure 13c). GUS activity was predominantly present in microspores that had taken on a slightly swollen appearance (Figure 13b). This swelling response is characteristic of microspores that are most likely to develop into embryos (Kott *et al.*, 1988). These results parallel the original observations made by Northern blot analysis and further suggest that the BngNAP1 promoter is activated specifically in microspores that have the greatest potential for developing into embryos in culture.

The spatial pattern of BngNAP1-directed GUS activity was also examined in developing microsporic embryos. GUS activity was detected as early as the globular stage (Figure 13d). Initially, GUS activity was confined to the basal region of the globular embryo, but spread part way up the lower axis during the heart (Figure 13e) and torpedo (Figure 13f) stages. During the early cotyledon stage staining was observed in the cotyledons but was still absent from the uppermost region of the axis (Figure 13g). As embryo development proceeded GUS activity was observed throughout the embryo (Figure 13h). At the latest stage of embryo development, the entire embryo stained a homogeneous dark blue (Figure 13i). These results are consistent with the temporal expression pattern obtained by RNA gel blot analysis using the BnmNAP subfamily oligonucleotide probe.

The same spatial pattern of GUS expression was observed for developing zygotic embryos (Figure 14). In zygotic embryos GUS activity was first detected in globular stage embryos in the region corresponding to the micropylar pole of the embryo (Figure 14a). GUS activity was also detected in the lower region of the suspensor at this and later stages. BngNAP1-GUS expression remained restricted to the axis during the heart (Figure 14b) and torpedo stages

(Figure 14c); however, cells corresponding to the future root apical meristem did not exhibit GUS activity. Zygotic embryos begin to exhibit GUS activity in the vascular tissue of the cotyledons during the early cotyledon stage, but expression in the axis was still restricted to the lower regions (Figure 14d). During subsequent stages GUS staining spread throughout the cotyledons and the axis (Figure 14e), so that by the mid-cotyledon stage the entire embryo stained dark blue (Figure 14f). During development small amounts of blue precipitate were often observed in the liquid endosperm, but not in leaf, stem or root tissues. GUS staining was often observed in the nectaries which lie at the base of the style (data not shown).

The spatial patterns of GUS activity directed by the BngNAP1 promoter are almost identical in microsporic and zygotic embryos. This observation suggests that the factors directly responsible for generating spatial and temporal patterns of napin expression originate within the embryo itself and not from the surrounding maternal tissues.

Taken together these results lend support to the possibility that the inability to detect napin mRNA during the early stages of zygotic embryo development by RNA gel blot analysis was due to the dilution of the embryo tissue. A more sensitive assay, such as RNAse protection, RT-PCR or

Figure 13 Histochemical Localization of GUS Activity in Microspores and Microsporic Embryos of Transgenic *B. napus* cv. Westar Plants Carrying the pBngNAP1-*gusA*-3'*nos* Fusion.

Late uninucleate and early binucleate microspores isolated from preconditioned donor plants grown at 10°/5°C (day/night) were assayed for GUS activity at **(a)** the time of collection (Mo sample), **(b)** after 4 days in culture at 32.5°C (Mi sample) and **(c)** after 4 days in culture at 25°C (Mu sample).

Microsporic embryos were assayed for GUS activity at the globular **(d)**, heart **(e)**, torpedo **(f)**, 21 d cotyledon **(g)**, 28 d cotyledon **(h)** and 35 d cotyledon **(i)** stage of development. Scale bars for **(a)**, **(b)** and **(c)** = 25 µm, for **(d)** and **(e)** = 100 µm, for **(f)** = 500 µm and for **(g)**, **(h)** and **(i)** = 1000 µm

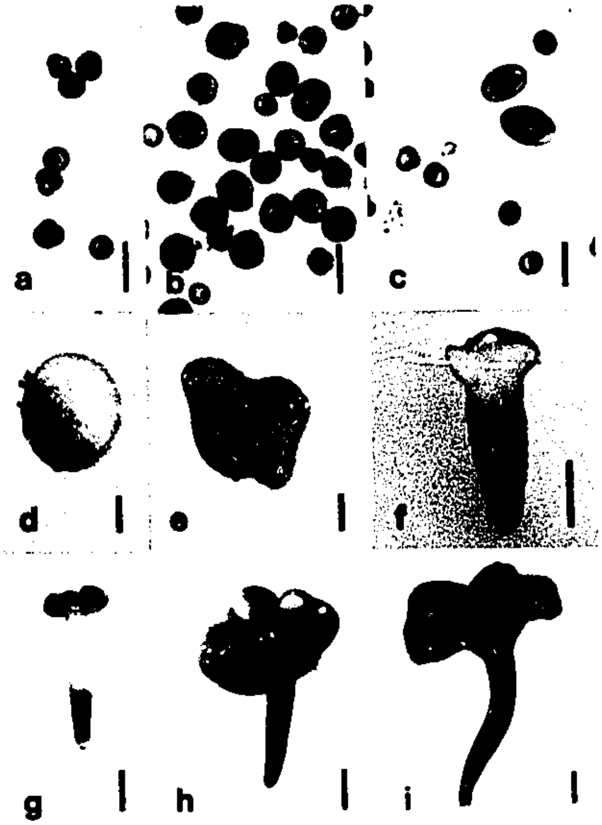
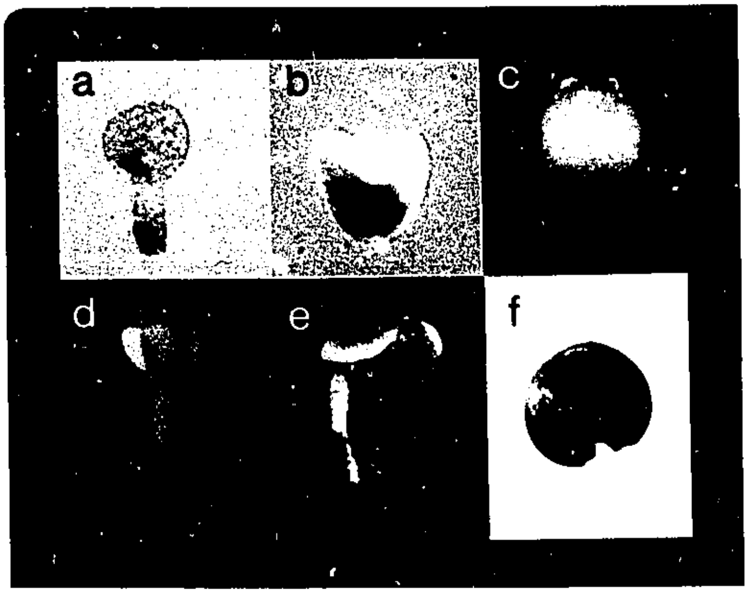

a
b
c
d
e
f
g
h
i

Figure 14 Histochemical Localization of GUS Activity in Zygotic Embryos of Transgenic *B. napus* cv. Westar Plants Carrying the BngNAP1 Promoter-*gusA*-3'*nos* Fusion

(a) globular stage zygotic embryo (10 DAP)
(b) heart stage zygotic embryo (12 DAP)
(c) torpedo stage zygotic embryo (14 DAP)
(d) cotyledon stage zygotic embryo (18 DAP)
(e) cotyledon stage zygotic embryo (28 DAP)
(f) cotyledon stage zygotic embryo (35 DAP)


a
b
c
d
e
f

in situ hybridization, is required to detect BnmNAP transcripts during these stages.

3.3.2 Analysis of BngNAP1 Expression in Transgenic Tobacco

Tobacco is frequently used as a heterologous host for gene expression studies, primarily due to the ease with which it can be transformed. The expression of a number of seed protein genes, including those from both monocots and dicots, has been shown to be developmentally regulated in tobacco seeds. These results have been used to suggest that the mechanisms which govern seed-specific gene expression are highly conserved among evolutionarily diverged plant species.

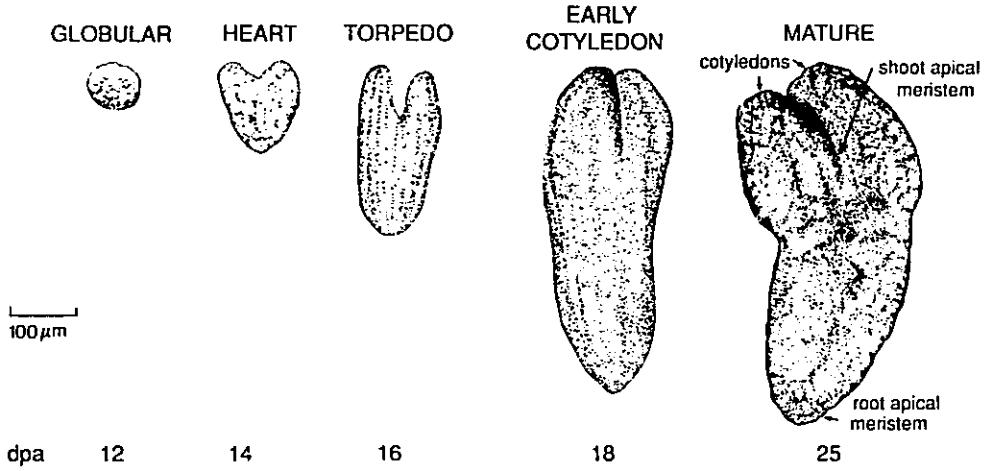
Embryo development in tobacco takes place over a shorter period of time than in *B. napus,* but still proceeds through the same major phases (Figure 15). Tobacco seeds also differ from *Brassica* seeds in that they contain a persistant endosperm. Although storage reserves accumulate in both the embryo and the endosperm, the major storage tissue is the endosperm.

The BngNAP1-GUS construct was introduced into tobacco to determine whether the spatial and temporal patterns of

Figure 15 Embryo Development in *N. tabacum*

dpa, days post anthesis

Figure adapted from DeMoor (1992)


GLOBULAR
HEART
TORPEDO
EARLY COTYLEDON
MATURE
cotyledons
shoot apical meristem
root apical meristem
100μm
dpa
12
14
16
18
25

BnmNAP gene expression were conserved in a more distantly related plant species.

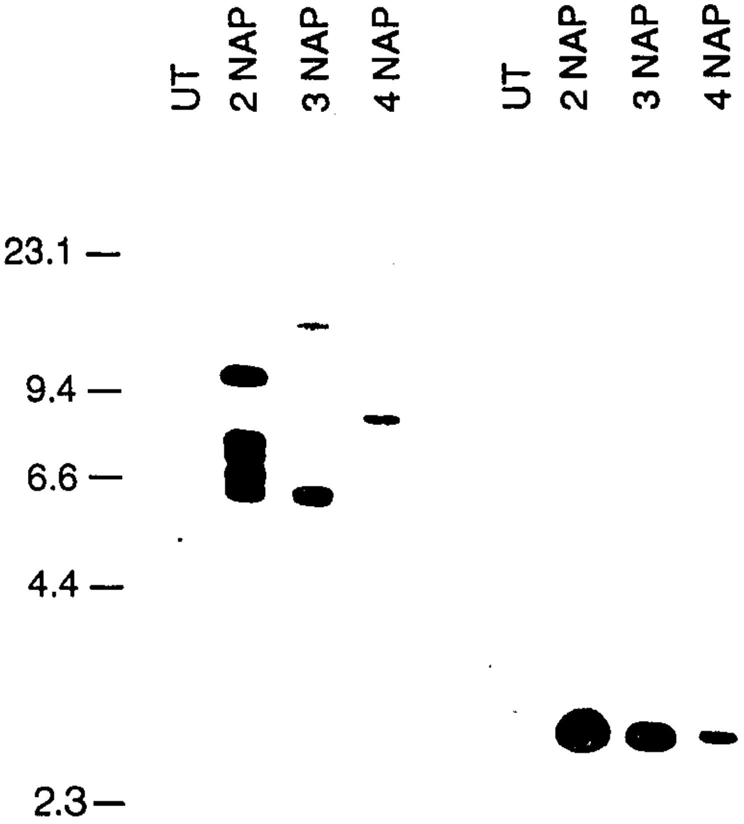
3.3.2.1 Gel Blot Analysis and Segregation Analysis

Three independent tobacco transformants, termed 2NAP, 3NAP and 4NAP, each carrying the same BngNAP1-GUS construct as in the previous section, were generated by *Agrobacterium*-mediated transformation (Horsch *et al.*, 1985). Gel blot analysis, using the GUS coding region as a probe, was carried out on DNA isolated from the original transformants. The isolated DNA was digested with either *Sst* I or *Eco* RI. As shown in Figure 10, *Sst* I cuts once within the integrated T-DNA, outside both the *gusA* and *nptII* genes, and outside the T-DNA, within the flanking plant genomic DNA. As with the *Hind* III digest, the size of the *Sst* I fragments hybridizing to the GUS probe will vary depending on the position of the first *Sst* I site flanking the integrated T-DNAs.

The results of the gel blot analysis are shown in Figure 16. The pattern of T-DNA integration was different for all three lines. In all cases the size of the bands is larger than the distance between the internal *Sst* I site and the end of the T-DNA. The same banding pattern was observed when the *npt* II coding region was used as a probe (data not

Figure 16 DNA Gel Blot Analysis of Transgenic tobacco *tabacum* Plants Carrying the pBngNAP1-*gusA*-3'*nos* Construct

DNA samples were digested with either SstI (left) or EcoRI (right) and hybridized with a ^{32}P-labeled probe derived from the *gusA* coding region. The individual plants listed across the top are: **UT**, untransformed *N. tabacum* cv Petit Havana SR1; **2NAP**, **3NAP**, **4NAP**, independent transformants of *N. tabacum* cv Petit Havana SR1. The molecular size markers are lambda HindIII restriction fragments.


UT
2 NAP
3 NAP
4 NAP
UT
2 NAP
3 NAP
4 NAP
23.1
9.4
6.6
4.4
2.3

shown). The band sizes do not correspond to either tandem (5.1 kb) or inverted (0.2 kb) repeats of the integrated T-DNA. The GUS gene does not appear to have undergone any major rearrangements as digestion with *Eco* RI generated a single band of the correct size (approximately 2.6 kb).

Gene segregation analysis of seeds derived from each of the three selfed plants was carried out by following the segregation of the kanamycin antibiotic resistance marker. Mature tobacco seeds were plated onto a germination medium containing 100 µg/ml kanamycin sulfate and the numbers of white, kanamycin-sensitive seedlings as well as the numbers of green, kanamycin-resistant seedlings were counted and subjected to chi^2 analysis (Table III). In all cases the ratios of km^r:km^s seedlings did not agree with the predicted ratios based on the observed number of segregating loci. However, the χ^2 values for the 2 NAP and 3NAP segregation data are not significantly different from a 63:1 ratio (at a 95% confidence level), suggesting that these two lines contain linked copies of the integrated T-DNAs. In the case of 3NAP this observation could be accounted for by the presence of a direct repeat that was accompanied by a 1 kb insertion of the plant target DNA.

TABLE III Segregation Analysis of Transgenic Tobacco Seeds Carrying the pBngNAP1-*gusA*-3'*nos* Fusion

transgenic line	**no. T-DNA copies**[a]	**no. km^r progeny**[b]	**no. km^s progeny**[c]	**km^r:km^s observed**	**km^r:km^s expected**[d]	**χ^2 value**
2NAP	5	388	10	39:1	5	9.4[e]
3NAP	2	444	6	74:1	2	18.5[e]
4NAP	1	515	126	4:1	1	8

[a] the number of integrated T-DNA copies was determined by counting the number of hybridizing bands in Figure 16

[b,c] seeds from individual tobacco capsules were germinated on plates containing 100 µg/ml kanamycin. The number of kamamycin-resistant (km^r) and kanamycin-sensitive (km^s) seedlings from at least three capsules were counted.

[d] the expected segregation ratio is based on the observed number of integrated T-DNAs.

[e] observed ratio not significantly different from a 63:1 ratio at 95% confidence level

3.3.2.2 Fluorogenic Analysis of BngNAP-GUS Expression

The temporal pattern of GUS expression directed by the BngNAP1 promoter was examined during tobacco seed development. Three samples, comprising whole seeds from individual tobacco capsules, from each of the three transgenic plants were assayed in duplicate for each of the timepoints.

The results of this analysis are shown in Figure 17. GUS activity was first detected in young seeds containing embryos at the early globular stage of development. This activity continued to increase, peaked in seeds around the mid-cotyledon stage and then began to decline. Although the levels of GUS activity differed among the three transformants, all three exhibited a similar temporal pattern of expression. The differences in levels of GUS activity among the transformants does not appear to be correlated with the number of integrated T-DNAs. GUS activity in untransformed seed tissues and in somatic tissues from transformed plants was neglible (Appendix II, Table II).

3.3.2.3 Histochemical Analysis of BngNAP-GUS Expression

The spatial pattern of GUS activity directed by the BngNAP1 promoter was also examined during tobacco zygotic embryo development. Tobacco embryos at the globular, heart, torpedo and developing cotyledon stages were excised from seeds and assayed histochemically for GUS activity. GUS staining was first detected in early heart-stage embryos where it began to accumulate as a band in the middle of the embryo, just under the lobes of the emerging cotyledons (Figure 18a). GUS activity spread rapidly in both directions as development proceeded through to the torpedo and early cotyledon stages, but generally remained absent from the regions corresponding to the cotyledon and root tips (Figs. 18b, 18c). The embryo stained completely blue by the late cotyledon stage (Figure 18d).

GUS staining was also detected in the endosperm of developing tobacco seeds. Although GUS activity was not detected in globular stage embryos, it was detected in the endosperm of seeds containing globular stage embryos (Figure 18e). This activity often began at the micropylar end of the seed, but spread very quickly so that by the time the embryos reached the early cotyledon stage the endosperm was evenly stained throughout (Figure 18f). GUS activity did not

Figure 17 GUS activity in Developing Seeds of *N. tabacum* cv. Petit Havana SR1 Plants Transformed with the pBngNAP1-*gusA*-3'*nos* Construct

Seeds from individual capsules at various timepoints throughout development were collected. Samples were collected in triplicate for each timepoint from the independently transformed lines 2NAP, 3NAP and 4NAP. Samples were assayed in duplicate and the average value plotted.

