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**MOLECULAR CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF A
POLLEN-SPECIFIC GENE IN *BRASSICA NAPUS***

by

© BRIAN K. TREACY, B.Sc

Thesis submitted to the
School of Graduate Studies and Research
University of Ottawa
in partial fulfillment of the requirements for the
Ph.D. degree in the

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Ottawa, Ontario
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I would like to dedicate this thesis to my family. To both my Mom and Dad for making me the person I am today. To my brother Sean who is my best friend on the whole planet! Finally Jocelynn, thank you for being my soul mate and my favorite person with whom to share all the fun adventures which lie ahead!

ABSTRACT

cDNA and genomic clones of a new pollen-specific gene, *Bnm1*, have been isolated from *Brassica napus* L. cv. Topas. The gene contains an open reading frame of 546 bp and a single intron of 362 bp. A comparison of the deduced amino acid sequence with sequences in data banks did not show similarity with known proteins. Northern blot analysis of developing pollen showed that *Bnm1* mRNA was first detected in bicellular pollen and accumulated to higher levels in tricellular pollen. *Bnm1* mRNA was not detected in leaves, stems, roots, pistils, seeds or pollen-derived embryos. RNA *in situ* hybridization of whole flower buds confirmed that *Bnm1* was pollen-specific and expressed late in development. A promoter fragment of the *Bnm1* gene fused to the *gusA* reporter gene yielded similar patterns of tissue specificity and developmental regulation in transgenic *B. napus* cv Westar plants; however, the promoter was also active during the early stages of pollen development. The *Bnm1* gene, cloned in this study, was derived from the A genome of the allotetraploid species *B. napus* (AACC). Southern blot analysis indicated that sequences similar to the *Bnm1* gene were found in both A and C *Brassica* genomes. Related sequences were found in all 10 members of the Brassiceae tribe examined, but were not present in all tribes of the Brassicaceae family. Antisera raised against the BNM1 protein detected a single band of predicted size in tricellular and germinating pollen. BNM1 protein was not detected in other developmental stages including; tetrad, unicellular microspores and bicellular pollen. Protein samples extracted from somatic tissues, leaf and stem were not recognized by the anti-BNM1 primary antibody. To investigate the function of BNM1 in pollen, *B. napus* plants were transformed with a construct containing the *Bnm1* promoter, fused to the complete cDNA in reverse orientation. Seven transgenic plants showed lower levels of *Bnm1* when compared with wild-type. No obvious morphological lesions in transgenic antisense plants were observed. No differences in viability or *in vitro* germinations were observed between these downregulated plants and wild-type. *In vivo* germination assays suggested that, although BNM1 is not essential to

germination and pollen tube growth, BNMI may modulate the growth/guidance of the pollen tube towards the ovule.

RESUME

L'ADNc et le clone génomique d'un nouveau gène spécifique au pollen, *Bnm1*, ont été isolés de *Brassica napus* L. cv. Topas. Le gène possède une région codante de 546 pb et un intron de 362 pb. Une comparaison de la séquence d'acide aminés prédite avec les séquences dans les banques de données n'ont pas démontré une similarité avec des protéines connues. L'analyse de transfert Northern d'ARNs de pollen en voie de développement démontre que l'ARN de *Bnm1* est détecté premièrement dans le pollen bicellulaire et s'accumule à un niveau supérieur dans le pollen tricellulaire. L'ARN de *Bnm1* n'a pas été détecté dans la feuille, tige, racine, pistil, graine ou embryon dérivé du pollen. L'hybridation *in situ* à l'ARN de bourgeons de fleurs entières ont confirmé que *Bnm1* est spécifique au pollen et est exprimé tard durant le développement. Un fragment du promoteur de *Bnm1* fusionné au gène reporteur *gusA* a donné des résultats semblables pour la spécificité de tissu ainsi que le contrôle durant le développement dans les plantes transgéniques de *B. napus* L. cv. Westar; par-contre, le promoteur était actif aussi lors des stades immatures du pollen. Le gène *Bnm1* isolé dans cette étude est dérivé du génome A de l'espèce allotetraploïde *B. napus* (AACC). L'analyse de transfert Southern a indiqué que des séquences similaires à *Bnm1* ont été détectées dans les deux génomes A et C de *Brassica*. Des séquences ayant rapport ont été trouvées chez les 10 membres étudiés de la tribu Brassiceae, mais la similarité n'était pas présente dans toutes les tribus de la famille Brassicaceae. Un antiserum contre la protéine BNM1 a détecté une seule bande, de taille prédite, dans le pollen tricellulaire et le germé. La protéine BNM1 n'a été détectée dans aucun stade de développement incluant tétrade, microspore unicellulaire et pollen bicellulaire. Les échantillons de protéines extraites des

tissus somatiques, feuille et tige, n'ont pas été reconnus par l'anticorps primaire anti-BNM1. Pour examiner la fonction de BNM1 dans le pollen des plantes transgéniques de *B. napus* transformées avec un vecteur ayant le promoteur de *Bnm1* fusionné à l'ADNc entier en sense inverse. Sept plantes transgéniques ont démontré des niveaux inférieurs de *Bnm1* lorsque comparées avec les plantes non-transformées. Aucune lésion évidente n'a été observée au niveau de la morphologie dans les plantes transgéniques antisense. Aucune différence de viabilité ou germination *in vitro* du pollen n'ont été observées entre les plantes antisense et les plantes non-transformées. Les expériences de germination *in vivo* ont suggéré que, bien que BNM1 n'est pas essentiel à la croissance et à la germination du tube pollinique, BNM1 pourrait moduler la croissance/direction du tube pollinique vers l'ovule.