MU, 4-methylumbelliferone
DAP, days after pollination

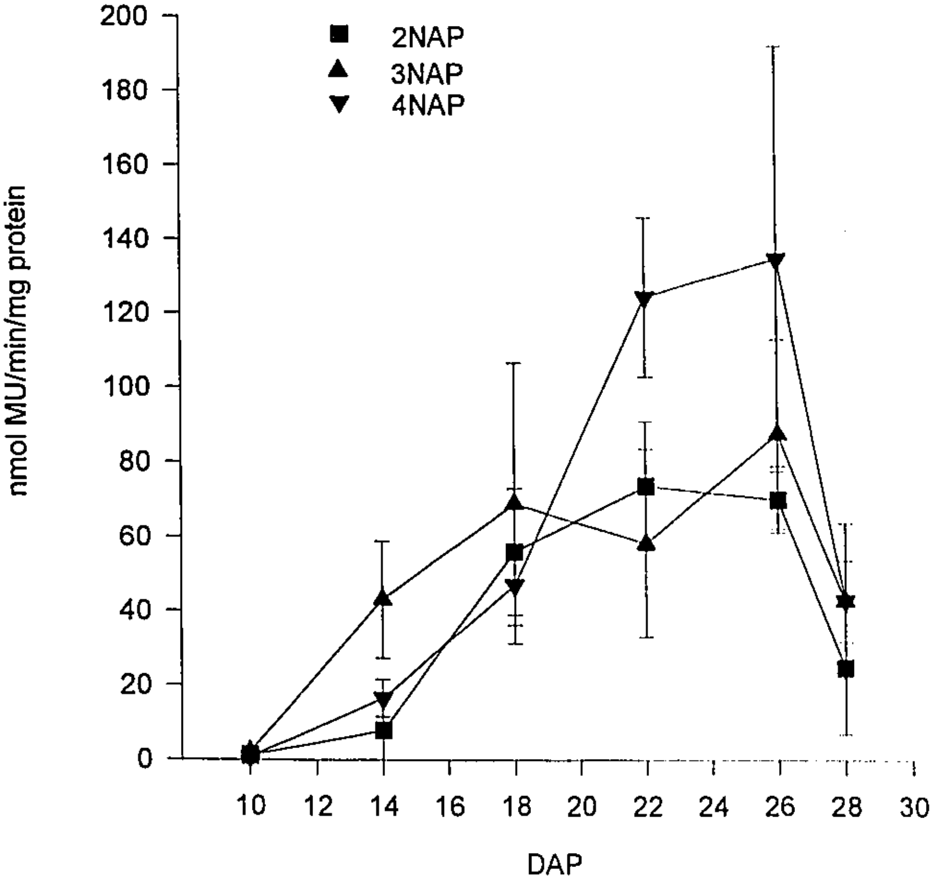
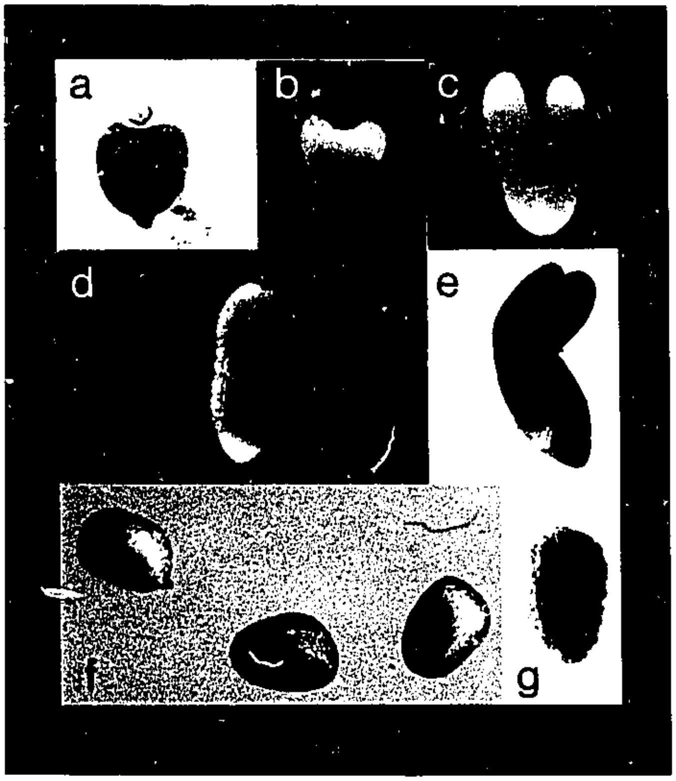

200
180
160
140
120
100
80
60
40
20
0
nmol MU/min/mg protein
■ 2NAP
▲ 3NAP
▼ 4NAP
10
12
14
16
18
20
22
24
26
28
30
DAP

Figure 18 Histochemical Localization of GUS Activity in Transgenic *N. tabacum* Petit Havana SR1 Seeds Carrying the BngNAP1 Promoter-*gusA* nos3' Fusion

(a) early heart stage embryos (10 DAP)
(b) heart stage embryos (14 DAP)
(c) torpedo stage embryos (16 DAP)
(d) early cotyledon stage embryos (18 DAP)
(e) late cotyledon stage embryos (28 DAP)
(f) whole seeds approximately 10 DAP
(g) whole seeds approximately 28 DAP


a
b
c
d
e
f
g

accumulate in the seed coat (Figure 18e,f) or in any other somatic tissue of the mature plant (data not shown).

These results indicate that although the seed specificity and timing of induction of BngNAP1-GUS expression in *B. napus* and tobacco were maintained, the spatial patterns of expression differed between the two species. It is unlikely that the differences observed in this study were the result of position effects as a number of independent tobacco transformants, each carrying different numbers of integrated T-DNAs at different chromosomal loci, gave the same results. Rather it appears that the results relate to inherent differences in the modes of seed storage protein accumulation and embryo development in the two species.

In summary, the results presented above have shown that napin gene expression in *B. napus* microspores is correlated with the induction of microspore embryogenesis and as such provides the earliest molecular marker for this process. This work represents the first detailed characterization of a molecular marker for the transition from pollen to embryo development.

CHAPTER 4

DISCUSSION

4.1 Mechanism of Induction of *B. napus* Microspore Embryogenesis

Under the appropriate culture conditions isolated microspores and pollen from a wide variety of plants can be induced to form haploid embryos *in vitro.* In *Brassica napus,* the transition from pollen to haploid embryo development can be separated into three phases. In the first phase, microspores are made competent for embryogenesis by growing the donor plants under low temperatures. In the second phase, a heat-stress treatment is used to block pollen development and induce embryogenesis. In the third phase, the induced microspores become irreversibly committed to embryo development.

4.1.1 Establishment of Competence

It has been suggested that the pollen grains of a number of species become competent to form embryos during their development in the anther and that subsequent culture events provide an environment for the expression of this embryogenic potential. This suggestion is based on the observation that many plant species exhibit a phenomenon known as pollen dimorphism (Raghavan, 1986). Pollen dimorphism refers to the existence of two populations of pollen grains within the anther. One population consists of normal, fertile pollen and the other of smaller pollen grains that, although viable, are thought to be functionally sterile. In addition to their small size, these pollen grains are generally starch-free and display a clear cytoplasm. This type of pollen grain has been referred to as S or P pollen (Dunwell, 1985; Raghavan, 1986). In a number of species a direct correlation has been observed between the frequency of microspore/pollen embryogenesis and the presence of P grains (Dunwell, 1985; Raghavan, 1986).

Although P grains occur naturally, their frequency can be increased by manipulating the growth conditions of the donor plants. For example, in tobacco, the proportion of P-grains can be increased by growing donor plants under short days or low temperatures, by the application of various feminizing agents, or by the application of growth substances or gametocides that are known to increase male sterility (Heberle-Bors, 1982a; Heberle-Bors, 1982b; Heberle-Bors, 1983). Interestingly, exposure of donor plants to low growth temperatures and short days also results in an increase in male sterility (Heberle-Bors, 1982). In all instances the increase in P grain formation results in an accompanying increase in the frequency of tobacco pollen embryo formation.

The observation that many plant species do not exhibit an obvious pollen dimorphism argues against the generality of the theory that P-grains are the direct progenitors of pollen embryos. Yet many of the plants that do not exhibit an obvious pollen dimorphism still exhibit an increase in the frequency of embryogenesis when donor plants are grown under low temperatures (Raghavan, 1986 and references therein). It is possible then, that pollen dimorphism does in fact occur in these species, but that it is more subtle, and difficult to detect by morphological features alone. This possibility needs to be further explored through more refined physiological and cytological analyses.

The mechanisms whereby various donor plant pretreatments, such as cold chilling, lead to increased P-grain formation and an accompanying increase in pollen embryogenesis is not known. However, it is thought that these effects are mediated through the destruction of the tapetum. Heberle-Bors (1882a; 1982b; 1985) has suggested that pollen embryogenesis is an induced form of apogamy (sporophyte formation via embryogenesis from somatic cells). He has suggested that microspores inherit high amounts of sporophytic determinants in the form of polysomes from the microspore mother cells during the prophase of meiosis. These sporophytic determinants may reactivate the sporophytic gene program in pollen grains making them competent for sporophytic embryogenesis in culture. This early switch toward a sporophytic mode of development blocks further gametophytic development, leaving the pollen grains viable, but functionally sterile. Interestingly, a shift in the sex balance of flowers toward femaleness is correlated with the reinforcement of sporophytic competence of gametophytes.

A similar mechanism may be occuring during the low temperature preconditioning treatment required to induce embryogenesis in *B. napus*. Although *B. napus* does not exhibit any obvious pollen dimorphism (Raghavan 1986), recent flow cytometry studies carried out with microspores from donor plants grown under different growth temperatures suggest that cytological differences do exist between microspores from low versus higher temperature-grown plants (Lo and Pauls, 1992). In particular, microspores isolated from plants grown under lower temperatures appear more translucent than microspores from higher temperature grown plants. It was suggested that this dimorphism may result from a reduction in the cytoplasmic granularity and/or exine density of the microspores. Most importantly, the frequency of microspore embryogenesis was found to be higher when there was a high proportion of this type of microspore in the cultures. Interestingly, higher frequencies of microspore embryogenesis have been reported for certain male-sterile lines of *B. napus* (Seguin-Swartz, 1984; Chuong et al., 1988) suggesting that the establishment of embryogenic competence involves a shift away from the normal pollen developmental pathway.

Interestingly, both napin mRNA and BngNAP1-driven GUS activity were also often observed at low levels in preconditioned microspores grown under low temperatures but not in same stage microspores from plants grown at higher temperatures. This observation suggests that some type of shift toward an embryogenic pathway is occuring at this early stage and that napin gene expression, in addition to being a marker for the induction process, may also provide a marker for the embryogenic competence of microspores. In this respect, it may be possible to utilize napin gene expression, in conjunction with cytological and physiological techniques, as a tool to characterize specific cell types that acquire embryo-forming competence in the anther.

4.1.2 Induction Phase of Microspore Embryogenesis

The critical step in the induction of *B. napus* microspore embryogenesis is the application of a heat-stress treatment at or near the time of microspore mitosis. The mechanism whereby heat stress blocks pollen development and induces embryogenesis is not presently known. In most organisms, exposure to high temperatures has the following effects (1) the transient synthesis of heat stress mRNAs and proteins (HSPs) (2) the suppression of the majority of normal RNA and protein synthesis and (3) the preservation of most pre-existing mRNAs (Brodl, 1991; Vierling, 1991). Thus heat stress may act to block pollen development by interfering with developmental processes that are essential for continued pollen development. This interference may occur through the inhibition of pollen mRNA or protein synthesis or through the rapid turnover of preexisting pollen proteins.

Changes in *in-vitro*-translated mRNA and 2-D gel protein profiles have been observed during the induction phase of microspore embryogenesis (Pechan *et al.*, 1991). In particular there is general reduction in the number of proteins expressed in embryogenically-induced microspores (8h, 32.5 ^{O}C), as compared to microspores continuing pollen development *in vitro* (8h, 25 ^{O}C). Furthermore, many of the proteins which are normally expressed during subsequent stages of pollen development are absent from or severely reduced in embryogenically-induced microspores. Results from our lab indicate that the expression of at least one pollen-specific gene, Bnm1, which is normally highly expressed during the binucleate and trinucleate stages of pollen development, decreases substantially during the induction of microspore embryogenesis (Brian Treacy, personal communication).

Interestingly, exposure to nonheat stresses such as ethanol, arsenite, heavy metals and glucose starvation can also induce a heat stress response (Vierling, 1991). In *B. napus* exposure to ethanol and gamma irradiation can also be used to induce microspore embryogenesis, suggesting that the induction process is mediated through the same pathway. This further suggests that the model presented above also applies to other systems, such as tobacco, that use non-heat stresses to induce embryogenesis.

If heat stress inhibits or eliminates the specific gene expression and/or protein synthesis programs of the gametophyte then gametic development will not continue and an alternative, sporophytic developmental program may proliferate. In most species, including *B. napus,* this new developmental program is driven by the sporophytic vegetative cell (Fan et al., 1988; Kott et al., 1988). The heat-stress treatment may act to release the vegetative nucleus from its arrested state by reactivating cell division activity. It has been suggested that the reinitiation of vegetative cell proliferation takes place through *de novo* sporophytic gene expression or through the translation of stored sporophytic mRNAs (Garrido *et al.*, 1993). However, results with napin, and preliminary results obtained from the analysis of subtraction library clones, suggest the former is more likely, i.e. that a new, embryo-based program of gene expression is initiated (Appendix IV). At present, it is not known whether the mRNAs corresponding to these newly-expressed genes are translated or whether, as in tobacco, the messages are stored for translation at a later point in development.

The induction of microspore or pollen embryogenesis bears a number of similarities to the phenocopy-induced mutations of *Drosophila.* Phenocopies are defined as nonheritable developmental defects which are induced by exposure to environmental stresses such as chemicals or high temperatures (reviewed in Peterson and Mitchell, 1991). Phenocopies in *Drosophila* are generally induced during periods of rapid change in gene expression and/or cell shape. Each specific phenocopy is induced at a different point in development within a very narrow developmental window. The observation that phenocopies can be prevented, through thermoprotection, by a brief exposure to a mild heat shock, supports the theory that some gene or protein essential for development at that time is inactivated.

The induction of microspore/pollen embryogenesis in plants is strikingly similar to the phenocopy-induced mutations of *Drosophila* in that microspore embryogenesis can only be induced during a narrow developmental window and appears to result from an inactivation of normal pollen developmental processes. The analogous "developmental defect" that may occur during microspore/pollen embryogenesis appears to be much more extreme in that it involves a switch from a gametophytic to a sporophytic mode of development. This is not completely surprising given the phenotypic plasticity of plants. One area that must be addressed, especially in plant systems that use heat stress to induce embryogenesis, is the role that thermotolerance plays in the prevention of developmental defects.

4.1.2.1 Mechanism of Induction of Napin Gene Expression in Embryogenically-Induced Microspores

The induction of napin gene expression in heat-stressed microspores does not appear to be a typical heat-stress response in that napin gene expression remains elevated for a prolonged period of time. Previous experiments have shown that the heat-stress response in plants is transient, with HSPs generally declining within 4 to 10 hours after the initiation of heat stress treatment, even during prolonged exposure to high temperatures (Cooper and Ho, 1983; Barnett et al., 1980; Altschuler and Mascarenhas, 1982).

Exposure of developing seeds to heat stress has previously been shown to result in increases in seed storage mRNAs and/or proteins in other species (Altschuler and Mascarenhas, 1985; Chrispeels and Greenwood, 1987; Blumenthal et al., 1990, 1993). For example, in isolated soybean embryos, the mRNA and protein for the 7S storage proteins are preferentially synthesized in seeds under heat-stress conditions (Altschuler & Mascarenhas, 1985; Mascarenhas & Altschuler, 1985). Similarly, in wheat, heat stress has been shown to result in the increase of α- and β-gliadins in grains as well as in roots and leaves (Blumenthal *et al.*, 1990). Examination of gliadin promoters indicates that the conserved heat stress consensus element, HSE(CTNGAANNTTCNAG), can be detected in the promoters of these genes (Blumenthal *et al.*, 1990). No sequences matching this consensus element or the more recently refined HSE NGAANNTTCN (Xiao and Lis, 1988) could be found in the BngNAP1 promoter or in the promoter region of any other *B.*

napus napin gene. This suggests that if napin gene expression is induced by heat stress then this response must be mediated by other *cis*-acting elements.

A more likely possibility is that it is not heat stress *per se* that induces napin expression in microspores, but rather the induction of embryogenesis by heat stress may lead to some secondary event which then results in the induction of napin gene expression. This possibility is supported by previous experiments in our lab which suggested that napin gene expression may be induced by elevated temperatures, but only in embryos and microspores with embryogenic potential (Appendix II). Heat stress may lead to the expression of a protein factor that, in conjunction with embryo-expressed factors, may then result in the elevation of napin gene expression in embryogenic tissues.

It should be possible then, to identify the *cis*- and *trans*-acting factors that direct this response. One approach that could be used to narrow down the DNA regions involved in this response is to construct deletion derivatives of the BngNAP1 gene, reintroduce them back into *B. napus*, and then assay for napin gene expression. Once the DNA region involved in this response has been more precisely defined, it should be possible to use DNase footprinting and South-Western analysis to identify the specific *cis*-acting DNA sequences and *trans*-acting protein factors involved.

The profile of napin gene expression in embryogenically-induced microspores and microsporic embryos is similar to that of the carrot *lea* genes ECP31 and ECP40 (Kiyosue et al., 1992, 1993). ECP31 and ECP40 mRNA accumulates in proembryogenic masses (PEMs) that give rise to somatic embryos in culture and to a lesser extent in somatic embryos up to the torpedo stage. It has been suggested that ECP31 and ECP40 expression in PEMs occurs in response to the high levels of abscisic acid (ABA) found in these cultures and is supported by the observation that ABA can also lead to elevated ECP31 and ECP40 expression in developing somatic embryos. However, ECP31 and ECP40 gene expression could not be induced in ABA-treated seedlings (Kiyosue et al., 1992, 1993). The response to ABA is therefore embryo specific. The expression of these specific carrot *lea* genes during somatic embryogenesis is similar to napin gene expression during *Brassica* napus microspore embryogenesis in that both napin and *lea* genes appear to be sensitive to induction by environmental stresses, but only in embryogenic tissues.

4.2 The Utility of Napin Gene Expression as a Marker for Microspore Embryogenesis

Histochemical analysis of transgenic microspores containing a GUS marker gene fused to the promoter of one member of the BnmNAP subfamily illustrated that this promoter was strongly induced in microspores that possessed the morphological characteristics associated with the induction of embryogenesis. This suggests that napin is predominantly expressed in those microspores that have initiated embryo development in culture. In general, 10 to 30% of heat-stressed microspores exhibit the swelling response; however, only 1 to 10% of the cells in a given culture go on to form embryos (K. Pomeroy, personal communication). The morphological and cytological characteristics of embryogenic microspores that develop past the swelling stage have not been examined in detail. Assays for BnmNAP gene expression could prove useful in this respect. Unfortunately, the GUS assays used in the preceeding analyses were lethal to the cells and thus cannot be used to follow the developmental fate of these swollen microspores. An alternative approach may be to use a non-lethal marker gene, such as luciferase, in conjunction with the BngNAP1 promoter, to assay for napin gene expression in embryogenic microspores. Using this approach it may be possibly to identify, and to follow the fate of individual cells as they become competent, induced and then committed to forming embryos in culture.

The BngNAP1-GUS construct used in this study also has potential application as a marker for embryogenic capacity and induction in a variety of *B. napus* cultivars (and in other *Brassica* species) that have been identified as being recalcitrant in terms of their ability to form embryos in culture. Although this approach requires the development of transgenic populations, it may prove to be worthwhile in the long run as this reporter gene construct can be used to quickly (and relatively inexpensively) assess the effect of various plant growth and culture conditions on embryogenicity well in advance of the appearance of actual embryos.

It is also possible, despite differences in the nature of the donor tissue and culture conditions used to induce embryogenesis, that common mechanisms operate for the induction of embryogenesis from cultured plant tissues. If this is true, then napin gene expression may also have utility as a marker for the induction of embryogenesis in other pollen/microspore systems or even in a number of somatic embryo culture systems. In fact, preliminary experiments, using microprojectile bombardment, indicate that the BngNAP1-GUS construct is expressed in embryogenic alfalfa calli, but not in non-embryogenic soybean calli; a CaMV 35S promoter-GUS fusion construct is expressed in both embryogenic and nonembryogenic callus tissues (L. Tian and D.C.W Brown, personal communication). Although these results are preliminary they do suggest that napin gene expression is correlated with the ability to form embryos in somatic cell cultures.

Tobacco pollen culture represents a highly efficient system for the study of *in vitro* embryogenesis; however, no molecular markers for the induction of tobacco pollen embryogenesis have been characterized. During the course of this thesis I attempted to determine if the BngNAP1 promoter was also a marker for the induction of tobacco pollen embryogenesis. Initially, pollen from transgenic tobacco plants carrying the BngNAP1-GUS construct was analysed histochemically for GUS activity. Unfortunately, high levels of endogenous GUS activity were observed in pollen from both transformed and untransformed plants (data not shown). Neither increasing the pH of the assay buffer (Martin *et al.*, 1992) nor the addition of 20% methanol (Kosugi *et al.*, 1990) were sufficient to eliminate background activity and the experiments were not carried any further. High-level GUS activity in tobacco pollen has previously been observed (Plegt and Bino, 1989). Nonetheless, it would be interesting to determine if BnmNAP gene expression occurs in tobacco P-grains, prior to culture, and during the starvation treatment used to induce embryogenesis. The use of a different reporter gene, such as *luciferase*, would facilitate such a study.

A recent report by Koning *et al.* (1992) provides support, although indirectly, that napin gene expression may be a marker for the embryogenic competence of tobacco pollen. This group observed that expression of a construct containing the promoter region of a *B. rapa* napin gene fused to the coding region of the cytotoxic *Psuedomonas aeruginosa* exotoxin A gene lead to male sterility in transgenic tobacco, but not in transgenic *B. napus* (Koning *et al.*, 1992). Interestingly, this *B. rapa* napin gene, BcNA1, is orthologous to the previously described *B. napus* Na gene, which was also isolated from the embryogenically induced microspore cDNA library. The discrepancy between expression profiles in *Brassica* and tobacco may be explained by assuming that the Na gene is also expressed in embryogenically-competent, tobacco pollen. Embryogenically-competent pollen grains occur naturally in tobacco (as P grains), but are possibly only seen in *B. napus* when donor plants are grown under low temperatures. If the napin-exotoxin A construct is expressed in embryogenically-competent microspores/pollen it may lead to male sterility in tobacco, but would only lead to male sterility in *Brassica* when the donor plants are grown under low temperatures. This is an intriguing possibility that should be examined in more detail.

4.3 BnmNAP Expression in Developing Microsporic and Zygotic Embryos

A number of studies have examined the temporal and spatial patterns of total napin gene expression in developing zygotic embryos of *B. napus*. However, relatively little is known about napin gene expression during microsporic embryo development. Furthermore, in both embryo systems, the spatial and temporal expression profiles of specific napin gene family members during development has not been examined in detail. In this study, the expression profile of the BnmNAP subfamily of napin genes has been examined in both developing microsporic and zygotic embryos of *B. napus*. The expression of the BnmNAP subfamily was examined by RNA gel blot analysis, using a subfamily-specific probe, and by histochemical and fluorogenic analysis of transgenic plants expressing a BngNAP1 promoter-ß-glucuronidase gene fusion.

The temporal pattern of BnmNAP expression detected by the BnmNAP subfamily oligonucleotide probe was shown to differ between microsporic and zygotic embryos with respect to the developmental stage at which napin mRNA is first detected. In microsporic embryos napin mRNA can be detected by RNA gel blot analysis as early as the globular stage, but is only detected at the early cotyledon stage of zygotic embryo development. This difference in the temporal pattern of napin gene expression between microsporic and zygotic embryos is likely due to the dilution of zygotic embryo tissue in seed samples. Both GUS fluorogenic and GUS histochemical assays indicated that the promoter of one BnmNAP family member, BngNAP1, is active in globular stage zygotic embryos.

Nonetheless, these results suggest that at least one member of the BnmNAP subfamily of napin genes, BngNAP1, is expressed earlier, and in a different spatial pattern than that reported for the entire napin gene family. Fernandez *et al* (1991) have shown that total napin mRNA begins to accumulate in late heart/early torpedo stage of development throughout the cortical cells of the axis, and to a lesser extent in the outer face of the cotyledons. During the torpedo and cotyledon stages, napin mRNA synthesis progresses in a wave-like fashion from the outer to the inner faces of the cotyledons. Napin mRNA cannot be detected in the cells of either the shoot or root apical meristems during the early stages of embryo development. However, during the maturation stage, high levels of napin mRNA accumulate in the shoot apical meristem and its associated leaf primordia and vascular tissue, but not in the cells of the root apical meristem. In this study, GUS expression directed by the BngNAP1 promoter was detected in both microsporic and zygotic embryos as early as the globular stage of development. GUS activity was initially confined to the lower region of axis (excluding the root cap) and remained absent from the upper region of the axis until the cotyledon stage. GUS activity was also detected in the vascular tissue at a slightly earlier stage of development than in the study by Fernandez *et al*. Together these results suggest that the BngNAP1 promoter is activated earlier and is initially restricted to a subset of the cells expressing napin.

Fernandez and co-workers (1991) have suggested that in *B. napus* an internal clock, set in response to one or more factors that exist in a gradient centered on the apex, determines when storage protein gene expression begins in different parts of the embryo. Cells exposed to low concentrations of this factor, such as those in the axis, begin storage protein synthesis earlier than cells exposed to increasingly higher levels of the factor, such as the cotyledons and the epiphysis respectively.

The observation that at least one member of the BnmNAP subfamily directs a pattern of expression that differs from other members of the napin multigene family indicates that individual members within a multigene family respond differently, both in time and in space, to these spatial gradients. The widespread observation that individual members within seed storage protein multigene families are often differentially expressed within the embryo (Guerche et al., 1990; Sjödahl *et al.*, 1993) supports this hypothesis. Alternatively, the expression of individual genes, in addition to being controlled by a single gradient of spatial factors, may also be influenced by additional smaller sub-gradients of factors.

Gene specific probes were not used to differentiate among BnmNAP subfamily members, so the relative contributions of each member to the total cannnot be determined. Furthermore, it is difficult to make any quantitative statements about the pattern of BnmNAP expression during development as an internal standard was not used to ensure that equal amounts of poly(A)$^+$ were present in each of the total RNA samples. It is also difficult to quantify with the GUS fluorogenic assays due to the stability of the GUS protein (Martin *et al.*, 1992).

It is likely, given the results obtained from the cloning of napin cDNAs from the induced microspore library, that other napin genes may are also expressed early in *Brassica* embryo development. At least two other napin genes, an Na-like and a napl/napA-like gene, were also found to be expressed at this time. A previous study by Blundy *et al.* (1991) demonstrated that expression of one of these genes, Na, peaked and declined earlier in embryo development than the other members of the napin multigene family. Unfortunately, seed samples earlier than 27 DAP were not analysed so it cannot be determined when Na gene expression begins. No detailed expression studies have been carried out with the Napl/napA gene in *B. napus;* however, the napA gene has been shown to be expressed at the torpedo stage of embryo development in transgenic tobacco. The possibility that these genes are also expressed early in development cannot be excluded.

The pattern of BngNAP1-GUS expression was shown to differ between embryos of *Brassica napus* and tobacco. Although the seed specificity and timing of induction were maintained, the spatial patterns of expression differed significantly between the two species. As in *B. napus,* the BngNAP1 promoter appears to be activated early in tobacco seed development; however, BngNAP1 directed GUS expression was first detected in the endosperm of tobacco seeds containing globular stage embryos, rather than in the embryo. BngNAP1 expression did not begin in the embryo until the early heart stage of development. BngNAP1-directed GUS expression in *B. napus* embryos began in the lower axis and spread upward, whereas in tobacco, GUS expression began in the middle of the embryo, just under the lobes of the developing cotyledons and spread outward in both directions. Similar spatial and temporal patterns of expression have been observed in transgenic tobacco for the *Arabidopsis thaliana* 2S albumin storage protein gene, At2S1 (DeMoor, 1992) and the soybean Kunitz trypsin inhibitor gene, KTi3 (Perez-Grau and Goldberg, 1989). In *Arabidopsis,* the At2S1 gene is expressed specifically in the axis of the embryo (Guerche *et al.*, 1990). In soybean, KTi3 gene expression begins at the globular stage, in the micropyle end of the axis, spreads throughout the axis from the heart to the cotyledon stage and then accumulates in a wave-like fashion from the outer to the inner margins of the developing cotyledons during the maturation phase (Perez-Grau and Goldberg, 1989). Taken together, these results suggest that the expression of seed protein genes, although confined to similar tissues, is not spatially regulated in the same manner in other species.

The results presented in this thesis describe the isolation and characterization of napin cDNA clones expressed during the induction phase of *B. napus* microspore embryogenesis. Three members of a subfamily of napin seed storage protein genes (BnmNAP) were shown to be highly expressed in embryogenic microspores, but not in microspores undergoing pollen development. Other, as of yet unidentified, clones have also been shown to be differentially expressed during the induction phase. This work represents the first detailed characterization of molecular markers for the induction phase of microspore embryogenesis.

APPENDIX I

SEED STORAGE PROTEIN GENE EXPRESSION IN MICROSPORES AND MICROSPORIC EMBRYOS

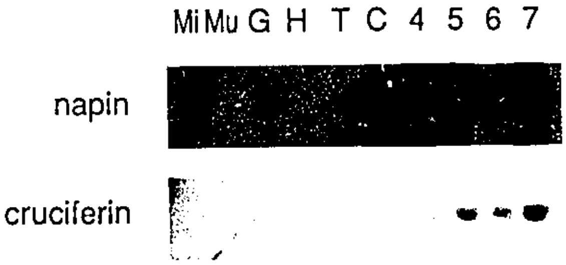
The results presented in Appendix I were obtained by M.-J. Gines and J. DeMoor and provide the experimental basis for my Ph.D thesis. These results, together with results presented in Appendix II and elsewhere in this thesis, have been accepted for publication.

B. napus cv. Topas microspores were collected at the late uninucleate and early binucleate stages of development and either induced to undergo embryogenesis by culture at 32.5°C for 4 days (Mi sample) or allowed to continue pollen development by culture at 25°C for the same amount of time (Mu sample). Heat-treated microspores were transferred to 25°C and microsporic embryos were collected at the globular, heart, torpedo and cotyledon stages of development. The expression pattern of seed storage protein genes in microspores and developing microsporic embryos was determined by Northern blot analysis using napin (pN2; Crouch et al., 1983) and cruciferin (pC1; Simon et al., 1985) cDNA probes. Figure 1 shows that both napin and cruciferin mRNA accumulated throughout the cotyledon stage and were either low or undetectable during earlier stages of embryo development. Cruciferin gene expression was initiated at a slightly later stage of development and cruciferin mRNA accumulated to a lower level than napin mRNA. The lower intensity of the 6-week cotyledon samples could be due to a smaller amount of mRNA in these lanes. Hybridization with a 26S rRNA probe indicated that the 6-week samples contained slightly less rRNA than the other samples (results not shown). In zygotic embryos, cruciferin gene expression is also initiated later in development; however, cruciferin mRNA accumulates to higher levels than napin mRNA (Finkelstein *et al.*, 1985; DeLisle and Crouch, 1989).

While cruciferin mRNA was absent from both microspore samples, we detected high levels of napin mRNA in microspores that had been induced to undergo embryogenesis (Mi sample), but not in microspores that were cultured to develop as pollen (Mu sample). The amount of napin mRNA declined considerably by the globular stage.

Figure 1. Seed Storage Protein mRNA Accumulation in Microspores and Microsporic Embryos

RNA gel blots containing 5 µg of total RNA per lane were probed with ^{32}P-labeled napin (pN2) or cruciferin (pC1) cDNAs. Exposure times were identical for the napin and cruciferin blots. Total RNA was isolated from late uninucleate and early binucleate microspores, collected from preconditioned donor plants grown at 10°/5°C (day/night), after 4 days in culture at 32.5°C (Mi) or after 4 days in culture at 25°C (Mu) and from microsporic embryos at the globular (G), heart (H), torpedo (T), 21 day cotyledon (C), 28 day cotyledon (4), 35 day cotyledon (5), 42 day cotyledon (6) and 49 day cotyledon (7) stage of development.


Mi Mu G H T C 4 5 6 7
napin
cruciferin

APPENDIX II

RELATIONSHIP BETWEEN NAPIN GENE EXPRESSION AND MICROSPORE EMBRYOGENESIS

The possibility that napin gene expression is an early marker for the induction of *B. napus* microspore embryogenesis was further explored by examining the relationship between the induction of napin gene expression and the developmental stage of donor microspores, the plant growth conditions, and the microspore culture conditions. This work was also carried out by M.-J. Gines and J. DeMoor.

Correlation with Stage of Pollen Development

The frequency of *B. napus* microspore embryogenesis has been shown to vary with the stage of microspore development (Kott et al., 1988; Fan et al., 1988; Pechan and Keller, 1988; Telmer et al., 1992). Particular stages of microspore development can be greatly enriched by collecting flower buds of specific lengths. We examined the relationship between napin mRNA accumulation, the stage of microspore development and the induction of embryogenesis.

Small flower buds, 1.5 to 3 mm in length, contained approximately equal amounts of microspores in tetrad and early uninucleate stages (E samples). Embryo yields of approximately 4% were obtained when these microspores were isolated and induced to undergo embryogenesis in culture (Ei sample). Under nonembryogenic culture conditions (Eu sample) microspore development continued and some pollen-like structures were observed. Figure 1A shows that napin mRNA accumulated to high levels when microspore embryogenesis was induced (Ei sample, Figure 1A), but was present at very low levels in microspores that were allowed to develop as pollen (Eu sample, Figure 1A). Small amounts of napin mRNA could often be detected in microspores at the time of collection (Eo sample, Figure 1A). The low levels of napin mRNA detected in freshly isolated microspores suggests that napin genes are transcriptionally active prior to the induction of embryogenesis, and that expression is greatly enhanced upon induction.

Flower buds, 3 to 5 mm in length, contained microspores at the late uninucleate and early binucleate stages of development (M samples). Upon induction of embryogenesis, this sample (Mi sample, Figure 1A) yielded the highest percentage of embryos (approximately 20%). When the microspores were cultured at 25^{o}C (Mu sample), approximately 40% of the microspores developed through the first asymmetric division typical of pollen development (Kott et al., 1988) and embryo formation was not observed. Napin transcripts accumulated in microspores induced to undergo embryogenesis (Mi sample, Figure 1A) but not in microspores that developed as pollen in culture (Mu sample, Figure 1A).

Again, small amounts of napin message were present in microspores at the time of collection (Mo sample, Figure 1A).

Large flower buds, 5 to 9 mm in length, contained a mixture of microspores from the late binucleate to late trinucleate stages (P samples). All microspore samples, whether induced to undergo embryogenesis (Pi sample) or allowed to continue pollen development (Pu sample) became elongated and swollen and eventually burst. A very low level of napin gene expression was observed in microspores induced to undergo embryogenesis (Pi sample, Figure 1A), but not in microspores cultured at 25°C (Pu sample, Figure 1A) or in microspores at the time of isolation (Po sample, Figure 1A). Embryos were not obtained from any of these samples.

The data indicate that the induction of high levels of napin gene expression is developmentally regulated in microspores and coincides with the stages of microspore development that are optimal for embryogenesis.

Correlation with Induction of Embryogenesis

The induction of *B. napus* microspore embryogenesis by heat is also dependent on the preconditioned growth of donor plants at low temperatures (10°C day/5°C night) (Keller et al., 1987). Omission of either the low temperature growth of donor plants or the heat treatment of isolated microspores leads to a severe reduction in or abolition of embryo formation. We examined the relationship between the donor plant growth temperatures, microspore culture temperatures and the induction of napin gene expression in late uninucleate and early binucleate microspores.

The induction of napin gene expression and subsequent embryo formation were both eliminated when microspores from donor plants grown on a 25°C/20°C day/night temperature cycle were heat treated (mi sample, Figure 1B) or allowed to continue pollen development (mu sample, Figure 1B). Furthermore, microspores from donor plants grown under low temperatures simultaneously lost the ability to form embryos and express napin if cultured for 24 hours at 25°C prior to the heat treatment (Mx sample, Figure 1B). Napin gene expression was only induced in microspores under conditions that resulted in microspore embryogenesis (Mi sample, Figure 1B).

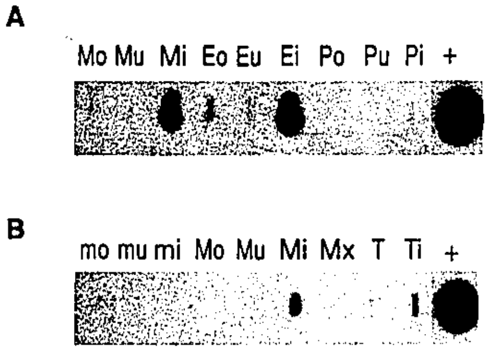
Microsporic embryos which usually express low levels of napin mRNA revealed small increases in napin gene expression when the culture temperature was increased from 25° to 32.5°C. Torpedo-stage embryos from 12-day cultures treated at 32.5°C for 2 days showed elevated napin mRNA levels (Ti sample, Figure 1B) relative to torpedo stage embryos that were cultured at 25°C (T sample, Figure 1B). The same result was observed for heart stage embryos (data not shown). Heat treatment of other tissues, including leaf, stem and root, collected from donor plants grown under either low or normal temperatures, failed to induce napin gene expression (data not shown). These results suggest that the induction of napin gene expression is not a direct response to the plant growth or microspore culture conditions alone. Napin gene expression may be induced by elevated temperatures, but only in embryos and microspores with embryogenic potential.

Figure 1 Relationship Between Microspore Stage, Microspore Culture Conditions and Napin mRNA Accumulation

RNA slot blots containing 6 µg of total RNA per slot were hybridized to a ^{32}P-labeled napin (pN2) cDNA.

(A) Effect of microspore developmental stage on the induction of napin gene expression.
Microspores were collected from flower buds 1.5 to 3.0 mm (E), 3.0 to 5.0 mm (M) and 5.0 to 9.0 mm (P) in length from preconditioned donor plants grown at 10°/5°C (day/night). Total RNA was isolated from these microspores either at the time of microspore collection (Eo, Mo, Po), after 4 days in culture at 25°C (Eu, Mu, Pu) or after 4 days in culture at 32.5°C (Ei, Mi, Pi). Total RNA from 28 days post anthesis zygotic embryos (+) was used as a hybridization control.

(B) Effect of plant growth and culture conditions on the induction of napin gene expression.
Late uninucleate and early binucleate microspores were collected from preconditioned donor plants grown at 10°/5°C (day/night) (M) or from donor plants grown under nonembryogenic temperatures (25°/20°C, day/night) (m). Total RNA was isolated from these microspores either at the time of microspore collection (Mo, mo), after 4 days in culture at 25°C (Mu, mu), after 4 days in culture at 32.5°C (Mi, mi) or after 24 hrs in culture at 25°C, followed by 3 days at 32.5°C (Mx). Total RNA was also isolated from torpedo stage microsporic embryos growing at 25°C (T) or 32.5°C (Ti).


A
Mo Mu Mi Eo Eu Ei Po Pu Pi +
B
mo mu mi Mo Mu Mi Mx T Ti +

APPENDIX III

GUS ACTIVITY IN UNTRANSFORMED SEEDS AND TRANSFORMED SOMATIC TISSUES OF *B. NAPUS* AND TOBACCO

TABLE I GUS Activity in Developing Seeds and Somatic Tissues of Untransformed *B. napus* cv. Westar and Transgenic *B. napus* cv. Westar Carrying the pBngNAP1-*gusA*-3'*nos* Construct

	GUS activity (nmol/MU/min/mg protein)			
	untransformed	**transgenic**		
sample		**C18D-1D**	**C18D-2C**	**C18D-2E**
LEAF	-	0.011	0.008	0.023
STEM	-	0.002	0.002	0.009
SEEDS-10 DAP[a]	0.003	-	-	-
-14 DAP	0.001	-	-	-
-18 DAP	0.002	-	-	-
-21 DAP	0.001	-	-	-
-28 DAP	0.001	-	-	-
-35 DAP	0.001	-	-	-
-42 DAP	0.002	-	-	-
-49 DAP	0.003	-	-	-
-56 DAP	0.011	-	-	-

[a] DAP, days after pollination

TABLE II GUS Activity in Seeds and Somatic Tissues of Untransformed *N. tabacum* cv. Petit Havana SR1 and Transgenic *N. tabacum* cv. Petit Havana SR1 Carrying the pBngNAP1-*gusA*-3'*nos* Construct

	GUS activity (nmol/MU/min/mg protein)			
	untransformed	**transgenic**		
sample		**2NAP**	**3NAP**	**4NAP**
LEAF	-	0.008	0.026	0.029
STEM	-	0.015	0.030	0.016
SEEDS-10 DAP[a]	0.025	-	-	-
-14 DAP	0.005	-	-	-
-18 DAP	0.014	-	-	-
-22 DAP	0.013	-	-	-
-26 DAP	0.005	-	-	-
-28 DAP	0.007	-	-	-

[a] DAP, days after pollination

APPENDIX IV

ISOLATION AND CHARACTERIZATION OF MARKER GENES FOR THE INDUCTION OF *B. NAPUS* MICROSPORE EMBRYOGENESIS

Appendix IV describes the preliminary characterization of additional molecular markers for the induction of *B. napus* microspore embryogenesis. This work represents the combined efforts of a number of different researchers including Thérèse Ouellet (construction of subtracted cDNA probe and screening of embryogenically induced microspore cDNA library) and Jiro Hattori (subcloning of lambda clones), both of the Plant Research Centre, Agriculture Canada, and myself (sequencing, DNA and RNA gel blot analysis).

Subtractive hybridization is a technique used to isolate cDNA clones that are differentially expressed in one cell type as compared to another. In this method, poly $(A)^+$ RNA from one cell type is used as a template to synthesize radiolabelled first strand cDNA which is then hybridized to an excess of poly$(A)^+$ mRNA isolated from the second cell type in which the gene(s) of interest is not expressed. cDNAs and mRNAs common to both cell types will form DNA:RNA hybrids that can be separated from the unhybridized single stranded cDNAs by hydroxyapatite column chromatography.