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LIST OF ABBREVIATIONS

Amp ^r	ampicillin resistance
ATP	adenosine-5'-triphosphate
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
cpm	counts per minute
CTP	cytidine-5'-triphosphate
cv	cultivar
DAPI	4', 6-deamindine-2-phenyl indole
ddH ₂ O	double-distilled water
dCTP	2'-deoxycytidine-5'-triphospahte
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dT	thymidine
DTT	dithiothreitol
<u>E. coli</u>	<u>Escherichia coli</u>
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis (β-amino ethyl ether)- N, N, N', N'-tetraacetic acid
g	gram(s)
GST	glutathione-S-transferase
FDA	fluorescein diacetate
hr	hour
IPTG	X-glucose 5-bromo-4-chloro-3-indolyl- β-D-glucuronide
kDa	kilodalton
l	litre(s)
LB	luria broth, left border

M	Molar
min	minute(s)
mg	milligram(s)
ml	millilitre(s)
mm	millimeter(s)
mM	millimoles
MOPS	morpholinopropanesulfoonic acid
MU	methylumbelliferone
MUG	4-methyl umbellsferyl- β-D-glucuronide
MW	molecular weight
nm	nanometers
N	normality
nos	nopaline synthase
npt II	neophosphotransferase II
nt	nucleotide(s)
OD	optical density
ORF	open reading frame
PAGE	poly acrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
POD	horseradish peroxidase
PROM	promotor
RB	right border
RNA	ribonucleic acid
RPM	revolutions per minute
SDS	sodium dodecyl sulfate
SSC	sodium chloride sodium citrate
SSPE	sodium chloride sodium phosphate
	EDTA

TAE	tris acetate EDTA
TBE	tris boric EDTA
TE	tris EDTA
TEMED	NNN'N'-tetramethylethylene-diamide
Tris	tris(hydroxymethyl)aminomethane
μg	microgram(s)
μm	micrometer(s)
u.v.	ultra violet
v/v	volume per volume
w/v	weight per volume
W	watts

INTRODUCTION

The first step in male gametogenesis (McCormick, 1993) is the division of sporophytic cells within the anther into tapetal initials and pollen mother cells. The pollen mother cell, through meiotic divisions, gives rise to a tetrad of haploid microspores which are encased in a callose wall. Unicellular microspores are released by dissolution of the callose wall and each divides mitotically to form a pollen grain consisting of a vegetative and a generative cell. In *Brassica napus* L., the generative cell undergoes a second mitotic division prior to anthesis, yielding tricellular pollen with two male gametes. In tissue culture, late unicellular and early bicellular *B. napus* pollen can be experimentally blocked from further development and diverted to embryo development by heat treatment at 32.5°C (Fan *et al.*, 1988; Keller *et al.*, 1987). Without the heat treatment pollen continues to develop to the bicellular and tricellular stages. The switch in developmental pathways is accompanied by immediate changes in cell morphology (Telmer *et al.*, 1992; Zaki and Dickinson, 1990) and gene expression (Boutilier *et al.*, 1994).

Pollen development is associated with the expression of both sporophytic and gametophytic genes, of which approximately 2,000 different transcripts or 10% of mature pollen mRNAs, represent genes specifically expressed in the male gametophyte (Mascarenhas, 1990). Transcripts appearing during male gametophyte development can be broadly classified into two groups for convenience (Mascarenhas, 1990). The first set (early genes) is present during microspore development (after tetrad release) reaching a maximal level at the haploid mitosis and then decreasing until pollen maturity. A few genes which are expressed specifically at the early stages of pollen development are expressed in both developing microspores and in the tapetum (Roberts *et al.*, 1993; Shen and Hsu, 1992; Theerakulpisut *et al.*, 1991), the only exception being the *NTM19* gene, recently cloned in tobacco (Odenhof *et al.*, 1996) which is specifically expressed in unicellular microspores. The second set (late genes) appears with the primary mitosis and reaches a maximal level at maturity. Some late pollen-specific genes have

been isolated but their potential functions have been determined in only a few cases. For instance, the *NeIF-4A8* gene in tobacco (Brander and Kuhlmeier, 1995) which is expressed in bicellular and tricellular pollen encodes a translation initiation factor that may play a role in mediating translational control during post-mitotic pollen development and perhaps during pollen germination and pollen tube growth. It has been shown that germination and early pollen tube growth are dependent on *de novo* translation but not on transcription of the mature pollen grain (Mascarenhas *et al.*, 1975).