Single stranded cDNA will pass through the column while the cDNA-mRNA hybrids, as well as any unhybridized mRNA, will remain bound to the column. The unhybridized cDNAs represent an enriched population of sequences expressed in the cell type of interest. The single stranded subtracted probe can then be used to screen a cDNA library for the gene(s) of interest. Using this technique it is possible to detect rare cDNAs that comprise only 0.005 to 0.01% of the total mRNA population (Sambrook *et al.*, 1989). However, this technique is not well suited for the detection of differentially expressed members of multigene families; nor can it be used to isolate genes whose level of expression is either up- or down-regulated in one of the two cell types.

The subtractive hybridization technique outlined above was used to identify genes that were expressed in microspores undergoing embryo development in culture, but absent from nonembryogenic microspores. Poly(A)$^+$ mRNA was extracted from two cell types. The first sample consisted of a population of late uninucleate microspres/early binucleate pollen, isolated from donor plants grown under low temperatures, that was cultured for 4 days at 32.5 ^{o}C to induce embryogenesis (Mi sample). The second sample consisted of the same cell population cultured for 1 day at 25 ^{o}C followed by 3 days at 32.5 ^{o}C (Mx sample). The Mx population is not embryogenic. Poly(A)$^+$ mRNA from the Mi sample was used to make radiolabelled first strand cDNA that was then hybridized to an excess of Mx poly(A)$^+$ mRNA. The two RNA samples were exhaustively hybridized and then subjected to hydroxyapatite column chromatography. The resulting radiolabelled, single stranded cDNA was used as a probe to screen the previously constructed Mi cDNA library (section 2.10). Filter lifts, from approximately 100,000 pfus were screened with the subtracted probe, as well as a negative control probe consisting of radiolabelled first strand Mx cDNA and a napin cDNA probe. Four plaques, designated 6A1, 9A1, 42A1 and 60B1, which hybridized strongly to the subtracted probe, but not to the Mx or napin probes, were selected for further analysis. DNA from each of the four plaques was digested with *Eco* RI and *Xba* I. The resulting fragments, containing the cDNA insert, were purified and then ligated to *Eco*R I, *Xba* I double digested pGEM4Z vector DNA. DNA sequencing, DNA probe synthesis and DNA/RNA gel blot analysis were carried out according to methods previously described in this thesis.

Clone 6A1

The sequence of cDNA insert 6A1 is shown in Figure 1. The 6A1 cDNA clone is 362 bp in length, excluding the poly(A) tail. The 6A1 cDNA clone contains an *Eco*R1 adaptor (used during the cDNA manipulation procedure) between positions 310 to 325 (in bold, Figure 1) suggesting that it is a chimera of two cDNA sequences. Examination of the *Eco* RI, *Xba* I double digest of DNA isolated from the original lambda clone indicates that a single insert of approximately the

Figure 1 DNA Sequence of the 6A1 cDNA Insert

The nucleotides are numbered on the left. The position of the *Eco* R1 adaptor sequence is indicated in bold.

1 ACATTGATTT CAACAACGGA TGCAAACTGA GCAAATAAGA AACGTTAATA

51 AAAAGTAGAA ATACTTCTAA TTCATGTTTC ATATTCATAG CATCATCAAA

101 CAATAACACT TTGAGGCTAC CAAGTACCAA ACAATAACAA AAACTATGGA

151 ATGATGATTA TTGATTAATA CTGAAATTGG ACGTGTTGAA TGCAGGATAT

201 GGGAGGCTTT CCTAGTGGTT CTTCTTGTGG TTTATACTGC ATGGGTTTCC

251 CTTTTCGAGT TTGGGTTCTT GAGAAAGCCA AGGCCACCAC TTTCTATCAC

301 CGATAACAT**C GACAGCAACG GAATT**CTGCA CATTCAACAT ATCATTTATT

351 ATTTTTCCCC AC

same size as the 6A1 cDNA *Eco* RI/*Xba* I fragment was obtained. This suggests that the observed rearrangement occured during prior manipulation of the induced microspore cDNA. Incomplete digestion with *Eco*RI during the subcloning procedure would account for the persistence of this fragment.

Comparison of the nucleotide sequence of 6A1 to published sequences indicates that nucleotides 188 to 309 are highly similar to the previously described *A. thaliana* cDNA clones AKT1 (Sentenac *et al.*, 1992) and KAT1 (Anderson *et al.*, 1992)(Figure 2). The AKT1 and KAT1 cDNA clones are 2.65 and 2.2 kb in length, respectively. The two cDNAs were cloned by their ability to complement *Saccharomyces cerevisiae* mutants deficient in K^+ uptake and appear to encode inward rectifying K^+ channel proteins. (Sentenac *et al.*, 1992; Schachtman *et al.*, 1992; Anderson et al., 1992). Although similar, ATK1 and KAT1 are not allelic. The predicted amino acid sequences of AKT1 and KAT1 share similarity in a region that includes a cluster of six putative transmembrane helices (S1-S6) with conserved amino acids corresponding to a presumptive voltage sensing region (S4) as well as a pore forming region (H5). AKT1 contains additional intracellular domains consisting of both a cyclic nucleotide binding site and an ankyrin like region.

Comparison of the nucleotide sequence of 6A1 with those of AKT1 and KAT1 indicates that 6A1 cDNA insert is more similar to AKT1 than to KAT1. As a group these three sequences are

Figure 2 Comparison of the DNA sequence of the 6A1 cDNA insert with the AKT1 and KAT1 sequences (Sentenac *et al.*, 1992; Anderson, *et al.*, 1992).

The EcoR1 adaptor is shown in bold. The numbering is for the 6A1 cDNA insert. A dash was used to indicate where a nucleotide was the same as that in 6A1. The nucleotide is indicated in positions where the sequence differed from 6A1. Positions where gaps were introduced are indicated with an asterisk.

```
                                                                                53
6A1                    ACATTGATTTCAACAACGGATG**CAAACTG**AGC***AAATAAGAAACGTTAATAAAA
AKT1                   -A-AG--CAAA--AAAG-A-CCAT-A--GA-GA---CAAG--A--A------CGG-GATG
KAT1                   GAGC-*-CG---GGG-AAAGATGT-G-TC******TCTTGG-CTC----TT-CTTC****

                                                                               101
6A1                    AGTAGAAATACTTC*TAATTC****ATGTTTCATAT****TC**ATAGCAT*CATCAAAC
AKT1                   --AG--GGGG---TG-T--GCGGAC-A--CCA-G--GAGA-TGA-C---T-T-GAG-G-G
KAT1                   ****----G-*TTC*TGCG-CGAGGA******---CAATA-AGACA*****C---A----

                                                                               157
6A1                    AATAA*CACTTTGAGGCTACCA**AGTACCAAACAATAACAAAAACTATGGAATGAT*GA
AKT1                   -G--GT--T---AGTCT-T-T-CTG-A-TTTT--CT-C--TTGGTGC-A-A-G-A--C--
KAT1                   -G*-GT*-G---C**CTCT-TGCCGA-CTTCT--CA-CT-TTGG-GCCA-G-TCA-CCA-

                                                                               205
6A1                    TTA*TTGA**TTAA******TACTGAAATTGGACGTGTT***GAATGCAGGATATGGGAG
AKT1                   CG-G--A-GC---GGAGATTCGT----TC-CCTTATGA-CACA---A-------------
KAT1                   -CTAC-A-GC-CCGCAAACAC-TAATCTC-CCTTT-AA-CCACGT-A---AGCG------

                                                                               265
6A1                    GCTTTCCTAGTGGTTCTTCTTGTGGTTTATACTGCATGGGTTCCCTTTTCGAGTTTGGG
AKT1                   ------T-----------***--------C--A--T--------GCC-------------
KAT1                   ATG-GGC----A***T-A--A--TA----CT-A--T---A---G--AA--TC-A----CT

                                                                               324
6A1                    TTC*TTGAGAAAGCCAAGGCCACCACTTTCTATCACCGATAACATCGACAGCAACGGAAT
AKT1                   ---*-----------------------------------------T-TG-AC****-C--
KAT1                   ---A-CACCT-TAAA--A-A-G-G-T----*----T---C-----T-TTA-TGG-****-

                                             362
6A1                    TCTGCACATTCAACAT*ATCATT*TATTATTT*TTCCCCAC
AKT1                   ---T-G-CA--G-T--C-----GAC---C---G--GGTT-C
KAT1                   ---T-G-CA-TG-T--T--TC-CACC--C--CG-CG-TT-T
```

most similar in the region comprising the S1 and S2 membrane spanning regions. The 5' amino terminal ends of all three sequences are not well conserved.

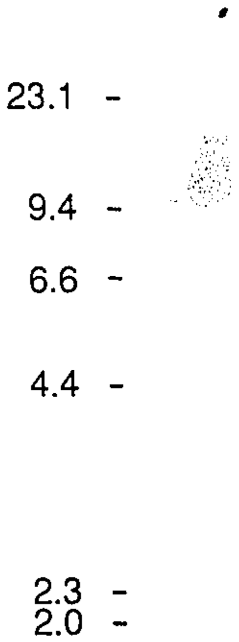
The 3' portion of clone 6A1 does not show significant nucleotide similarity to any previously identified genes, suggesting that the two portions of 6A1 might not be derived from the same mRNA.

Two attempts to isolate a full length K^+ channel cDNA from the induced microspore cDNA library were unsuccessful. Weak hybridization signals were obtained when filter lifts from the induced microspore library were screened with a radioactively labelled *Eco*RI insert from clone 6A1; however, no subsequent hybridization signals were detected when individual positive plaques were rescreened. Furthermore, repeated attempts to isolate a full length 6A1 clone from induced microspore cDNA using PCR were also unsuccessful. An internal primer, specific to the 5' end of the 6A1 cDNA, and a primer complementary to the poly(A) tail of the cDNA clone, generated PCR products of approximately the same size as the original 6A1 cDNA clone (data not shown).

DNA gel blot analysis of *Eco*RI digested *B. napus* cv. Topas DNA, using the 5' *Eco*RI fragment of clone 6A1 as a probe, indicates that the putative K^+ channel portion of this cDNA clone is present once per haploid genome (Figure 3). The small size of the hybridizing bands suggests that the 6A1 probe may be detecting only the 5' portion of these genes, either due to the presence of an internal *Eco*RI site or due

Figure 3 DNA Gel Blot Analysis of 6A1 Related Genes in the *B. napus* Genome

B. napus cv. Topas DNA was digested with *Eco* R1, separated electrophoretically on 0.7% agarose gels and transferred to nylon membranes. The gel blots were hybridized overnight to a ^{32}P-labeled *Eco* RI insert from the 6A1 cDNA clone. The final wash was in 0.1X SSC, 0.1% SDS at 50 °C. The molecular size markers are lambda-*Hind* III restriction fragments.


23.1 -
9.4 -
6.6 -
4.4 -
2.3 -
2.0 -

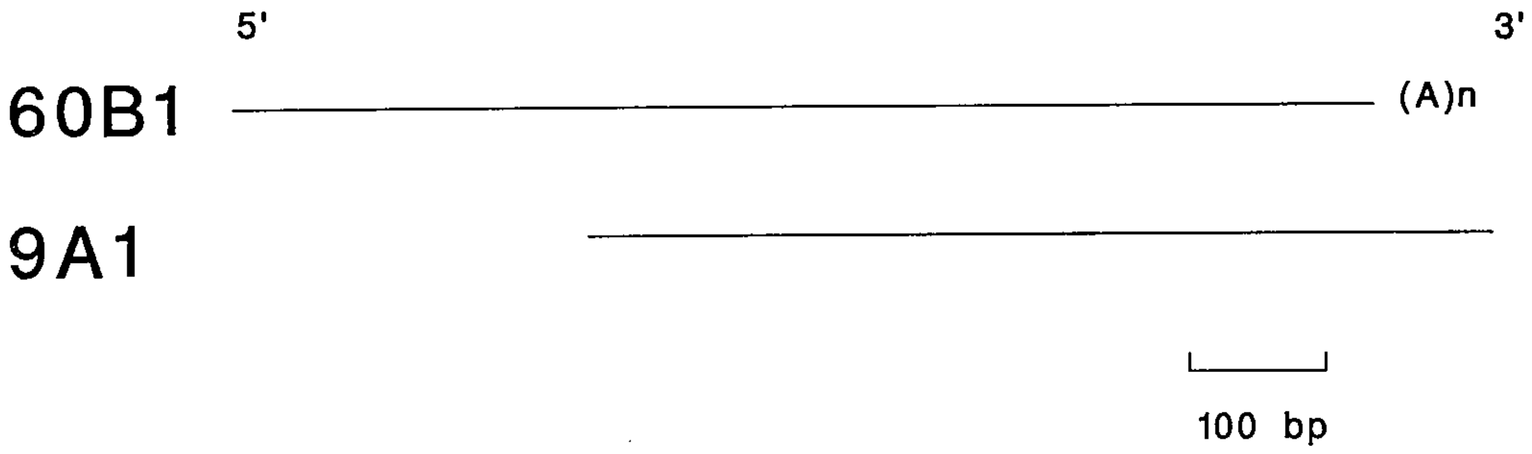
to the presence of an intron also with an internal *Eco*RI site. Alternatively, the genomic fragment corresponding to 6A1 may be closely flanked by *Eco*RI sites. The 3' non K^+ channel portion of clone 6A1 has not been used to probe *B. napus* genomic DNA.

The temporal pattern of expression of the K^+ channel portion of the 6A1 cDNA insert was examined by RNA gel blot analysis of microspores and developing microsporic and zygotic embryos, however no positive hybridization signals were obtained. The inability to detect 6A1 gene expression may simply be a consequence of the low abundance of K^+ channel proteins (Cao *et al.*, 1992). A more sensitive assay, such as reverse transcriptase PCR, RNAse protection or *in situ* hybridization could be used to increase the sensitivity of detection. A second possibility is that the K^+ channel portion of clone 6A1 was derived from a DNA contaminant. Both reverse transcriptase PCR and/or *in situ* hybridization could potentially resolve this problem.

9A1 and 60B1

Preliminary nucleotide sequence analysis of the 9A1 and 60B1 cDNA clones suggests that they are members of the same multigene family (data not shown). 9A1 and 60B1 are 660 and 880 nt in length respectively. The two clones overlap by approximately 620 nt (Figure 4). 9A1 appears to be truncated at the 5' end and does not contain a poly(A) tail. The 60B1

Figure 4 Schematic Representation of The Region of Sequence Overlap Between the 9A1 and 60B1 cDNA inserts


5'
3'
60B1
(A)n
9A1
100 bp

cDNA clone however, does contain a poly(A) tail, suggesting that alternative polyadenylation addition sites exist for these two clones. A significant open reading frame has not been established for either of these clones due to the incompleteness of the sequencing data. No nucleotide similarity was found between these two sequences and other published sequences in the literature.

DNA gel blot analysis of *B. napus* cv. Topas DNA digested with *Eco*RI, using either the 9A1 or 60B1 cDNA insert as a probe, indicates that these two sequences are also present once per haploid genome and are therefore allelic (Figure 5). Again, the small size of the hybridizing fragments suggests that either the genomic fragments corresponding to these sequences are closely flanked by *Eco*RI restriction sites or, the fragments contain an internal *Eco*RI site. Clone 60BI does, in fact, contain an *Eco*RI restriction site approximately 75 bp from the 3' end of the cDNA clone.

Figure 6 shows the temporal pattern of 9A1 and 60B1 gene expression detected in microspores, developing microsporic and zygotic embryos as well as in a number of somatic tissues. Both cDNA inserts hybridize to a 950 nt message and both exhibit the same temporal pattern of gene expression. The 9A1/60B1 cDNA clones are expressed in embryogenically induced microspores, but not in microspores continuing pollen develoment in culture. Expression of clones 9A1 and 60B1 continues during subsequent microspore embryo development, from the globular through to the 21-day

Figure 5 DNA Gel Blot Analysis of 9A1/60B1 Related Genes in the *B. napus* Genome

B. napus cv. Topas DNA was digested with *Eco* R1, separated electrophoretically on 0.7% agarose gels and transferred to nylon membranes. The gel blots were hybridized overnight to a ^{32}P-labeled *Eco* RI/*Xba* I double digested insert from the 9A1 cDNA clone. The same pattern of hybridizing bands was observed when 60B1 cDNA insert DNA was used as a probe. The final wash was in 0.1X SSC, 0.1% SDS at 50 °C. The DNA size markers are lambda-*Hind*-III restriction fragments.

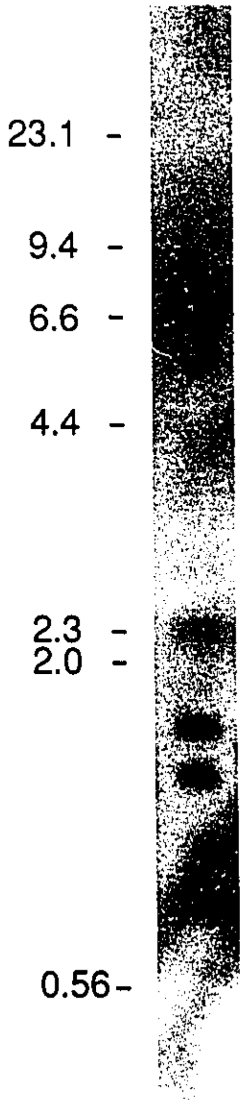

23.1
9.4
6.6
4.4
2.3
2.0
0.56

Figure 6 RNA Gel Blot Analysis of 9A1/60B1 Expression in Microspores, Microsporic and Zygotic Embryos, and Somatic Tissues

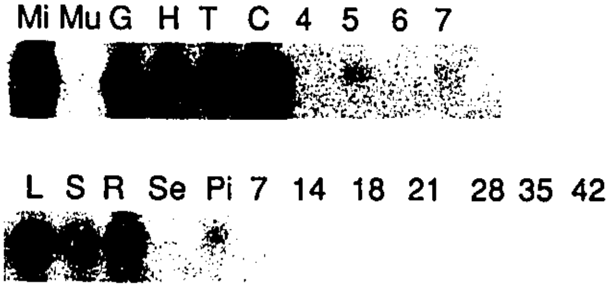
RNA gel blots containing 5 µg of total RNA per lane were probed with a ^{32}-P labeled 9A1 *Eco* RI/*Xba* I, double digested cDNA insert. The same pattern of expression was observed when 60B1 cDNA insert DNA was used as a probe. The final wash was carried out at 50 °C in 0.1X SSC, 0.1% SDS.

(A) 9A1/60B1 expression in microspores and developing microsporic embryos.

Total RNA was isolated from late uninucleate and early binucleate microspores, collected from preconditioned donor plants grown at 10°/5°C (day/night), after 4 days in culture at 32.5°C (Mi) or after 4 days in culture at 25°C (Mu) and from microsporic embryos at the globular (G), heart (H), torpedo (T), 21 day cotyledon (C), 28 day cotyledon (4), 35 day cotyledon (5), 42 day cotyledon (6) and 49 day cotyledon (7) stage of development.

(B) 9A1/60B1 expression in zygotic embryos and somatic tissues

Total RNA was collected from developing seeds at 7, 14, 18, 21, 28, 35 and 42 DAP and from leaf (L), stem (S), root (R), sepal (Se) and pistil (Pi) somatic tissues.


Mi Mu G H T C 4 5 6 7
L S R Se Pi 7 14 18 21 28 35 42

cotyledon stage (Figure 6A). A lower level of expression was detected in older cotyledon stage embryos. No expression was observed in developing zygotic embryos, however 9A1/60B1 expression was detected in leaf, stem and root somatic tissues and as well as in whole pistils (Figure 6B).

42A1

The 42A1 cDNA clone contains a 1.2 kb cDNA insert. The nucleotide sequence of this cDNA clone has not been completely determined, but a preliminary search of the DNA and protein databases suggests that similar sequences have not been previously characterized.

DNA gel blot analysis of *Eco* RI digested *B. napus* cv. Topas genomic DNA indicates that the 42A1 cDNA insert also belongs to a small multigene family (Figure 7). One of the hybridizing bands appears darker than the others and may represent two comigrating fragments. Alternatively, the two lighter fragments may represent gene family members with less nucleotide similarity.

The developmental expression of the 42A1 cDNA clone was also examined by RNA gel blot analysis of microspores, developing microsporic and zygotic embryos and a number of somatic tissues (Figure 8). The 42A1 cDNA clone hybridizes to a mRNA that is approximately 2kb in length, suggesting that the cDNA insert does not represent a full length clone.

Figure 7 DNA Gel Blot Analysis of 42A1 Related Genes in the *B. napus* Genome

B. napus cv. Topas DNA was digested with *Eco* R1, separated electrophoretically on 0.7% agarose gels and transferred to nylon membranes. The gel blots were hybridized overnight to a ^{32}P-labeled *Eco* RI/*Xba* I double digested insert from the 42A1 cDNA clone. The final wash was in 0.1X SSC, 0.1% SDS at 50 °C. The DNA size markers are lambda-*Hind* III restriction fragments.

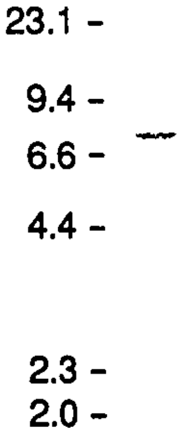

23.1 -
9.4 -
6.6 -
4.4 -
2.3 -
2.0 -

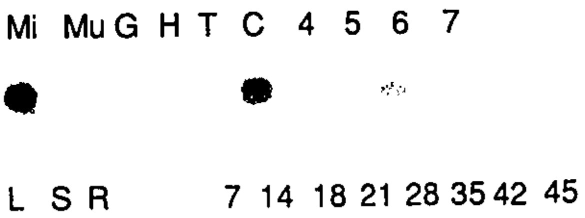
Figure 8 RNA Gel Blot Analysis of 42A1 Expression in Microspores, Microsporic and Zygotic Embryos, and Somatic Tissues

RNA gel blots containing 5 μg of total RNA per lane were probed with a ^{32}-P labeled *Eco* RI/*Xba* I, double digested 42A1 cDNA insert. The final wash was performed at 50 ^{o}C in 0.1X SSC, 0.1% SDS.

(A) 42A1 expression in microspores and developing microsporic embryos.

(B) 42A1 expression in zygotic embryos and somatic tissues

Legend as in Figure 6.


Mi Mu G H T C 4 5 6 7
L S R 7 14 18 21 28 35 42 45

42A1 mRNA was detected in embryogenically induced microspores, but not in microspores undergoing pollen development (Figure 8A). 42A1 mRNA was also observed to accumulate throughout microspore embryo development, but appeared stronger up to and including the 21 cotyledon stage of development (Figure 8A). 42A1 mRNA was not detected during zygotic embryo development or in any of the somatic tissues analysed (Figure 8B).

Discussion

A subtracted probe has been used to isolate four cDNA clones which appear to be differentially expressed in *B. napus* microspores that have been induced to undergo embryogenesis in culture. Preliminary characterization of these cDNA clones indicates three of the clones are present once per haploid genome, while one clone is encoded by a small multigene family. At least three of these clones, 9A1, 60B1 and 42A1, appear to mark the induction phase of *B. napus* microspore embryogenesis. The expression of these three cDNAs in embryogenic microspores does not appear be a direct result of the heat-stress treatment used to induce embryogenesis, as these genes are also expressed during subsequent stages of microsporic embryo development. Rather, their expression patterns suggest that they are induced in response to a new, embryo-based mode of development. All three cDNA clones appear to be predominately expressed during the early stages of microsporic embryo development;

however, expression of these clones was not observed at similar stages in zygotic embryos. This observation may be the result of the low levels of embryo mRNA in total seed RNA collected from samples during the early stages of embryo development (see section 3.2).

The expression of clones 9A1 and 60B1 was not restricted to embryos, suggesting that they encode proteins that function during other stages of plant development. Nonetheless, the expression of these clones is still a useful marker for the induction of microspore embryogenesis.

It is not presently known whether any of these genes are also expressed in developing microspores and pollen. If they are expressed in pollen then they are likely to fall into the "early" class of pollen expressed genes as their expression was not detected in cultured microspores at the late binucleate/early trinucleate stage of development (Mu sample).

Although we have not ruled out the possibility that the *Eco*RI fragment of the 6A1 cDNA clone was isolated as a DNA contaminant, it is still intriguing to speculate on the role that this gene plays at such an early stage of microsporic embryo development.

Potassium channel proteins have been found in a wide variety of plant cells and tissues (Hedrich and Schroeder, 1989; Tester, 1990). The two major classes of potassium channels that have been identified in plant cells include an inward-rectifying channel that mediates K^+ influx upon membrane hyperpolarization and an outward-rectifying channel that mediates K^+ efflux upon membrane depolarization (Schroeder *et al.*, 1987). Potassium transport across the plasma membrane contributes significantly to the control of turgor pressure, the opening and closing of stomata, leaf movements, ion uptake and transport and electrochemical homeostatsis in the cell. Potassium transport across the plasma membrane has also been implicated in the establishment of embryo polarity in cultures of carrot somatic embryos. Gorst *et al.*(1986) and Brawley *et al.*, (1984) have demonstrated that globular stage carrot somatic embryos exhibit an electrical polarity. Current enters the presumptive apical pole and exits near the presumptive radicle pole. This current continues, and increases in magnitude, during subsequent stages of embryo development. Potassium influx is likely to be a major component of the inward current in developing embryos (Rathore *et al.*, 1988). This electrical polarity precedes the differentiation of the vascular tissue and cotyledons and it has been suggested that development of embryo polarity is associated with these endogenous currents. Similar observations have been made for the establishment of polarity and polar growth in other plant, microbial and animal systems (Kropf, 1992).

The possibility that the 6A1 cDNA clone may be expressed in microspores that have been induced to undergo embryogenesis is intriguing. In analogy with other systems, the protein encoded by this gene could be involved in the establishment of subsequent embryo polarity, or in the maintenace of turgor pressure.

In conclusion it has been shown that a number of "embryo-expressed" genes are differentially expressed during the induction phase of *B. napus* microspore embryogenesis. Further characterization of the DNA sequence of these clones, as well as a more detailed analysis of their expression patterns, will be required in order to assign a functional role to the proteins encoded by these genes.

APPENDIX V"

EVOLUTION OF THE NAPIN MULTIGENE FAMILY

2S albumin seed storage proteins have been described in a wide range of species (Youle and Huang, 1981) but have been most fully characterized in members of the Cruciferae family. Cruciferae 2S albumin sequence data are available for *Brassica napus*, *Brassica oleracea*, *Brassica rapa*, *Brassica juncea*, *Rapahanus sativus* and *Arabidopsis thaliana* (Table I). The taxonomic relationship among these species has been inferred from morphological, cytological, interspecific hybridization, isozyme and molecular analyses (reviewed in Warwick and Black, 1991, 1993) and is shown in Figure 1.

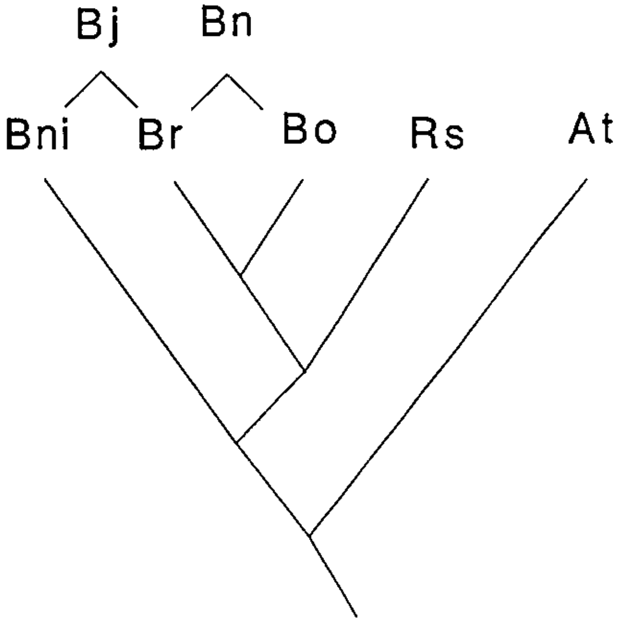
The availability of 2S albumin DNA sequence data provides an opportunity to examine the evolutionary history of the 2S albumin genes, and, in particular, the relationship of the BnmNAP subfamily to the other members of *B. napus* napin gene family. A preliminary phylogenetic analysis of the 2S albumin genes was carried out in collaboration with Bernard Baum (Centre for Land and Biological Resources Research, Agricuture Canada) and is presented below.

TABLE I Cloned 2S Albumin Sequences of the Cruciferae

GENE	**SPECIES**	**TYPE OF CLONE**	**ACCESSION NUMBER**	**REFERENCE**
At2S1	*A. thaliana*	genomic	M22032	Krebbers *et al.*, 1988
At2S2	*A. thaliana*	genomic	M22034	Krebbers *et al.*, 1988
At2S3	*A. thaliana*	genomic	M22035	Krebbers *et al.*, 1988
At2S4	*A. thaliana*	genomic	M22033	Krebbers *et al.*, 1988
napA	*B. napus*	genomic	J02798	Josefsson *et al.*, 1987
Na	*B. napus*	genomic	J02782	Scofield and Crouch, 1987
napB	*B. napus*	genomic	X58142	Baszczynski and Fallis, 1990
napB'	*B. napus*	genomic	X14492	unpublished
N1	*B. napus*	cDNA	K01544	Crouch *et al.*, 1983
N2	*B. napus*	cDNA	K01545	Crouch *et al.*, 1983
Bc2Sc	*B. rapa*	PCR amplified protein coding region	X65037	unpublished
Bo2SC	*B. oleracea*	PCR amplified protein coding region	X65038	unpublished
Bj2SC	*B. juncea*	PCR amplified protein coding region	X65040	unpublished
radnapA	*R. sativus*	cDNA	M36630	Raynal *et al.*, 1991
radnapB	*R. sativus*	cDNA	M36629	Raynal *et al.*, 1991
radnapC	*R. sativus*	cDNA	M36630	Raynal *et al.*, 1991

Figure 1 Taxonomic Relationship Among Members of the Family Cruciferae

B. napus and *B. juncea* are amphidiploid species. *B. napus* is derived from an interspecific hybridization between *B. rapa* and *B. oleracea* while *B. juncea* is derived from an interspecific hybridization between *B. nigra* and *B. rapa*.



GENUS-SPECIES	TRIBE	SUBTRIBE
At=Arabidopsis thaliana	Sisymbrieae	Arabidopsidinae
Rs=Raphanus sativus	Brassiceae	Brassicinae
Bo=Brassica oleracea		
Br=Brassica rapa		
Bni=Brassica nigra		
Bn=Brassica napus		
Bj=Brassica juncea		

The phylogenetic relationships of the 2S albumin genes were investigated using the distance matrix method (see Swofford and Olsen, 1990 for a review). Phylogenetic trees were generated by separately analyzing the DNA sequence of the protein coding, 5' flanking and 3' untranslated regions. However, as indicated in Table I, a number of the 2S albumin clones do not contain DNA sequence information corresponding to the 5' flanking and/or 3' untranslated regions and therefore could not be included in all of the analyses.

The DNA sequences of the 2S albumins were aligned using CLUSTAL V (Higgins *et al.*, 1992) modified to run on the VAX/VMS (Version 5.3.1). The DNA sequence of the protein coding region of the 2S albumin genes was aligned using sequence information located between the putative translational start and stop codons. The DNA sequence alignment of the 3' untranslated region of the 2S albumin genes included approximately 140 nt downstream of the translatoional stop codon. The DNA sequence alignment of the 5'flanking region of the 2S albumin genes included approximately 250 nt upstream of the translational start codon. Phylogenetic analyses were conducted with the aligned DNA sequences as follows: Distance matrices were computed using the Jukes-Cantor model (Jukes and Cantor, 1969) which assumes the independent changes at all sites with equal probability. Positions containing gaps in any sequence were ignored. The matrix of distances obtained was subjected to a cluster analysis using the neighbour-joining method (Saitou and Nei, 1987). Bootstrap analysis (Felsenstein, 1985) was used to establish the confidence limit of the various branches of the tree.

Analysis of the Coding Region

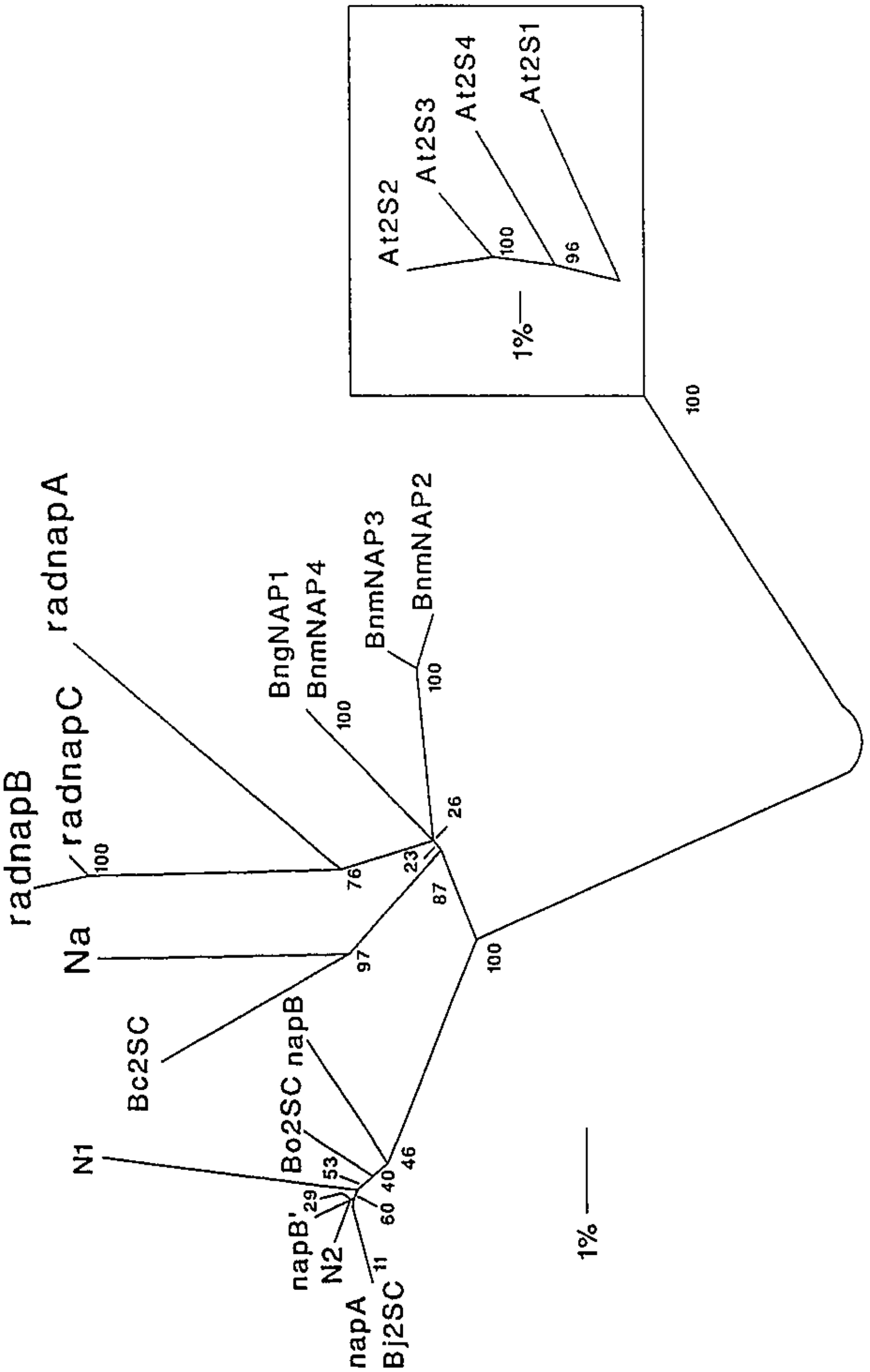
The DNA sequence of the protein coding region was used to determine the phylogenetic relationship among the three BnmNAP sequences and the other available members of the napin multigene families in *B. napus*, *B. oleracea*, *B. rapa*, *B. juncea*, *R. sativus* and *A. thaliana*. The phylogenetic tree obtained using the neighbour joining method is shown in Figure 2.

The neighbour joining method generated a tree consisting of two main groups of 2S albumin sequences. One group consists entirely of the four *Arabidopsis* sequences, while the second group contains all of the *Brassica* and *Raphanus* sequences. The *Brassica* sequences seem to cluster into three subfamilies: one subfamily consists of the four BnmNAP sequences; the second subfamily is composed of one sequence each from *B. napus* (Na) and *Brassica rapa* (Bc2SC); the third subfamily contains the remaining *B. napus* sequences as well as one sequence each from *B. oleracea* (Bo2SC) and *B. juncea* (Bj2SC).

The analysis supports the existence of two distinct groups of 2S albumins, with one group containing all the *Arabidopsis* sequences and the other group containing all the *Brassica* and *Raphanus* sequences. The exact relationships of

Figure 2 Phylogenetic Analysis of the DNA Sequence of the Coding Region of 2S Albumin Genes.

Neighbour joining analysis of the coding regions of the *Brassica napus, B. rapa, B. oleracea, B. juncea, R.sativus* and *A. thaliana* napin DNA sequences. Branch lengths are drawn proportional to the distances. The bars indicate 1% divergence. Confidence estimates (%) obtained by 1000 bootstrap repeats are shown for each branch below the fork. Gene nomenclature is as in Table I.


radnapB
radnapC
100
radnapA
76
23
26
87
BngNAP1
BnmNAP4
100
BnmNAP3
BnmNAP2
100
100
At2S2
At2S3
At2S4
At2S1
100
96
1%
100
Na
97
Bc2SC
Bo2SC napB
N1
53
29
40
46
60
napB'
N2
11
napA
Bj2SC
1%

the members of each subfamily to each other, however, cannot be determined. For example, the confidence level for the internal node connecting the four *B. napus* BnmNAP sequences to the *R. sativus* sequences is low and indicates that the position of the node, and therefore the relationship with the BnmNAP sequences, is not certain. However, when only 2S albumin sequences from *B. napus* and *Arabidopsis* are analysed the BnmNAP sequences form a distinct subfamily (data not shown). Similarly, the remaining *Brassica* sequences in the second subfamily are clearly different from the other 2S albumin sequences, but the branching order among these sequences remains unresolved. The inability of the analysis to resolve these relationships may be due to the high degree of sequence similarity between members within the same group.

Interestingly, one 2S albumin gene from each putative parental species of *B. napus* can be found in each of the two subfamilies containing *B. napus* sequences: one subfamily contains a single *B. rapa* 2S albumin sequence (Bc2SC) while the other subfamily contains a single *B. oleracea* sequence (Bo2SC). This observation suggests that each of the putative parental species may have contributed a distinct subfamily of 2S albumin sequences to *B. napus*. This interpretation is rather speculative as the 2S albumins of both *B. oleracea* and *B. rapa* are encoded by multigene families (Ericson, 1988 and Kim Boutilier, personal observation). Only a single 2S albumin sequence is available for each.of the progenitor species and different members of these gene families may exist that belong to one of the other subfamilies of 2S albumins. Firm conclusions cannot be made until more sequence data become available.

Analysis of the 3' Untranslated and 5' Flanking Regions

Phylogenetic trees were also constructed for the 3' untranslated region of the *Brassica napus, R.sativus* and *A. thaliana* 2S albumin genes.

The tree generated using the DNA sequence of the 3' untranslated region is shown in Figure 3. Many of the *B. napus* sequences and all of the *A. thaliana* sequences remain clustered in two separate groups. However, the *R. sativus* radnapA and the *B. napus* N1 3' untranslated regions, as observed for the DNA sequence of the protein coding region, appear to be evolving separately from the majority of 2S albumin sequences.

Interestingly, the *Arabidopsis* sequences are joined by a common node to two of the *R. sativus* and one of the *B. napus* sequences. The observation that the 3' untranslated region of the *B. napus* Na sequence and the *R. sativus* radnapB and radnapC sequences appear closer to each other than to any of the other sequences from their respective species suggest that these three gene regions are orthologous.