Pollen-specific genes may be conserved at the DNA level across diverse taxa. For example, the *P2* gene which encodes a putative polygalacturonase in *Oenothera* (Brown and Crouch, 1990) hybridized to similar transcripts in both monocot and dicot families. Other genes may be restricted to a single family of plants. The *Bcp1* gene from *B. rapa* L., Syn. *B. campestris* L. (Theerakulpisut *et al.*, 1991) was found to encode transcripts that were only present in members of the Brassicaceae (Syn. Cruciferae) family. Another late pollen-expressed gene encoding a receptor-like kinase (*PRK1*) was restricted to members of the Solanaceae family (Mu *et al.*, 1994). In both cases it was hypothesized that the restricted distribution of the gene was related to the specificity of pollen-stigma interactions.

Many pollen-specific genes have been isolated, however, little is known about the proteins they encode. Sequence similarities to genes of known function only infer a putative role for that protein. Questions concerning the timing of protein synthesis and location within the cell may provide clues to their biological function. Mutational analysis by way of antisense technology may assign function to these proteins when combined with the above analyses. If a pollen-specific protein plays an important role during these processes, an altered phenotype would be expected. For example, the antisense approach showed that the pTOM13 protein is involved in ethylene synthesis as part of the ripening process of the tomato (Hamilton *et al.*, 1990). Examples of the antisense approach to study pollen-expressed genes include; *PRK1*, *Bcp1* and *LAT52* (Lee *et al.*, 1996; Xu *et al.*, 1995; Muschietti *et al.*, 1994). Both *PRK1* and *Bcp1* proved to be essential to the development of the pollen grain as the pollen grains

aborted development prior to reaching maturity. In contrast, the LAT52 protein appears to play a role later in development, during germination of the pollen grain and subsequent pollen tube growth down the style, as indicated by an altered phenotype during these processes (i.e. a lack of swelling in 50% of the pollen grains in *in vitro* germination media and knotted pollen tubes in *in vivo* germination assays). Swelling of the pollen grain prior to germination represents the hydration process which occurs during a compatible pollination. The lesion observed in *LAT52* pollen could be rescued by incubating the pollen grains in a medium with a higher water potential. Taken together, these observations suggested that *LAT52* may be involved in the hydration process. Localization of the *LAT52* protein at the apertures of the pollen grain would support the proposed function of *LAT52* during the hydration process.

The original intent of this research was to isolate and characterize genes which may be involved in the transition from pollen to embryo development during the induction phase of microspore embryogenesis in *B. napus*. Previous studies revealed that the expression of napin seed storage protein genes is a molecular marker for embryogenic microspores (Boutilier, 1994). As part of this effort, a study was initiated to identify genes that were highly expressed in embryogenic microspore cultures. The results from this study provided the basis for my thesis. Here, I report the isolation, characterization and functional analysis of a new gene, *Bnm1* (Brassica napus microspore 1). *Bnm1* is expressed at high levels in pollen samples cultured at 24°C to allow continued pollen development, whereas samples cultured at 32.5°C to block pollen development and initiate pollen embryogenesis had reduced levels of *Bnm1* mRNA. However, it was still high enough to represent the predominant mRNA cloned. Expression of *Bnm1* could not be detected in pollen-derived embryos that subsequently developed in culture. Taken together with the spatial and temporal expression profiles of *Bnm1* as determined by Northern blot analysis, *in situ* hybridization, GUS histochemical and fluorogenic assays in transgenic plants, it was concluded that *Bnm1* is pollen-specific and expressed only in bicellular and tricellular pollen. This was unexpected, considering *Bnm1* was the most abundant message (except for ribosomal and napin gene transcripts) in the cDNA

library prepared from *B. napus* microspores and pollen induced to undergo embryogenesis in culture. Since *Bnm1* has no significant sequence similarity to cloned genes with known function, a strategy to characterize the biological role of the gene product BNM1 was developed. This functional analysis involved the production of antisera against the BNM1 protein and the use of antisense technology in transgenic plants. The antisense approach was undertaken to create a mutation specific to *Bnm1* to assess function and antisera were developed to determine the timing of BNM1 synthesis as well as to immunolocalize the BNM1 protein in the cell. Results from these studies, although incomplete, suggest that BNM1 is not involved in the development and/or maturation of the pollen grain and is not essential to pollen germination and pollen tube growth. However, BNM1 may be involved in mediating/modulating the growth/guidance of the pollen tube towards the ovule.