Analysis of the 5' flanking and untranslated regions of the 2S albumin sequences from *B. napus* and *A. thaliana* also generated a phylogenetic tree that differed from those obtained using DNA sequence data from both the coding and 3' untranslated regions (Figure 4).

The majority of *B. napus* and *A. thaliana* 5' flanking sequences are clustered into two separate groups, however the *B. napus* BngNAP1 and the *A. thaliana* At2S1 5' flanking sequences appear more similar to each other than to sequences within their own species. In fact, the two sequences exhibit at least 80% nucleotide similarity (excluding deletions and insertions) over the region analysed (data not shown). This high degree of sequence conservation among two species from different tribes is surprising considering that this gene region is often quite variable even within the same species. This result suggest that these two gene regions are orthologous.

The *B. napus* Na 5' flanking sequence, as with the DNA sequence of the coding and 3' untranslated regions of this gene, appears to be evolving separately from the other *B. napus* sequences.

Discussion

Phylogenetic analysis of the DNA sequences of the protein coding, 5' flanking and 3' untranslated regions of number of 2S albumin genes of the Cruciferae has been carried out. These analyses have shown that the DNA sequence of the coding regions within a species are more similar to each other than they are to the same region in other cruciferous species. This pattern of evolution, termed concerted

Figure 3 Phylogenetic Analysis of the DNA Sequence of the 3' Utranslated Region of 2S Albumin Genes.

Neighbour joining analysis of the 3' untranslated region of the *Brassica napus, Raphanus sativus* and *Arabidopsis thaliana* napin DNA sequences. Branch lengths are drawn proportional to the distances. The bar indicates 1% divergence. Confidence estimates (%) obtained by 1000 bootstrap repeats are shown for each branch below the fork. Gene nomenclature is as in Table I.

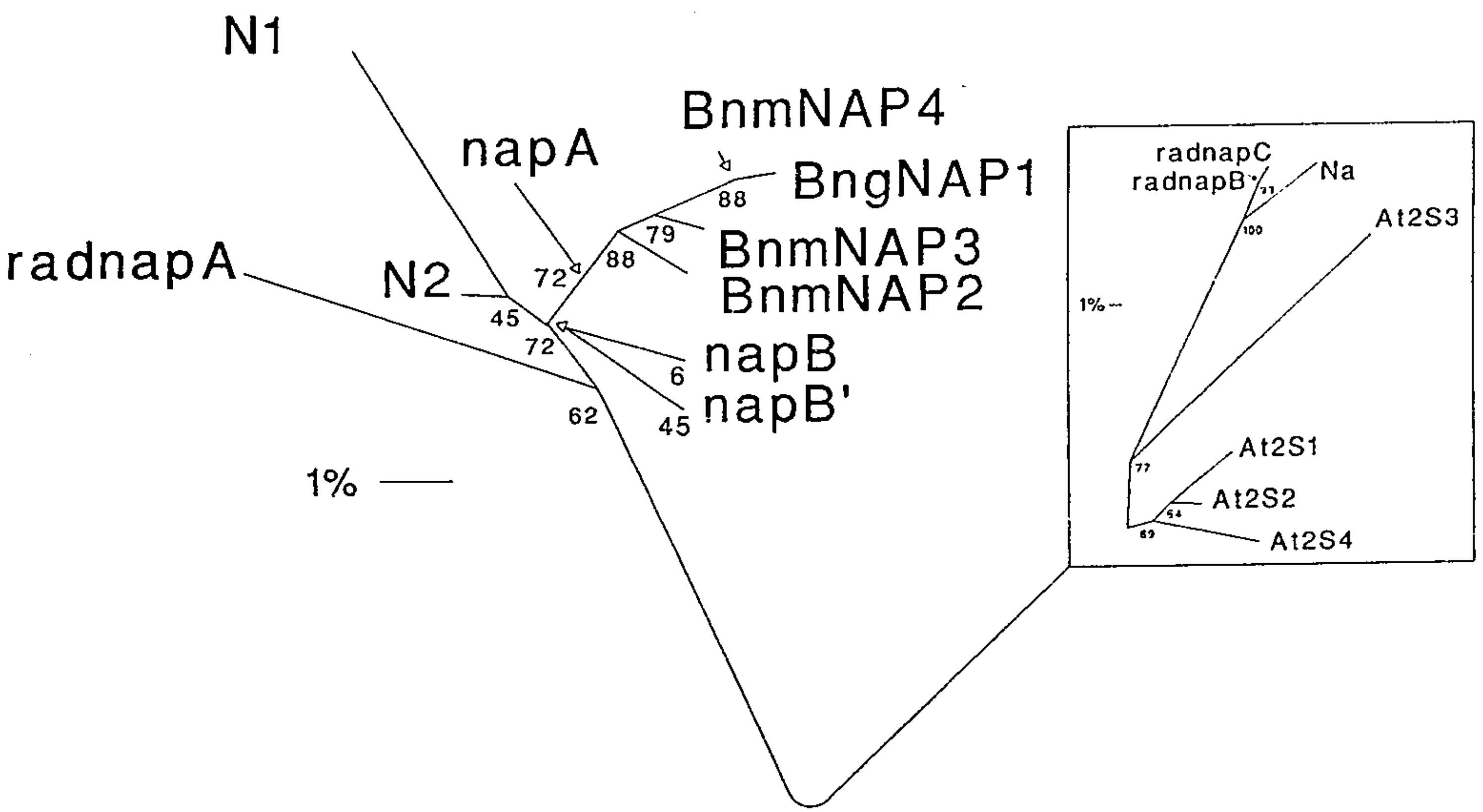
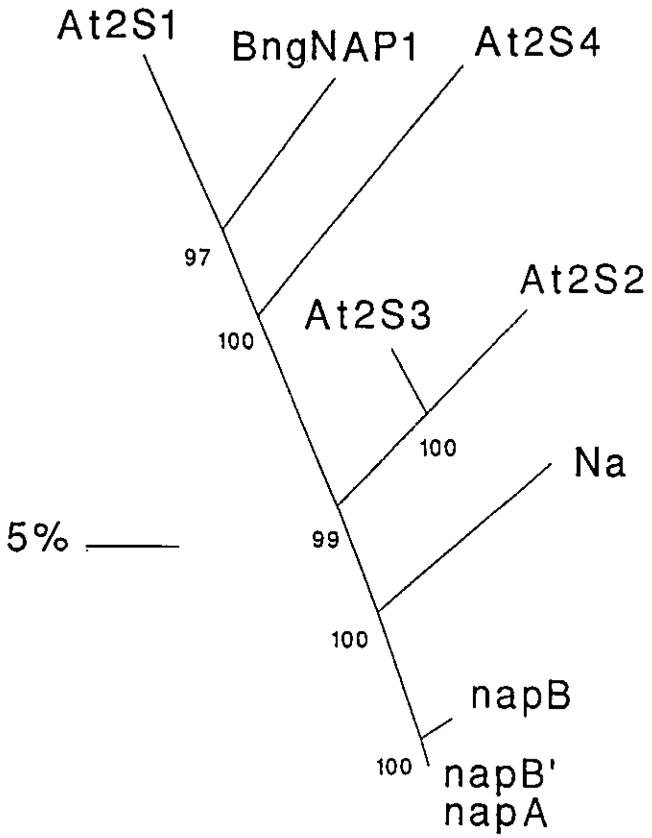

N1
BnmNAP4
napA
BngNAP1
88
79
radnapA
88
BnmNAP3
N2
72
BnmNAP2
45
72
napB
6
napB'
62
45
1%
radnapC
radnapB
71
Na
100
At2S3
1%
77
At2S1
At2S2
54
69
At2S4

Figure 4 Phylogenetic Analysis of the DNA Sequence of the 5' flanking Region of 2S Albumin Genes.

Neighbour joining analysis of the 5' flanking region of the *Brassica napus* and *A. thaliana* napin DNA sequences. Branch lengths are drawn proportional to the distances. The bar indicates 5% divergence. Confidence estimates (%) obtained by 1000 bootstrap repeats are shown for each branch below the fork. Gene nomenclature is as in Table I.


At2S1
BngNAP1
At2S4
97
100
At2S3
At2S2
100
Na
5%
99
100
napB
100
napB'
napA

evolution, is frequently observed among members of multigene families and is thought to result from a variety of gene conversion events that result in non-reciprocal DNA transfer within and between homologous and non-homologous chromosomes (Dover, 1982; 1986). Recombination involving gene conversion tends to decrease differences between gene family members by transferring sequence information from the donor to the recipient gene. Gene conversion would therefore eliminate many of the independent insertions, deletions and base-pair changes that normally accumulate in duplicated sequences, with the result that members of a gene family would become homogenized.

The *Brassica* sequences appear to cluster into three subfamilies. These subfamilies could have arisen within the larger multigene family if they were converted by different master sequences which had diverged before the first conversion event. Continued divergence of these subfamilies may prevent them from being homogenized against the other duplicated *B. napus* 2S albumin sequences (Smith, 1976).

The coding regions of the majority of 2S albumin sequences appear to be evolving in a concerted fashion. However, a number of sequences, including the *R. sativus* radnapA and *B. napus* N1 and Na sequences, appear to be evolving separately from the other members of their respective gene families. These sequences may have diverged enough to "escape" the conversion events responsible for their concerted evolution (Walsh, 1987).

The results obtained from analysis of the DNA sequence of the protein coding region appears to contrast with the results obtained from analysis of the DNA sequence of the 5' flanking and 3' untranslated regions, where it was shown that a number of these regions may be orthologous. This contradiction can be resolved by assuming that, for some genes, the protein coding sequences, along with the flanking sequences, have been homogenized as a result of gene conversion, whereas for other genes, only gene conversion events involving the protein coding region have occurred.

The similarity between the 5' flanking regions of BngNAP1 (*Brassica*) and At2S1 (*Arabidopsis*), and between the 3' untranslated region of Na (*Brassica*), radnapB and radnapC (*Raphanus*) raises the question as to whether orthologous flanking regions direct similar spatial and temporal patterns of gene expression during seed development.

Unfortunately, no detailed studies of this type exist, however, we have carried out a preliminary study, comparing the expression patterns directed by the *A. thaliana* At2S1 and *B. napus* BngNAP1 promoters (DeMoor, 1992; this thesis). In *A. thaliana*, the At2S1 gene is expressed in an axis specific manner (Guerche *et al.*, 1990). In transgenic *B. napus*, the BngNAP1 promoter, although highly expressed in the embryonic axis, is not restricted to this structure (this thesis). Both genes however, are expressed in a similar manner in developing transgenic tobacco seeds (DeMoor, 1992; this thesis). It is difficult to draw comparisons between the expression patterns of these two genes due to the differences in the length of promoters used in each of the analyses. Future studies, aimed at comparing the expression patterns of these orthologous flanking gene regions, as well as other orthologous flanking regions within the 2S albumins genes of the Cruciferae, will require the construction and reintroduction of deletion derivatives.

REFERENCES

Aleith, F., and Richter, G. (1990). Gene expression during induction of somatic embryogenesis in carrot cell suspensions. Planta **183**, 17-24.

Allen, R., Bernier, F., Lessard, P.A., and Beachy, R.N. (1989). Nuclear factors interact with a soybean ß-conglycinin enhancer. Plant Cell **1**, 623-631.

Altschuler, M., and Mascarenhas, J.P. (1982). Heat shock proteins and effects of heat shock in plants. Plant Mol. Biol. **1**, 103-115.

Altschuler, M., and Mascarenhas, J.P. (1985). Transcription and translation of heat shock proteins in seedlings and developing seeds of soybean exposed to a gradual temperature increase. Plant Mol. Biol. **5**, 291-297.

Ammirato, P.V. (1983). Embryogenesis. In Handbook of Plant Cell Culture, Vol I, D.A. Evans, W.R. Sharp, P.V. Ammirato, and Y. Yamada eds (New York: Macmillan).

Ampe, C., Van Damme, J., deCastro, L., Sampaio, M.J., Van Montagu, M. and Vandkerckhove, J. (1986). The amino acid sequence of the 2S sulfur rich proteins from seeds of the Brazil nut (*Bertholletia excelsa* H.B.K.). Eur. J. Biochem. **159**, 597-604.

Anderson, J.a., Huprikar, S.S., Kochian, L.v., Lucas, W.J., and Gaber, R.F. (1992). Functional expression of a probable *Arabidopsis thaliana* potassium channel in *S. cerevisiae*. Proc. Natl. Acad. Sci. USA **89**, 3736-3740.

Asker, S.E., and Jerling, L. (1992). Apomixis in Plants. (Boca Raton: CRC Press)

Aviv, H., and Leder, P. (1972). Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid cellulose. Proc. Natl. Sci. USA **69**, 1408-1412.

Barnett, T., Altschuler, M., McDaniel, C.N., and Mascarenhas, J.P. (1980). Heat shock induced proteins in plant cells. Dev. Genet. **1**, 331-340.

Baszczynski, C.L., and Fallis, L. (1990). Isolation and nucleotide sequence of a genomic clone encoding a new *Brassica napus* napin gene. Plant Mol. Biol. **14**, 633-635.

Bäumlein, H., Wobus, U., Pustell, J., and Kafatos, F.C. (1986). The legumin gene family: Structure of a B type gene of *Vicia faba* and a possible legumin gene-specific regulatory element. Nucl. Acids Res. **14**, 2707-2720.

Bäumlein, H., Nagy, I., Villaroel, R., Inzé, D., and Wobus, U. (1992). *Cis*-analysis of a seed protein gene promoter: The conservative RY repeat CATGCATG within the legumin box is essential for tissue specific expression of a legumin gene. Plant J. **2**, 233-239.

Bedinger, P.A. (1992). The remarkable biology of pollen. Plant Cell **4**, 879-878.

Bedinger, P.A., and Edgerton, M.D. (1990). Developmental staging of maize microspores reveals a transition in developing microspore proteins. Plant Physiol. **92**, 474-479.

Benito Moreno, R.M., Macke, F., Hauser, M.T., Alwen, A., and Heberle-Bors, E. (1988). Sporophytes and male gametophytes from *in vitro* cultured, immature tobacco pollen. In Sexual Reproduction in Higher Plants, M. Cresti, P. Gori and E. Pacini eds (New York: Springer-Verlag).

Berleth, T., and Jürgens, G. (1993). The role of the *monopteros* gene in organising the basal body region of the *Arabidopsis* embryo. Development **118**, 575-587.

Bewley, J.D., and Black, M. (1978). Physiology and Biochemistry of Seeds in Relation to Germination, Vol I. (New York: Springer-Verlag).

Blumenthal, C.S., Batey, I.L., Bekes, F., Wrigley, C.W., and Barlow, E.W.R. (1990). Gliadin genes contain heat-shock elements: Possible relevance to heat-induced changes in grain quality. J. Cereal Sci. **11**, 185-187.

Blumenthal, C.S., Barlow, E.W., and Wrigley, C.W. (1993). Growth environment and wheat quality: The effect of heat stress on dough properties and gluten proteins. J. Cereal Sci. **18**, 3-21.

Blundy, K.S., Blundy, M.A.C., and Crouch, M.L. (1991). Differential expression of members of the napin storage protein gene family during embryogenesis in *Brassica napus*. Plant Mol. Biol. **17**, 1099-1104.

Bögre, L., Stefanov, I., Ábrahám, M., Somogyi, I., and Dudits, D. (1990). Differences in responses to 2,4-dichlorophenoxyacetic acid (2,4-D) treatment between embryogenic and non-embryogenic lines of alfalfa. In Progress in Plant Cellular and Molecular Biology, H.J.J. Nijkamp, L.H.W. van der Plas, and J. van Aartrijk eds (Dordrecht: Kluwer Academic Publishers).

Bokird, C., Choi, J.H., and Sung, Z.R. (1986). Effect of 2,4-dichlorophenoxyacetic acid on the expression of embryogenic program in carrot. Plant Physiol. **81**, 1143-1146.

Bostan, R.S., Kodrzycki, R., and Larkins, B.A. (1986). Transcriptional regulation of maize genes. In Molecular Biology of Seed Storage Proteins and Lectins. Ninth Symposium in Plant Physiology. American Society of Plant Physiologists, L.M. Sharron and M.J. Chrispeels eds.

Bouman, F. (1984). The ovule. In Embryology of the Angiosperms, B.M. Johri ed (New York: Springer-Verlag).

Bowman, J.L., Drews, G.N., and Meyerowitz, E.M. (1991). Expression of an *Arabidopsis* floral homeotic gene *AGAMOUS* is restricted to specific cell types late in flower development. Plant Cell **3**, 749-748.

Brawley, S.H., Wetherell, D.F., and Robinson, K.R. (1984). Electrical polarity in embryos of wild carrot precedes cotyledon differentiation. Proc. Natl. Acad. Sci. USA **81**, 6064-6067.

Brodl, M.R. (1990). Biochemistry of the heat shock response in plants. In Environmental Injury to Plants, F. Katterman ed (San Diego: Academic Press).

Bryce, W.H., and Nelson, O.E. (1979). Starch synthesizing enzymes in the endosperm and pollen of maize. Plant Physiol. **63**, 312-317.

Bustos, M., Guiltinan, M.J., Jordano, J., Begum, D., Kalkan, F.A. and Hall, T.C. (1989). Regulation of ß-glucoronidase expression in transgenic tobacco plants by an A/T-rich, *cis* acting sequence found upstream of a french bean ß-phaseolin gene. Plant Cell 1, 839-853.

Cao, Y., Anderova, M., Crawford, N.M., and Schroeder, J.I. (1992). Expression of an outward-rectifying potassium channel from maize mRNA and complementary RNA in Xenopus oocytes. Plant Cell **4**, 961-969.

Chamberland, S., Daigle, N., and Bernier, F. (1992). The legumin boxes and the 3' part of soybean β-conglycinin promoter are involved in seed gene expression in transgenic tobacco plants. Plant Mol. Biol. **19**, 937-949.

Chirgwin, J.M., Przybyla, A.E., Macdonald, J., and Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**, 5294-5299.

Chrispeels, M.J., and Greenwood, J.S. (1987). Heat stress enhances phytohemagglutinin synthesis but inhibits its transport out of the endoplasmic reticulum. Plant Physiol. **83**, 778-784.

Chuong, P.V., and Beversdorf, W.D. (1985). High frequency embryogenesis through isolated microspore culture in *Brassica napus* L. and *B. carinata* Braun. Plant Sci. **39**, 219-226.

Chuong, P.V., DesLauriers, C., Kott, L.S., and Beversdorf, W.D. (1988). Effects of donor genotype and bud sampling on microspore culture of *Brassica napus*. Can. J. Bot. **66**, 1653-1657.

Cooper, P., and Ho, T. -H.D. (1983). Heat shock proteins in maize. Plant Physiol. **71**, 215-222.

Cordewener, J., Booij, H., Van Der Zandt, H., Van Engelen, F., Vanderkerckhove, J., Van Kammen, A., and de Vries, S.C. (1991). Tunicamycin-inhibited carrot somatic embryogenesis can be restored by secreted cationic peroxidase isoenzymes. Planta **184**, 478-486.

Coventry, J., Kott, L., and Beversdorf, W.D. (1988). Manual for microspore culture technique for *Brassica napus*. Department of Crops Science, Technical Bulletin, University of Guelph (OAC Publication 0489).

Crouch, M.L. (1982). Non-zygotic embryos of *Brassica napus* L. contain embryo specific-storage proteins. Planta **156**, 520-524.

Crouch, M.L. (1988). Regulation of gene expression during seed development in flowering plants. In Developmental Biology: A Comprehensive Synthesis, L.W. Browder ed. (New York, Plenum Press).

Crouch, M.L., Tenbarge, K.M., Simon, A.E., and Ferl, R. (1983). cDNA clones for *Brassica napus* seed storage proteins: Evidence from nucleotide sequence analysis that both subunits of napin are cleaved from a precursor polypeptide. J. Mol. Appl. Genet. **2**, 273-283.

de Jong, A.J., Cordewener, J., Lo Schiavo, F., Terzi, M., Vandekerckhove, J., van Kammen, A and de Vries, S.C. (1992). A carrot somatic embryo is rescued by chitinase. Plant Cell **4**, 425-433

de Jong, A.J., Schmidt, E.D.L., and de Vries, S.C. (1993). Early events in higher plant embryogenesis. Plant Mol. Biol. **22**, 367-377.

de Vries, S.C., Booij, H., Janssen, R., Vogels, R., Saris, L., Lo Schiavo, F., Terzi, M., van Kammen, A. (1988). Carrot somatic embryogenesis depends on the phytohormone-controlled expression of correctly glycosylated extracellular proteins. Genes Dev. **2**, 462-476.