CHAPTER ONE

LITERATURE REVIEW

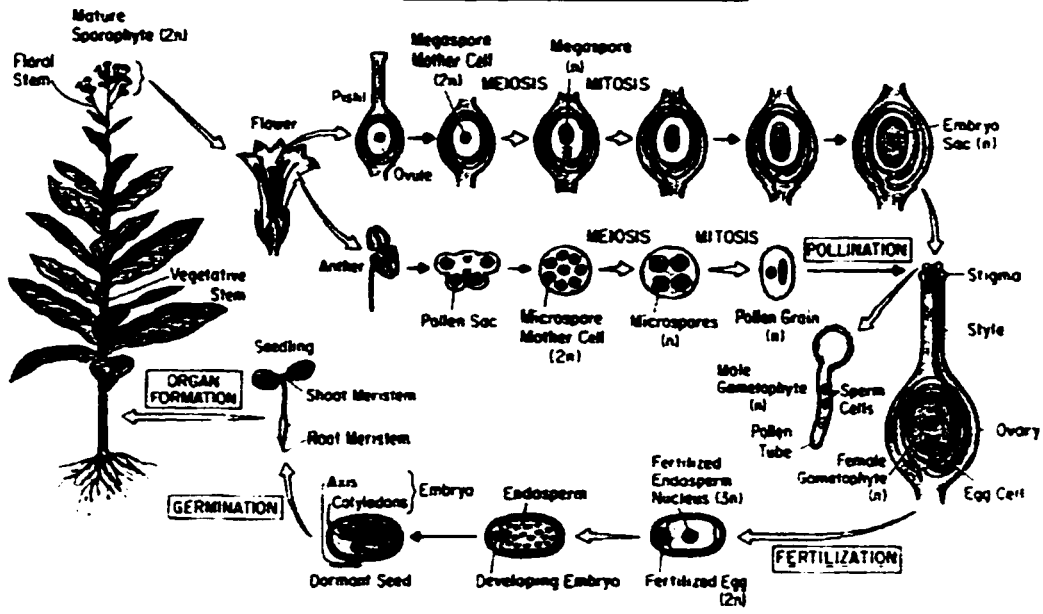
1.1. *Gametogenesis*

The life cycle of plants (Fig.1) is characterized by the alternation between diploid and haploid phases. In most plants, the dominant phase provides nutrition and physical support for the other less conspicuous phase (Fig.1). In mosses for instance, the haploid organism is the dominant phase, whereas in angiosperms this phase is represented by the diploid organism (referenced in Baltz, 1992). The diploid organism in angiosperms produces haploid spores as a result of meiosis and is therefore called the sporophyte. These spores do not function as gametes but undergo mitotic division giving rise to multicellular haploid individuals, referred to as gametophytes, which produce gametes (Raven, 1987). The gametophytes of angiosperms are extremely reduced in size and complexity and develop entirely within the sporophytic (stamen/carpel) tissues. The male gametophyte or the pollen grain in *Brassica* species consists of three cells, one vegetative cell containing within it two non-motile sperm cells. The female gametophyte or the embryo sac consists of seven cells of which the central cell is binucleate. In contrast, free living multicellular and photosynthetic "plant-like" gametophytes of lower plants produce motile male gametes. Thus, the primary function of the gametophyte, the production of gametes, has been conserved during evolution. In higher plants the gametophyte has become increasingly smaller and dependent upon the sporophyte for its development and function.

Fig.1. Schematic Representation of the Life Cycle of a Flowering Tobacco Plant.

Adapted from Goldberg (1988).

Gametogenesis



Embryogenesis

1.2 Microgametogenesis

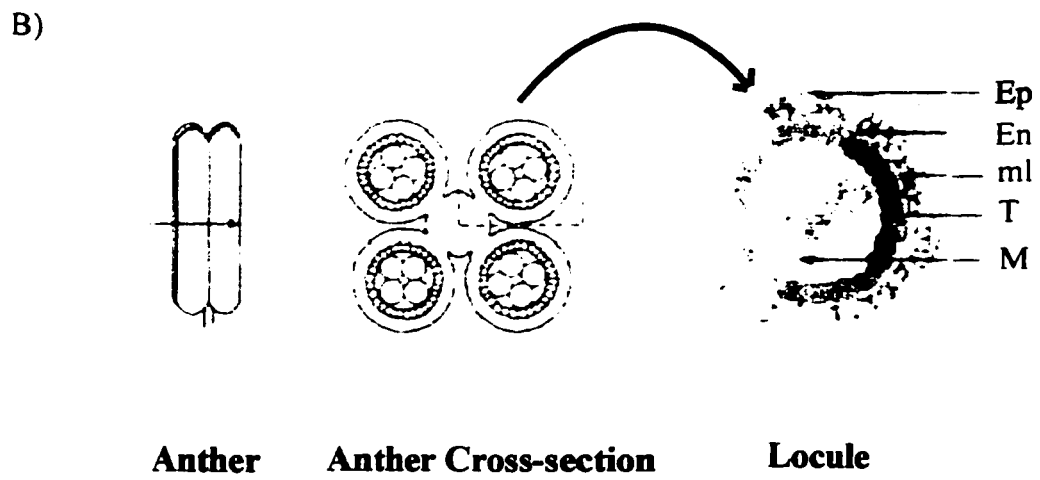
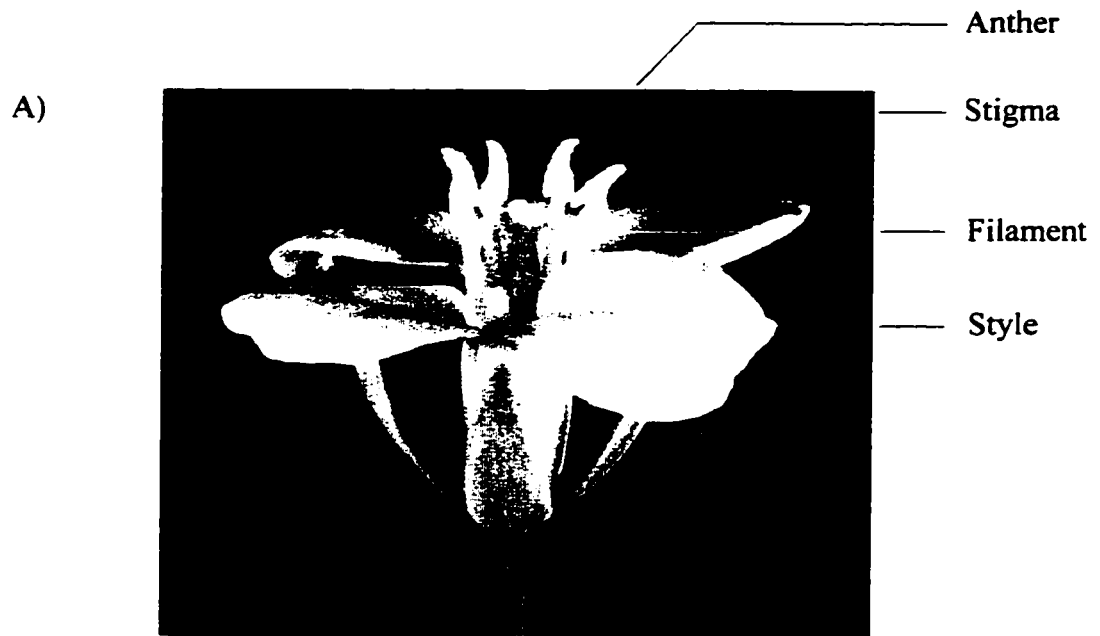
In angiosperms, formation of the haploid male gametophyte (microgametogenesis) or pollen takes place in the stamen. The stamen arises from the floral meristem and is composed of the filament and anther. The filament acts indirectly in microgametogenesis by supplying water and nutrients to the anther. Pollen grains differentiate from sporogenous cells in the anther. The anther initially consists of a uniform mass of cells, except for the partly differentiated epidermis. Eventually, four groups of sporogenous cells become discernible within the anther (Fig.1 and Fig.2) (Raven, 1987). Each group is surrounded by several layers of cells which develop into the wall of the pollen sac. The pollen sac includes nutritive cells which supply nutrition to the developing pollen. The innermost layer is called the tapetum (Fig.2) (Raven, 1987).

The first step in male gametogenesis (McCormick, 1993) is the division of sporogenous cells within the anther into tapetal initials and pollen mother cells (Fig.1). The pollen mother cell, through meiotic divisions gives rise to a tetrad of haploid microspores (Fig. 3). Each meiotic division may be followed by a callose cell wall formation. Alternatively, the walls may form simultaneously after the second meiotic division. The first condition is common to the monocots and the latter is most common to the dicots like *Brassica napus* (Raven, 1987).

The tapetal cells play a vital role in pollen development. During meiosis of the microspores, the tapetal cells undergo a dramatic differentiation into binucleate polar secretory cells which aid in the release of the young haploid microspores from the callose (β -1,3 glucan) wall via the secretion of a β -1,3 glucanase or callase (Bedinger, 1992). The microspores are released as free cells into the anther locule (Fig. 2). A second function of the tapetal cells is the production of precursors for the biosynthesis of the outer pollen wall called the exine. As the microspores develop, the tapetal cells degenerate and may deposit cell remnants called tryphines or pollenkit on the microspore surface (Fig. 3). These substances

Fig.2. A) A *Brassica napus* flower. B) An illustration of an anther cross-section including a micrograph of an anther locule or pollen sac. Ep, epidermis; En, endothecium; ml, middle layer; T, tapetum; M, microspore.

Adapted from Bedinger (1992).



are largely lipoidal and may protect the developing pollen from dehydration as well as aid pollinators in collection. Preuss *et al.* (1994) have also noted that these compounds play a vital role in successful hydration of the pollen grain which precedes proper germination on the stigma. In addition, mutation of tapetal cells has been correlated with defective pollen wall biosynthesis (Kaul, 1988). The inner wall or intine (Fig.3) is laid down by the microspore protoplast and is composed of cellulose and pectin while the outer wall or exine (Fig. 3) is apparently derived from both the tapetum and haploid microspores. (Bedinger, 1992).