DeLisle, A.J., and Crouch, M.L. (1989). Seed storage protein transcription and mRNA levels in *Brassica napus* during development and in response to exogenous abscisic acid. Plant Physiol. **91**, 617-623.

DeMoor, J.M. (1992). Expression of an *Arabidopsis thaliana* 2S albumin storage protein gene in transgenic *Nicotiana tabacum* and *Brassica napus*. Ph.D Thesis, Carleton University, Ottawa, Canada.

Diboll, A.G. (1968). Fine structural development of the megagametophyte of *Zea mays* following fertilization. Am. J. Bot. **55**, 787-806.

Dover, G.A. (1982). Molecular drive: a cohesive mode of species evolution. Nature **299**, 111-117.

Dover, G.A. (1986). Molecular dive in multigene families: how biological novelties arise, spread and are assimilated. Trends Genet. **2**, 159-165.

Dudits, D., Bögre, L., and Gyorgyey, J. (1991). Molecular and cellular approaches to the analysis of plant embryo development from somatic cells *in vitro*. J. Cell. Sci. **99**, 475-484.

Dunwell, J. M. (1986). Pollen, ovule and embryo culture as tools in plant breeding. In Plant Tissue Culture and Its Agricultural Applications, L.A. Withers and P.G. Alderson eds (Toronto, Butterworths).

Dure, L. (1985). Embryogenesis and gene expression during seed formation. Oxford Surv. Plant Mol. Cell Biol. **2**, 179-197.

Dure, L., Crouch, M., Harada, J., Ho, T.-H.D., Mundy, J., Quatrano, R., Thomas, T., and Sung, Z.R. (1989). Common amino acid sequence domains among the LEA proteins of higher plants. Plant Mol. Biol. **12**, 475-486.

Ericson, M.L. (1988). Seed storage proteins: Studies on the structure, molecular genetics and intracellular localization of napin from *Brassica napus*. Ph.D Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Ericson, M.L., Rödin, J., Lenman, M., Glimelius, K., Josefsson, L.-G.,and Rask, L. (1986). Structure of the rapeseed 1.7S storage protein, napin, and its precursor. J. Biol. Chem. **261**, 14576-14581.

Ericson, M.L., Rödin, J., Rodriguez-Martinez, H., Plöen, L., and Rask, L. (1989). An immunocytological localization of the *Brassica napus* storage protein napin in developing seed embryos. Eur. J. Cell Biol. **49**, 213-217.

Ericson, M.L., Murén, E., Gustavsson, H.O., Josefsson, L.-G., and Rask, L. (1991). Analysis of the promoter region of napin genes from *Brassica napus* demonstrates binding of nuclear proteins *in vitro* to a conserved sequence motif. Eur. J. Biochem. **197**, 741-746.

Fan, Z., Armstrong, K.C., and Keller, W.A. (1988). Development of microspores *in vivo* and *in vitro* in *Brassica napus* L. Protoplasma **147**, 191-199.

Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**, 6-13.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstraps. Evolution **39**, 783-791.

Fernandez, D.E., Turner, F.R., and Crouch, M.L. (1991). *In situ* localization of storage protein mRNAs in developing meristems of *Brassica napus* embryos. Development **111**, 299-313.

Finkelstein, R.R., Tenbarge, K.M., Shumway, J.E., and Crouch, M.L. (1985). Role of ABA in maturation of rapeseed embryos. Plant Physiol. **78**, 630-636.

Finklestein, R.R. and Crouch, M.L. (1984). Precociously germinating rapeseed embryos retain characteristics of embryogeny. Planta, **162**, 573-577.

Finlayson, A.J. (1976). The seed protein contents of some Cruciferae. In The Biology and Biochemistry of the *Cruciferae*, J.G. Vaughn, A.J. MacLeod and B.M.G. Jones eds (New York: Academiv Press).

Forde, B.G., Heyworth, A., Pywell, J. and Kreis, M. (1985). Nucleotide sequence of a B1 hordein gene and the identification of possible upstream regulatory elements in endosperm storage protein genes from barley, wheat and maize. Nucl. Acids Res. **13**, 7327-7339.

Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Gallupi, G.R., Goldberg, S.B., Hoffman, N.L., and Woo, S.C. (1983). Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. USA **80**, 4803-4807.

Frova, C., Binelli, G., and Ottaviano, E. (1987). Isozyme and hsp gene expression during male gametophyte development in maize. In Isozymes: Current Topics in Biological and Medical Research, Vol 15. Genetics, Development and Evolution, M.C. Rattazzi and J.G. Scadalios eds (New York: Allan R. Liss).

Galau, G.A., and Dure, L. III. (1981). Developmental biochemistry of cottonseed embryogenesis and germination: Changing messenger ribonucleic acid populations as shown by reciprocal heterologous complementary deoxyribonucleic acid-messenger ribonucleic acid hybridization. Biochemistry **20**, 4169-4178.

Galau, G.A., Bijaisoradat, N., Hughes, D.W. (1987). Accumulation kinetics of cotton late embryogenesis-abundant mRNAs and storage protein mRNAs: Coordinate regulation during embryogenesis and the role of abscisic acid. Dev. Biol. **123**, 198-212.

Galau, G.A., Jakobsen, K.S., and Hughes, D.W. (1991). The controls of late dicot embryogenesis and early germination. Physiol. Plant. **81**, 280-288.

Gamborg, O.L., Miller, R.A., and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. Exper. Cell Res. **20**, 151-158.

Garrido, D., Eller, N., Heberle-Bors, E., and Vincente, O. (1993). De novo transcription of specific mRNAs during the induction of tobacco pollen embryogenesis. Sex. Plant Reprod. **6**, 40-45.

Gasser, C.S., and Robinson-Beers, K. (1993). Pistil development. Plant Cell **5**, 1231-1229.

Gasser, C.S., Budelier, K.A., Smith, A.G., Shah, D.M., and Fraley, R.T. (1989). Isolation of tissue-specific cDNAs from tomato pistils. Plant Cell **1**, 15-24.

Gatehouse, J.A., Evans, I.M., Croy, R.R.D., and Boulter, D. (1986). Differential expression of genes during legume seed development. Phil. Trans. R. Soc. Lond. B **314**, 367-384.

Giuliano, G., Rosellini, D., and Terzi, M. (1983). A new method for the purification of the different stages of carrot embryoids. Plant Cell Rep. **2**, 216-218.

Gland, A., Lichter, R., and Schweiger, H.-G. (1988). Genetic and exogenous factors affecting embryogenesis in isolated microspore cultures of *Brassica napus* L. J. Plant Physiol. **132**, 613-617.

Glisin, V., Crkvenjakov, R., and Byus, C. (1974). Ribonucleic acid isolation by cesium chloride centrifugation. Biochemistry **13**, 2633-2637.

Goldberg, R.B., Hoschek, G., Tan, S.H., Ditta, G.S., and Breidenbach, R.W. (1981). Abundance, diversity and regulation of mRNA sequence sets in soybean embryogenesis. Dev. Biol. **83**, 201-217.

Goldberg, R.B., Barker, S.J., and Perez-Grau, L. (1989). Regulation of gene expression during plant embryogenesis. Cell **56**, 149-160.

Goldberg, R.B., Beals, T.P., and Sanders, P.M. (1993). Anther development: Basic principles and practical applications. Plant Cell **5**, 1217-1229.

Gómez, J., Sánchez-Martinez, D., Stiefel, V., Rigau, J., Puigdomènech, P., and Pages, M. (1988). A gene induced by the plant hormone abscisic acid in response to water stress encodes a glycine-rich protein. Nature **334**, 262-264.

Gorst, J., Overall, R.L., Wernicke, W. (1987). Ionic currents traversing cell clusters from carrot suspension cultures reveal perpetuation of morphogenetic potential as distinct from induction of embryogenesis. Cell Different. **21**, 101-109.

Grant, I., Beversdorf, W.D., and Peterson, R.L. (1986). A comparative light and electron microscopic study of microspore and tapetal development in male fertile and cytoplasmic male sterile oilseed rape (*Brassica napus*). Can. J. Bot. **64**, 1055-1068.

Guerche, P., Tire, C., Grossi de Sa, F., De Clerq, A., and Van Montagu, M. (1990). Differential expression of the *Arabidopsis* 2S albumin genes and the effect of increasing gene family size. Plant Cell **2**, 469-478.

Guha, S., and Maheshwari, S.C. (1964). *In vitro* production of embryos from anthers of *Datura*. Nature **204**: 497.

Gustavsson, H.O., Ellerström, M., Stålberg, K., Ezcurra, I., Koman, A., Höglund, A., Rask, L., and Josefsson, L.-G. (1991). Distinct sequence elements in a napin promoter interact in vitro with DNA-binding proteins from *Brassica napus*. Physiol. Plant. **82**, 205-212.

Halperin, W. (1966). Alternative morphogenetic events in cell suspensions. Am. J. Bot. **53**, 443-451.

Hanahan, D. (1985). Techniques for transformation of *E. coli*. In DNA Cloning (Vol I): A practical Approach, D.M. Glover, ed (Washington: IRL Press).

Harada, J.J., Barker, S.J., and Goldberg, R.B. (1989). Soybean β-conglycinin genes are clustered in several DNA regions and are regulated by transcriptional and post-transcriptional processes. Plant Cell **1**, 415-425.

Hause, B., Hause, G., Pechan, P., and Van Lammeren, A.A.M. (1993). Cytoskeletal changes and induction of embryogenesis in microspore and pollen cultures of *Brassica napus* L. Cell Biol. Int. **17**, 153-168.

Heberle-Bors, E. (1982a). In vitro pollen embryogenesis in *Nicotiana tabacum* L. and its realtion to pollen sterility, sex balance, and floral induction of the pollen donor plants. Planta **156**, 396-401.

Heberle-Bors, E. (1982b). On the time of embryogenic pollen grain induction during sexual development of *Nicotiana tabacum* L. plants. Planta **156**, 402-406.

Heberle-Bors, E. (1983). Induction of embryogenic pollen grains *in situ* and subsequent *in vitro* pollen embryogenesis in *Nicotiana tabacum* L. by treatments of the pollen donor plants with feminizing agents. Physiol. Plant. **59**, 67-72.

Heberle-Bors, E. (1989). Isolated pollen culture in tobacco: Plant reproductive development in a nutshell. Sex. Plant Reprod. **2**, 1-10.

Heberle-Bors, E., and Reinert, J. (1981). Environmental control and evidence of predetermination of pollen embryogenesis in *Nicotiana tabacum* pollen. Protoplasma **109**, 249-255.

Heberle-Bors. (1985). In vitro haploid formation from pollen: A critical review. Theor. Appl. Genet. **71**, 361-374.

Hedrich, R., and Schroeder, J.I. (1991). The physiology of ion channels and electrogenic pumps in higher plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. **40**, 539-569.

Herrera-Estralla, L., Depicker, A., Van Montagu, M., and Schell, J. (1983). Expression of chimaeric genes transferred into plant cells using a Ti-plasmid derived vector. Nature **303**, 209-213.

Higgins, E.G., Bleasby, A.J., and Fuchs, R. (1992). CLUSTAL V: Improved software for multiple sequence alignment. CABIOS **7**, 189-191.

Höglund, A.-S., Rödin, J., Larsson, E., Rask, L. (1991). The distribution of napin and cruciferin in developing rape seed embryos. Plant Physiol. **98**, 509-515.

Horsch, R.B., Fry, J.E., Hoffmann, N.l., Eichholtz, D.S. Rogers, S.G., and Fraley, R.T. (1985). A simple and general method for transferring genes into to plants. Science 227, 1229-1231.

Hughes, D.W., and Galau, G. (1989). Temporally modulated gene expression during cotyledon development. Genes Dev. **3**, 358-369.

Hussey, P.J., Lloyd, C.W., and Gull, K. (1988). Differential and developmental expression of β-tubulins in a higher plant. J. Biol. Chem. **263**, 5474-5479.

Jack, T., Brockman, L., and Meyerowitz, E.M. (1992). *APETALA*3 of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. Cell **68**, 683-697.

Jefferson, R.A., Burgess, S.M., and Hirsh, D. (1986). β-glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc. Natl. Acad. Sci. USA **83**, 8447-8451.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. **5**, 387-405.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6**, 3901-3907.

Jensen, W.A., Ashton, M.E., and Beasley, C.A. (1985). Pollen tube-embryo sac interaction in cotton. In Pollen: Biology and Implications for Plant Breeding, D.L. Mulcahy and E. Ottoviano, eds (New York: Elsevier Biomedical).

Jordano, J., Almoguera, C., and Thomas, T.L. (1989). A sunflower helianthinin gene upstream sequence ensemble contains an enhancer and sites of nuclear protein interaction. Plant Cell 1, 855-866.

Josefsson, L.-G., Lenman, M., Ericson, M.L., and Rask, L. (1987). Structure of a gene encoding the 1.7S storage protein, napin, from *Brassica napus*. J. Biol. Chem. **262**, 12196-12201.

Jukes, T.H., and Cantor, C.R. (1969). Evolution of protein molecules. In Mammalian Protein Metabolism, H.N Munroe ed (New York, Academic Press).

Jürgens, G., Mayer, U., Torres Ruiz, R.A., Berleth, T., and Miséra, S. (1991). Genetic analysis of pattern formation in the *Arabidopsis* embryo. Development (suppl.) **1**, 27-38.

Kamaly, J.C., and Goldberg, R.B. (1980). Regulation and structural gene expression in tobacco. Cell **19**, 935-946.

Kao, K.N., and Michayluk, M.H. (1980). Plant regeneration from mesophyll protoplats of alfalfa. Pflanzenphysiol. **96**, 135-141.

Keller, W.A., Fan, Z., Pechan, P., Long, N. and Grainger, J. (1987). An efficient method for culture of isolated microspores of *Brassica napus*. In Proceedings of the 7th International Rapeseed Congress, Poznan, Poland. Vol. I, 152-157.

Kirk, J.T.O. and Pylotis, N.A. (1976). Cruciferous oilseed proteins: The protein bodies of *Sinapis alba* seed. Aust. J. Plant Physiol. **3**, 731-746.

Kiyosue, T., Yamaguchi-Shinozaki, K., Shinozaki, K., Higashi, K., Satoh, S., Kamada, H., and Harada, H. (1992). Isolation and characterization of a cDNA that encodes ECP31, an embryogenic-cell protein from carrot. Plant Mol. Biol. **19**, 239-249.

Kiyosue, T., Yamaguchi-Shinozaki, K., Shinozaki, K., Kamada, H., and Harada, H. (1993). cDNA cloning of ECP40, an embryogenic-cell protein in carrot, and its expression during somatic and zygotic embryogenesis. Plant Mol. Biol. **21**, 1053-1068.

Koltunow, A., M. (1993). Apomixis: Embryo sacs and embryos formed without meiosis or fertilization. Plant Cell **5**, 1425-1437.

Koncz, C., Martini, N., Koncz-Kalman, Z., Olsson, O., Radermacher, A., Szalay, A., and Schell, J. (1987). Genetic tools for the analysis of gene expression in plants. In Tailoring Genes for Crop Improvement, G. Bruening, J. Harada, T. Kosuge, and A. Hollaender eds (New York: Plenum Press).

Koning, A., Jones, A., Fillatti, J., Comai, L., and Lassnar, M. (1992). Arrest of embryo development in *Brassica napus* mediated by modified *Pseudomonas aeruginosa* exotoxin A. Plant Mol. Biol. **18**, 247-258.

Kosugi, S., Ohashi, Y., Nakajima, K., and Arai, Y. (1990). An improved assay for β-glucuronidase in transformed cells: Methanol almost completely suppresses a putative endogenous β-glucuronidase activity. Plant Sci. **70**, 133-140.

Kott, L.S., Polsoni, L., and Beversdorf, W.D. (1988). Cytological aspects of isolated microspore culture of *Brassica napus*. Can. J. Bot. **66**, 1658-1664.

Krebbers, E., Herdies, L., De Clercq, A., Seurinck, J., Leemans, J., Van Damme, J., Segura, M., Gheysen, G., Van Montagu, M., and Vandekerckhove, J. (1988). Determination of the processing sites of an *Arabidopsis* 2S albumin and characterization of the complete gene family. Plant Physiol. **87**, 859-866.

Kropf, D.L. (1992). Establishment and expression of cellular polarity in fucoid zygotes. Microbiol. Rev. **56**, 316-339.

Kyo, M., and Harada, H. (1990). Phosphorylation of proteins associated with embryogenic dedifferentiation of immature pollen grains of *Nicotiana rustica*. J.Plant Physiol. **136**, 716-722.

Lakshmanan, K.K., and Ambegaokar, K.K. (1984). Polyembryony. In Embryology of the Angiosperms, B.M. Johri, ed (New York: Springer-Verlag).

LaRoche, M. Aspart, L., Delseny, M. and Penon, P. (1984). Characterization of radish (*Raphanus sativus*) storage proteins. Plant Physiol. **74**, 487-493.

Lehrach, H.D., Diamond, D., Wozney, J.M., and Boedtker, H. (1977). RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16**, 4743-4751.

Lelievre, J.-M., Oliviera, L.O., and Nielson, N.C. (1992). 5'-CATGCAT-3' elements modulate the expression of glycinin genes. Plant Physiol. **98**, 387-391.

Lo, K.-H., and Pauls, K.P. (1992). Plant growth environment effects on rapeseed microspore development and culture: A flow cytometric study. Plant Physiol. **99**, 468-472.

Lo Schiavo, F., Quesada-Allué, L.A., and Sung, Z.R. (1986). Tunicamycin inhibits somatic embryogenesis but not cell proliferation in carrot. Plant Sci. **44**, 65-

Lonnerdal, B.O. and Janson, J.-C. (1972). Studies on *Brassica* seed proteins. I. The low molecular weight proteins in rapeseed. Isolation and Characterization. Biochim. Biophys. Acta **278**, 175-183.

Lopes, M.A., and Larkins, B.A. (1993). Endosperm origin, development, and function. Plant Cell **5**, 1383-1399.

Lott, J.N.A. (1980). Protein bodies. In The Biochemistry of Plants, Vol I, N.E. Tolbert, ed (New York: Academic Press).

Ludwig, S.R., Oppenheimer, D.G., Silflow, C.D., and Snustad, D.P. (1988). The α_1-tubulin gene of *Arabidopsis thaliana*: Primary structure and preferential expression in flowers. Plant Mol. Biol. **10**, 311-321.

Ma, H., Yanofsky, M.F., Meyerowitz, E.M. (1991). *AGL1-AGL6*, an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. Genes Dev. **5**, 484-495.

Mandaron, P., Niogret, M.F., Mache, R., and Monegar, F. (1990). *In vitro* protein synthesis in isolated microspores of *Zea mays* at several stages of development. Theor. Appl. Genet. **80**, 134-138.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbour, Cold Spring Harbour Laboratory).

Martin, T., Wöhner, R.V., Hummel, S., Willmitzer, L., and Frommer, W.B. (1992). The GUS reporter system as a tool to study plant gene expression. In GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression, S.R. Gallagher ed (Toronto: Academic Press).

Mascarenhas, J.P. (1989). The male gametophyte of flowering plants. Plant Cell 1, 657-664.

Mayer, U., Torres, R.A., Berleth, T., Miséra, S., and Jürgens, G. (1993). Mutations affecting body organization in the *Arabidopsis* embryo. Nature **353**, 402-407.

Mogie, M. (1988). A model for the evolution and control of generative apomixis. Biol. J. Linn. Soc. **35**, 127-154.

Moloney, M.M., Walker, J.M., and Sharma, K.K. (1989). High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Rep. **8**, 238-242.

Morrison, R.A., and Evans, D.A. (1988). Haploid plants from tissue culture: New plant varieties in a shortened time frame. Bio/Tech. **6**, 684-690.

Motto, M., Maddaloni, M., Panziani, G., Brembilla, M. and Marotta, R. (1988). Molecular cloning of the o2-m5 allele of *Zea mays* using transposon marking. Mol. Gen. Genet. **212**, 488-494.