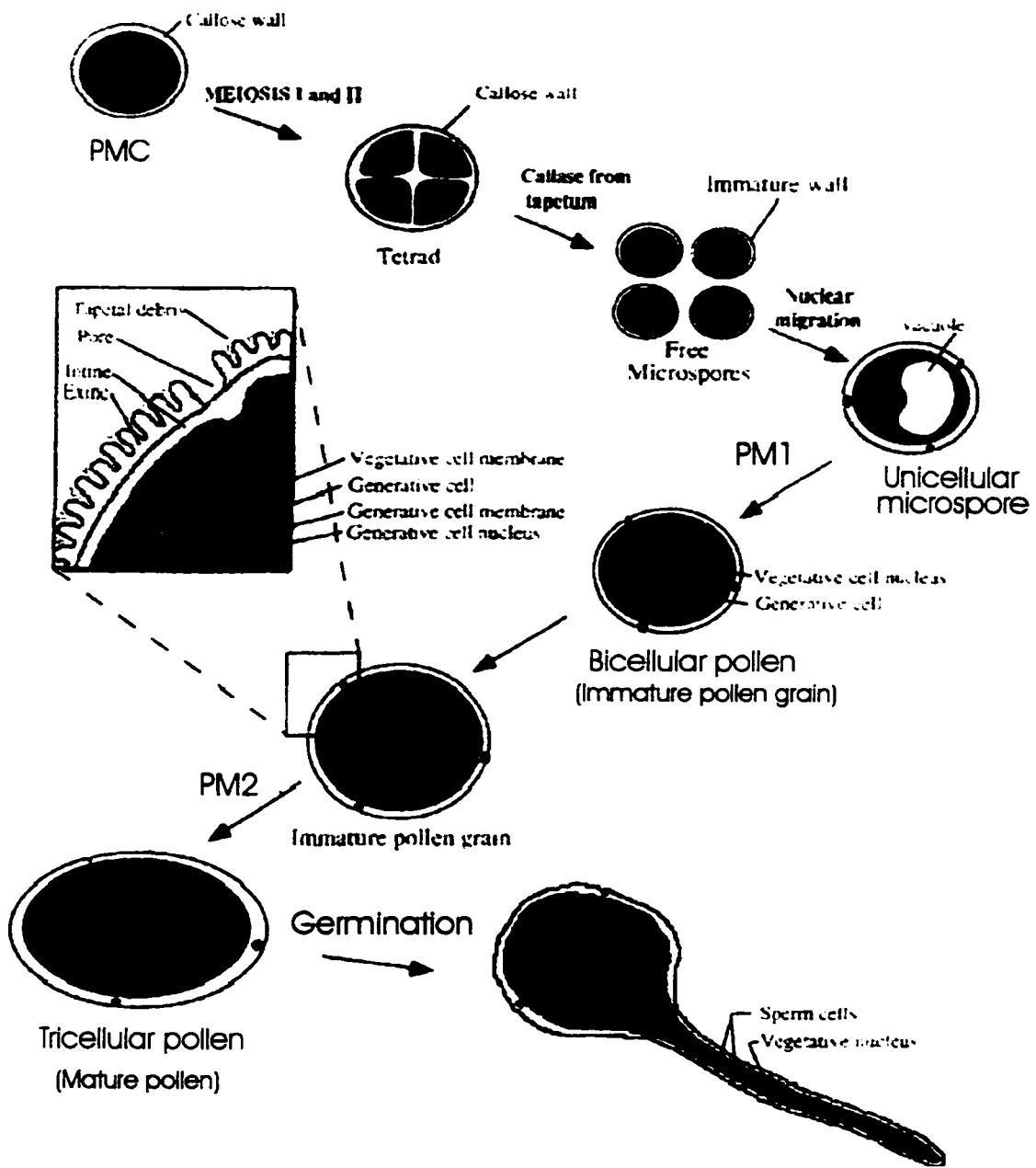
Once the unicellular microspores are released from the callose wall enclosing the tetrad, the centrally located microspore nucleus migrates to a position opposite the developing pollen pore (Fig. 3). Vacuolation of the microspore is initiated with the fusion of multiple small vacuoles into a single large vacuole that occupies the majority of the microspore volume (Fig. 3). At this time the tapetal cells begin to degenerate. An asymmetric nuclear division, microspore mitosis, produces a vegetative nucleus and a smaller generative nucleus (Fig. 3). The nucleus adjacent to the plasma membrane becomes cellularized, forming the generative cell (Bedinger, 1992). This nuclear division defines the transition of microspores into young pollen, which are now bicellular. This division can also be viewed as a determinative division in that the resulting two cells have very different fates (Horvitz and Herskowitz, 1992). The vegetative cell, or tube cell, nucleus does not undergo another mitosis. Its role is to serve in cell maturation for germination and pollen tube formation, hence the definition of tube cell. The generative cell must undergo another mitotic division to give rise to the two sperm cells. In 70% of the plant species studied (Brewbaker, 1967), tobacco being an example, the microgametophyte remains at the bicellular stage at anthesis. In other species, such as members from the Poaceae and Brassicaceae family, the generative cell undergoes a second mitotic division prior to anthesis yielding tricellular pollen with two male gametes or sperm cells. In addition, all taxa considered to be phylogenetically advanced were tricellular. Schurhoff (1926) first proposed that the tricellular condition was phylogenetically advanced. The most important observation emerging from Brewbaker's analysis (1967) of 265

Fig.3. Schematic Representation of Microsporogenesis.

(Development of the male gametophyte).

PMC, pollen mother cell; PMI, pollen mitosis I; PMII, pollen mitosis II.

Adapted from McCormick (1993).



families is that the tricellular taxa regularly trace back to bicellular taxa, while the reverse is not observed in a single instance. This character is, therefore, a derived character which has evolved independently on numerous occasions (Brewbaker, 1967). The mitotic divisions that occur between meiosis and gametic formation serve not only to generate essential accessory cells but also to provide a critical haplosufficiency test for basic cellular functions. Any spore that contains a deleterious recessive allele of a gene that is required during gametophyte development will fail to produce a functional gametophyte. Thus, although spores may arise from cells that have accumulated many mutations, the existence of the gametophyte generation allows selection against many of the most detrimental mutations, reducing the genetic load in the next sporophyte generation (Chasan and Walbot, 1993). It is interesting to note that, despite considerable divergence in floral morphology and pollen size between two model species, *Nicotiana tabacum* (bicellular) and *Arabidopsis thaliana* (tricellular), microspore and pollen development occur over similar time periods and major cytological processes, such as vacuolation and the accumulation of metabolic reserves, are similar between the two pollen types (Scott and Stead, 1994).

Studies of RNA and protein synthesis (Bedinger and Edgerton, 1990) in isolated microspores (Mandaron *et al.*, 1990) indicate that a major developmental switch in gene expression patterns occurs after this first asymmetric mitotic division. Studies of cloned pollen-specific genes indicate that the majority of such genes appear to become actively transcribed only after microspore mitosis (Stinson *et al.*, 1987; McCormick, 1991). The generative and sperm cells contain few mitochondria, sparse ER and condensed chromatin along with a lower nuclear pore density suggesting that these cells are less transcriptionally active than the vegetative cell nucleus (Wagner *et al.*, 1990). However, serial electron microscopy reconstructions of alfalfa microspores showed that there are more nuclear pores on the surface of the vegetative nucleus that is appressed to the generative cell than on the opposing surface, implying close association and communication between both cells (Shi *et al.*, 1991).

Following the first mitotic division, the young bicellular pollen begins to engorge with starch and the generative nucleus migrates laterally. The second division of the generative cell occurs giving rise to two sperm cells to form the tricellular pollen grain. During the final maturation process, lipid and proteinaceous remnants of the tapetal cells are deposited on the surface of the pollen grain, which desiccates (not in all species) and is shed from the anther upon anthesis (Fig. 3).

1.3 Megagametogenesis

As with the microgametophyte, the megagametophyte or embryo sac is highly dependent on the sporophytic tissue. Formation of the embryo sac takes place in the carpel which is defined as the whole female counterpart of the stamen. The carpel is composed of the stigma and style, which make up the pistil, and the placenta or ovule-bearing region (Fig. 1). The ovule itself is a complex structure, consisting of a stalk, called the funiculus that, like the filament of the stamen provides both a physical and vascular connection to the plant. Initially, the developing ovule is entirely nucellus which arises from the ovular tissue and differentiates to produce the diploid megasporocyte or megaspore mother cell as well as one or two enveloping layers, the integuments (Fig. 4). The integuments, which later become the seed coat, envelope the ovule, and leave a small opening, the micropyle, at one end. The micropyle marks the point of entry of the pollen tube during fertilization (Fig. 4D). The diploid megaspore mother cell divides meiotically to form four haploid megaspores. Three of these megaspores usually disintegrate and the one farthest from the micropyle survives and develops into the megagametophyte. The megagametophyte enlarges at the expense of the nucellus and subsequently undergoes three nuclear mitotic divisions. After the first division, both resulting nuclei migrate to opposing ends of the embryo sac (Fig. 1). At the end of the third mitotic division, the eight nuclei are arranged in two groups of four, one group near the micropylar end of the megagametophyte and the other at the opposite, or chalazal end. One

nucleus from each group migrates into the center to become known as the polar nuclei. The three remaining nuclei at the micropylar end become cellularized and organized as the egg apparatus, consisting of an egg cell and two cellular synergids which are thought to play an active role in chemotrophic attraction of the pollen tube (Russell, 1993). Cell wall formation also occurs around the other three nuclei left at the chalazal end, forming the so-called antipodals. Although the antipodal structure remains intact until fertilization, little is known about their function during megagametogenesis. The central cell containing the polar nuclei remains binucleate and may fuse to become diploid. The eight-nucleate, seven-celled structure is the mature female gametophyte or embryo sac (Fig.4D).