Muntz, K., Bassuner, R., Lichtenfeld, C., Scholz, G., and Weber, E. (1985). Proteolytic cleavage of storage proteins during embryogenesis and germination of legume seeds. Physiol. Veg. **23**, 75-94.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco cultures. Physiol. Plant. **15**, 473-497.

Murphy, D.J., and Cummins, I. (1989). Biosynthesis of seed storage products during embryogenesis in rapeseed, *Brassica napus*. J. Plant Physiol. **135**, 63-69.

Murphy, D.J., Cummins, I., and Kang, A.S. (1989). Synthesis of the major oil-body membrane protein in developing rapeseed (*Brassica napus*) embryos. Biochem. J. **258**, 285-293.

Neilson, N.C., Dickinson, C.D., Cho, T.-J., Thanh, V. H., Scallon, B. J., Fischer, R.L., Sims, T.L., Drews, G.N., and Goldberg, R.B. (1989). Characterization of the glycinin gene family in soybean. The Plant Cell 1, 313-328.

Newbigin, E.J., de Lumen, B.O., Chandler, P.M., Gould, A., Blagrove, R.J., March, J.F., Kortt, A.A., and Higgins, T.J.V. (1990). Pea convicilin: Structure and primary sequence of the protein and expression of a gene in the seeds of transgenic tobacco. Planta **180**, 461-470.

Nogler, G.A. (1984). Gametophytic apomixis. In Embryology of Angiosperms, B.M. Johri ed (New York: Springer-Verlag).

Nunberg, A.N., Zhuwen, L., Bogue, M.A., Vivekanada, J., Reddy, A.S., and Thomas, T.L. (1994). Developmental and hormonal regulation of sunflower helianthinin genes: Proximal promoter sequences confer regionalized seed expression. Plant Cell **6**, 473-486.

O'Farrell, P.H.O., Kutter, E., Nakanishi, M. (1980). A restriction map of the bacteriophage T4 genome. Mol. Gen. Genet. **179**, 421-435.

Osborne, T.B. (1924). The Vegetable Proteins. (New York: Longmans, Green and Co.).

Ow, D., Wood, K.V., DeLuca, M., de Wet, J.R., Helsinki, D.R., and Howell, S.H. (1986). Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Science **234**, 856-859.

Pechan, P.M., and Keller, W.A. (1988). Identification of potentially embryogenic microspores in *Brassica napus*. Physiol. Plant. **74**, 377-384.

Pechan, P.M., Bartels, D., Brown, D.C.W., and Schell, J. (1991). Messenger RNA and protein changes associated with induction of *Brassica* microspore embryogenesis. Planta **184**, 161-165.

Pennell, R.I., and Roberts, K. (1990). Sexual Development in the pea presaged by altered expression of an arabinogalactan protein. Nature **344**, 547-549.

Pennell, R.I., Janniche, L., Kjellbom, P., Scofield, G.N., Peart, J.M., and Roberts, K. (1991). Developmental regulation of a plasma membrane arabinogalactan protein epitope in oilseed rape flowers. Plant Cell **3**, 1317-1326.

Perez-Grau, L., and Goldberg, R.B. (1989). Soybean seed protein genes are regulated spatially during embryogenesis. Plant Cell **1**, 1095-1109.

Peterson, N.S., and Mitchell, H.K. (1991). Environmentally induced developmental defects in *Drosophila*. In Results and Problems in Cell Differentiation, Vol 17. Heat Shock and Development, L.E. Hightower and L. Nover eds (Berlin: Springer-Verlag).

Plegt, L., and Bino, R.J. (1989). β-glucuronidase activity during development of the male gametophyte from transgenic and non-transgenic plants. Mol. Gen. Genet. **210**, 321-327.

Pomeroy, M.K., Kramer, J.K.G., Hunt, D.J., and Keller, W.A. (1991). Fatty acid changes during development of zygotic and microspore-derived embryos of *Brassica napus*. Physiol. Plant. **81**, 447-454.

Proudfoot, N.J., and Brownlee, G.G. (1976). 3' non-coding region sequences in eukaryotic messenger RNA. Nature **263**, 211-214.

Pysh, L.D., Aukerman, M.J., and Schmidt, R.J. (1993). OHP1: A maize basic domain/leucine zipper protein that interacts with Opaque2. Plant Cell **5**, 227-236.

Quattrocchio, F., Tolk, M.A., Coraggio, I., Mol, J.N.M., Viotti, A., and Koes, R.E. (1990). The maize zein gene zE19 contains two distinct promoters which are independently activated in endosperm and anthers of transgenic Petunia plants. Plant Mol. Biol. **15**, 81-93.

Radke, S.E., Andrews, B.M., Moloney, M.M., Crouch, M.L., Kridl, J.C. and Knauf, V.C. (1988). Transformation of *Brassica napus* L. using *Agrobacterium tumefaciens*: Developmentally regulated expression of a reintroduced napin gene. Theor. Appl. Genet. **75**, 685-694.

Raghavan, V. (1986). Embryogenesis in Angiosperms. A Developmental and Experimental Study. (New York, Cambridge University Press).

Raleigh, E.A., Murray, N.E., Revel, H., Blumenthal, R.M., Westaway, D., Reith, A.D., Rigby, P.W.J., Elhai, J., Hanahan, D. (1988). McrA and McrB restriction phenotypes of some *E. coli* strains and implications for cloning. Nucl. Acids Res. **16**, 1563-1575.

Rathore, K.S., Hodges, T.K., and Robinson, K.R. (1988). Ionic basis of currents in somatic embryos of *Daucus carota*. Planta **175**, 280-289.

Raynal, M., Depigny, D., Grellet, F., Delseny, M. (1991). Characterization and evolution of napin-encoding genes in radish and related crucifers. Gene **99**, 77-86.

Reinert, J. (1959). Uber die kontrolle der morphogenese und die induktion von adventivembryoone an gewebekulturen aus karrotten. Planta **53**, 318-333.

Reiser, L., and Fischer, R.L. (1993). The ovule and the embryo sac. Plant Cell **5**, 1291-1301.

Reynolds, T.L., and Kitto, S.L. (1992). Identification of embryoid-abundant genes that are temporally expressed during pollen embryogenesis in wheat anther cultures. Plant Physiol. **100**, 1744-1750.

Roberts, M.R., Hodge, R., Ross, Jo H.E., Sorensen, A., Murphy, D.J., Draper, J., and Scott, R. (1993). Characterization of a new class of oleosins suggests a male gametophyte-specific lipid storage pathway. Plant J. **3**, 629-636.

Russell, S.D. (1993). The egg cell: Development and role in fertilization and early embryogenesis. Plant Cell **5**, 1349-1359.

Rychlik, W., and Rhoads, R.E. (1989). A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and *in vitro* amplification of DNA. Nucl. Acids. Res. **17**, 8543-8551.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**, 406-425.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, second edition (Cold Spring Harbor: Cold Spring Harbor Laboratory Press).

Sanders, P.R., Winter, J.A., Barnason, A.R., Rogers, S.G., and Fraley, R.T. (1987). Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. Nucl. Acids Res. **15**, 1543-1558.

Sanger, F., Nicklin, S., Coulsen, A.R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**, 5463-5467.

Schachtman, D.P., Schroeder, I.J., Lucas, W.J., Anderson, J.A., and Gaber, R.F. (1992). Expression of an inward-rectifying potassium channel by the *Arabidopsis* KAT1 cDNA. Science **258**, 1654-1658.

Schiavone, F.M., and Cooke, T.J. (1987). A geometric analysis of somatic embrtyo formation in carrot cell culture. Can. J. Bot. **63**, 1573-1578.

Schmidt, R.J., Burr, F.A., and Burr, B. (1987). Transposon tagging and molecular analysis of the maize regulatory locus opaque-2. Science 238, 960-963.

Schroeder, J.I., Raschke, K., and Neher, E. (1987). Voltage dependent K^+ channels in guard cell protoplasts. Proc. Natl. Acad. Sci. USA. **84**, 4108-4112.

Scofield S.R., and Crouch, M.L. (1987). Nucleotide sequence of a member of the napin storage protein family from *Brassica napus*. J. Biol. Chem. **262**, 12202-12208.

Scott, R.J., and Draper, J. (1987). Transformation of carrot tissues derived from proembryogenic suspension cells: A useful model system for gene expression studies in plants. Plant Mol. Biol. **8**: 265-274.

Seguin-Swartz, G. (1984). Androgenesis in cytoplasmic male sterile *Brassica napus*. In Proceedings of the International Association for Plant Tissue Culture. First Plant Genetic Engineering Workshop, Canada Section, Saskatoon, Canada.

Sentenac, H., Bonneaud, N., Minet, M., Lacroute, F., Salmon, J.-M., Gaymard, F., and Grignon, C. (1992). Cloning and expression in yeast of a plant potassium ion transport system. Science **256**, 663-665.

Sharief, F. and Li, S.S. (1982). Amino acid sequence of small and large subunits of seed storage protein from *Ricinus communis*. J. Biol. Chem. **262**, 14753-14759.

Shirsat, A.H. (1991). Control of gene expression in the developing seed. In Developmental Regulation of Plant Gene Expression, D. Grierson, ed (New York: Chapman and Hall).

Shutov, A.D., and Vaintraub, I.A. (1987). Degradation of storage proteins in germinating seeds. Phytochemistry **26**, 1557-1566.

Simon, A.E., Tenbarge, K.M., Scofield, S.R., Finkelstein, R.R., and Crouch, M.L. (1985). Nucleotide sequence of a cDNA clone of *Brassica napus* 12S storage protein shows homology with legumin from *Pisum sativum*. Plant Mol. Biol. **5**, 191-201.

Sjödahl, S., Gustavsson, H.-O., Rödin, J., Lenman, M., Höglund, A.S., and Rask, L. (1993). Cruciferin gene families are expressed coordinately but with tissue-specific differences during *Brassica napus* seed development. Plant Mol. Biol. **23**, 1165-1176.

Smith, G.P (1976). Evolution of repeated DNA sequences by unequal crossing over. Science **191**, 528-535.

Song, J., Sorensen, E.L., and Liang, G.H. (1990). Direct embryogenesis from single mesophyll protoplasts in alfalfa (*Medicago sativa* L.) Plant Cell Rep. **9**, 21-25.

Stålberg, K., Ellerström, M., Josefsson, L.-G., and Rask, L. (1993). Deletion analysis of a 2S seed storage protein promoter of *Brassica napus* in transgenic tobacco. Plant Mol. Biol. **23**, 671-683.

Steeves, T.A., and Sussex, I.M. (1989). Patterns in Plant Development, second edition. (Cambridge: Cambridge University Press).

Sterk, P., Booij, H., Schellekens, G.A., van Kammen, A., and de Vries, S.C. (1991). Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell **3**,907-921.

Sterk, P., and de Vries, S.C. (1993). Molecular markers for plant embryos. In SynSeeds: Applications of Synthetic

Seeds to Crop Improvement, K. Redenbaugh ed. (Boca Raton, CRC Press).

Steward, F.C., Mapes, M.O., Mears, K. (1958). Growth and organized development of cultured cells. I. Growth and division of freely suspended cells. Am. J. Bot. **45**, 693-703.

Stinson, J.R., Eisenberg, A.J., Willing, R.P., Pe, M.E., Hanson, D.D., and Mascarenhas, J.P. (1987). Genes expressed in the male gametophyte of flowering plants and their isolation. Plant Physiol. **83**, 442-447.

Sung, Z.R., Fienberg, A., Chorneau, R., Bokird, C., Furner, I., Smith, J., Terzi, M., Lo Schiavo, F., Giuliano, G., Pitto, L., and Nuti-Ronchi, V. (1984). Developmental biology of embryogenesis from carrot culture. Plant Mol. Biol. Rep. **2**, 3-14.

Swofford, D.L., and Olsen, G.J. (1990). Phylogeny reconstruction. In Molecular Systematics, D.M. Hillis and C. Moritz, eds (pub?)

Taylor, D.C., Weber, N., Underhill, E.W., Pomeroy, M.K., Keller, W.A., Scowcroft, W.R., Wilen, R.W., Moloney, M.M., and Holbrook, L.A. (1990). Storage protein regulation and lipid accumulation in microspore embryos of *Brassica napus* L. Planta **181**, 18-26.

Telmer, C.A., Simmonds, D.H., and Newcomb, W. (1992). Determination of developmental stage to obtain high frequencies of embryogenic microspores in *Brassica napus*. Physiol. Plant. **84**, 417-424.

Telmer, C.A., Newcomb, W., and Simmonds, D. (1993). Microspore development in *Brassica napus* and the effect of high temperature on division symmetry in vivo and in vitro. Protoplasma **172**, 154-165.

Templeman, T.S., Stein, D.B., DeMaggio, A.E. (1988). A fern storage protein is genetically similar to the 1.7S seed storage protein of *Brassica napus*. Biochem. Genet. **26**, 595-603.

Tester, M. (1990). Plant ion channels: Whole and single channel studies. New Phytol. **114**, 305-340.

Thomas, P.S. (1983). Hybridization of denatured RNA transferred or dotted onto nitrocellulose paper. Methods Enzymol. **100**, 255-267.

Thomas, T.L. (1993). Gene expression during plant embryogenesis and germination: An overview. Plant Cell **5**, 1401-1410.

Thomas, T.L., Vivekananda, J., and Bogue, M.A. (1991). ABA regulation of gene expression in embryos and mature plants. In Abscisic Acid: Physiology and Biochemistry, W.J. Davies and H.G. Jones eds (Oxford: Bios Scientific Publishers).

Thurling, N., and Chay, P.M. (1984). The influence of donor plant genotype and environment on production of multicellular microspores in cultured anthers of *Brassica napus* ssp. oleifera. Ann. Bot. **54**, 681-693.

Tsai, C.Y., Larkins, B.A., and Glover, D.V. (1978). Interaction of the opaque-2 gene with starch forming mutant genes on the synthesis of zein in maize endosperm. Biochem. Genet. **16**, 883-896.

Tsao, S.G.S., Brunk, C.F., and Pearlman, R.E. (1983). Hybridization of nucleic acids directly in agarose gels. Anal. Biochem. **131**, 365-372.

Ueng, P., Galili, G., Sapanara, V., Goldsbrough, P.B., Dube, P., Beachy, R.N., and Larkins, B.A. (1988). Expression of a maize storage protein gene in Petunia plants is not restricted to seeds. Plant Physiol. **86**, 1281-1285.

Van Engelen, F.A., Sterk, P., Booij, H., Cordewener, J.H.G., Rook, W., van Kammen, A., and de Vries, S.C. (1991). Heterogeneity and cell type-specific localization of a cell wall glycoprotein from carrot suspension cells. Plant Physiol. **96**, 705-712.

Van Engelen, F.A., and de Vries, S.C. (1992). Extracellular proteins in plant embryogenesis. Trends Genet. **8**, 66-70.

Van Larebeke, N., Engler, G., Holsters, M., Van der Elsacker, S., Zaenen, J., Schilperoot, R.A., and Schell, J. (1974). Large plasmid in *Agrobacterium tumefaciens* essential for crown gall-inducing ability. Nature **252**, 169-170.

Vierling, E. (1991). The roles of heat shock proteins in plants. Ann. Rev. Plant Physiol. Mol. Biol. **42**, 579-620.

Vijayaraghavan, M.R., and Prabhakar, K. (1984). The endosperm. In Embryology of the Angiosperms, B.M. Johri ed (New York: Springer-Verlag).

Walbot, V. (1978). Control mechanisms for plant embryogeny. In Dormancy and Developmental Arrest, Experimental Analysis in Plants and Animals. M.E. Clutter ed (New York: Academic).

Walling, L., Drews, G.N., and Goldberg, R.B. (1986). Transcriptional and posttranscriptional regulation of soybean seed protein mRNA levels. Proc. Natl. Acad. Sci. USA **83**, 2123-2127.

Walsh, J.B. (1987). Sequence dependent gene conversion: Can duplicated genes diverge fast enough to escape conversion. Genetics **117**, 543-557.

Warwick, S.I. and Black, L.D. (1991). Molecular systematics of *Brassica* and allied genera (subtribe Brassicinae, Brassiceae)- chloroplast genome and cytodeme congruence. Theor. Appl. Genet. **82**, 81-92.

Warwick, S.I. and Black, L.D. (1993). Molecular relationships in subtribe Brassicinae (Cruciferae, tribe Brassiceae). Can J. Bot. **71**, 906-918.

Werr, W., Frommer, W.-B., Maas, C. and Starlinger, P. (1985). Structure of the sucrose synthase gene on chromosome 9 of *Zea mays*. EMBO J. **4**, 53-58.

West, M.A.L., and Harada, J. (1993). Embryogenesis in higher plants: an overview. Plant Cell **5**,1371-1381.

Wiberg, E., Råhlen, L., Hellman, M., Tillberg, E., Glimelius, K., and Stymne, S. (1991). The microspore-derived embryo of *Brassica napus* L. as a tool for studying embryo-specific lipid biogenesis and regulation of oil quality. Theor. Appl. Genet. **82**, 515-520.

Wilde, H.D., Nelson, W.S., Booij, H., de Vries, S.C., and Thomas T.L. (1988). Gene expression programs in embryogenic and nonembryogenic carrot cultures. Planta, **176**, 205-211.

Wilen, R.W., Mandel, R.M., Pharis, R.P., Holbrook, L.A. and Moloney, M. (1990). Effects of abscissic acid and high osmoticum on storage protein gene expression in microspore embryos of *Brassica napus*. Plant Physiol, **94**, 875-881.

Williams, M.E., Foster, R., Chua, N.-H. (1992). Sequences flanking the hexameric G-box core CACGTG affect the specificity of protein binding. Plant Cell **4**, 485-496.

Willing, R.P., and Mascarenhas, J.P. (1984). Analysis of the complexity and diversity of mRNAs from pollen and shoots of *Tradescantia*. Plant Physiol. **75**, 865-868.

Willing, R.P., Bashe, D., and Mascarenhas, J.P. (1988). An analysis of the quantity and diversity of messenger RNAs from pollen and shoots of *Zea mays*. Theor. Appl. Genet. **75**, 751-753.

Wurtele, E.S. Wang, H., Durgerian, S., Nikolau, B.J., and Ulrich, T.H. (1993). Characterization of a gene that is expressed early in somatic embryogenesis of *Daucus carota*. Plant Physiol. **102**, 303-312.

Xiao, H., and Lis, J.T. (1988). Germline transformation used to define key features of heat shock response elements. Science **239**, 1139-1142.

Yang, H., Zhou, C., Cai, D., Yan, H., Wu, Y. and Chen. (1986). *In vitro* culture of unfertilized ovules of *Helianthus annus*. In Haploids of Higher Plants In Vitro. H. Hu and H. Yang eds (New York:Springer-Verlag).

Yeung, E.C., and Meinke, D.W. (1993). Embryogenesis in angiosperms: Development of the suspensor. Plant Cell **5**, 1371-1381.

Youle, R.J., and Huang, A.H.C. (1981). Occurrance of low molecular weight and high cysteine containing albumin storage proteins in oilseeds of diverse species. Am. J. Bot. **68**, 44-48.

Zaki, M.A.M., and Dickinson, H.G. (1990). Structural changes during the first divisions of embryos resulting from anther and free microspore culture in *Brassica napus*. Protoplasma **156**, 149-162.

Zaki, M.A.M., and Dickinson, H.G. (1991). Microspore-derived embryos in *Brassica*: The significance of division symmetry in pollen mitosis I to embryogenic development. Sex. Plant Reprod. **4**, 48-55.

Zarsky, V., Garrido, D., Rihova, L., Tupy, J., Vincente, O., and Heberle-Bors, E. (1992). Derepression of the cell cycle by starvation is involved in induction of tobacco pollen embryogenesis. Sex. Plant Reprod. **5**, 189-194.

Zhou, C., Yang, H., Tian, H., Liu, Z. and Yan, H. (1986). *In vitro* culture of pollinated ovaries of *Oryza sativa* L. In Haploids of Higher Plants In Vitro. H. Hu and H. Yang eds (Berlin: Springer-Verlag).

Zimmerman, L.J. (1993) Somatic embryogenesis: a model for early development in higher plants. Plant Cell **5**, 1411-1423.