1.4 Pollen Germination

The mature pollen rapidly germinates upon contact with the stigma to form pollen tubes (Fig.1). At the pollen-stigma interface, the establishment of a zone of adhesion between the pollen grain and the "dry" surface of the papillar cell (typical for species in the crucifer family) occurs. This involves spreading of the tryphine from the pollen grain along the proteinaceous coating of the stigma. Important tryphine components originating mostly from the tapetal cells include long chain alkanes (Aarts *et al.*, 1995) and oleosins (Ross *et al.*, 1996) among other fatty acids which appear to facilitate exine adhesion by organizing lipids into a matrix structure through which water is transferred from channels in the stigma cell wall. It is during this one-on-one interaction between a pollen grain and a papillar cell of the stigma that the Self-Incompatibility (SI) reaction is manifested (Nasrallah *et al.*, 1994). This process will be discussed later. Upon compatible signalling and hydration of the pollen grain, germination of one pollen tube per pollen grain by way of the aperture occurs (Fig.5).

Fig.4. Schematic Representation of Megagametogenesis.

(Development of the female gametophyte).

A: Initiation of development. nu, nucellus, ms, megasporocyte. B: After first meiotic division. ii, inner integument; oi, outer integument; fu, funiculus. C: After second meiotic division. dm, degenerate megaspores; fm, functional megaspore. D: Mature ovule.

Adapted from Reiser and Fisher (1993).

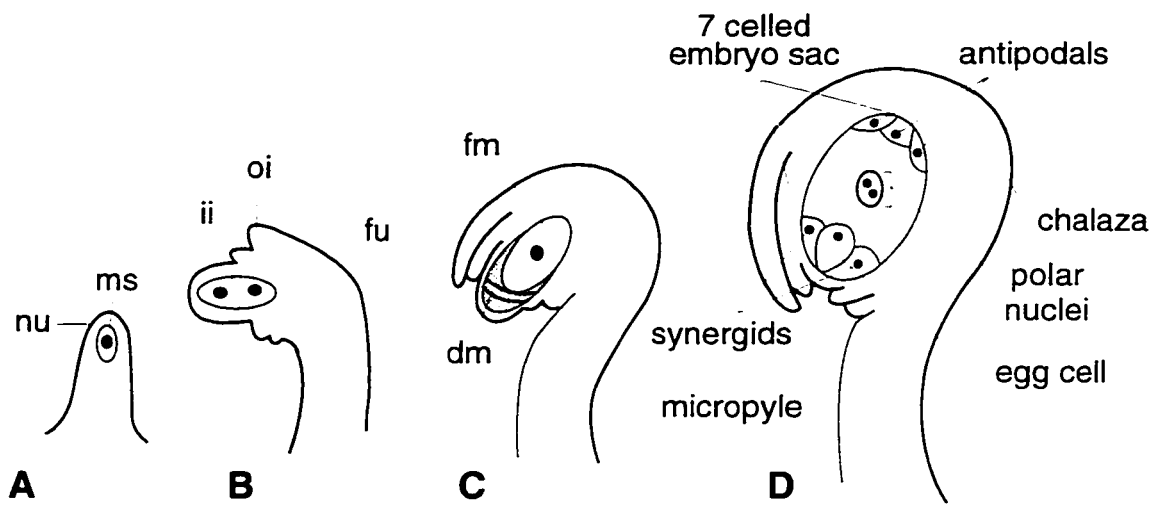
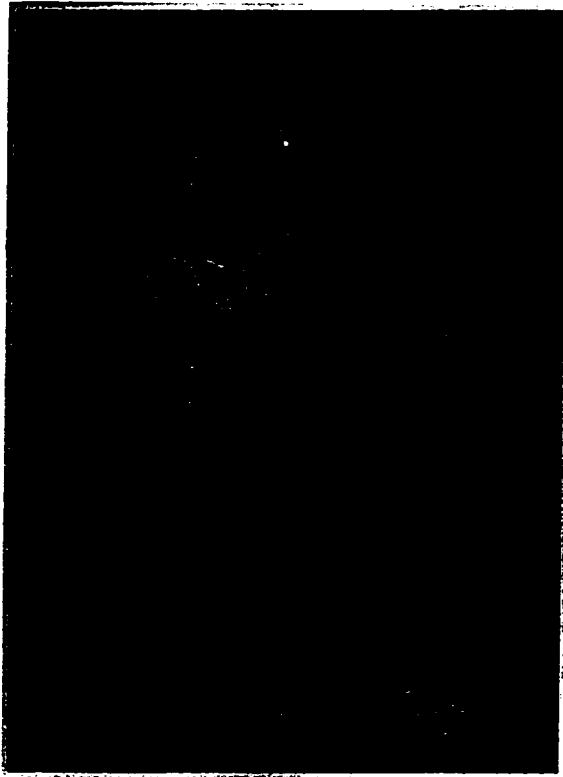


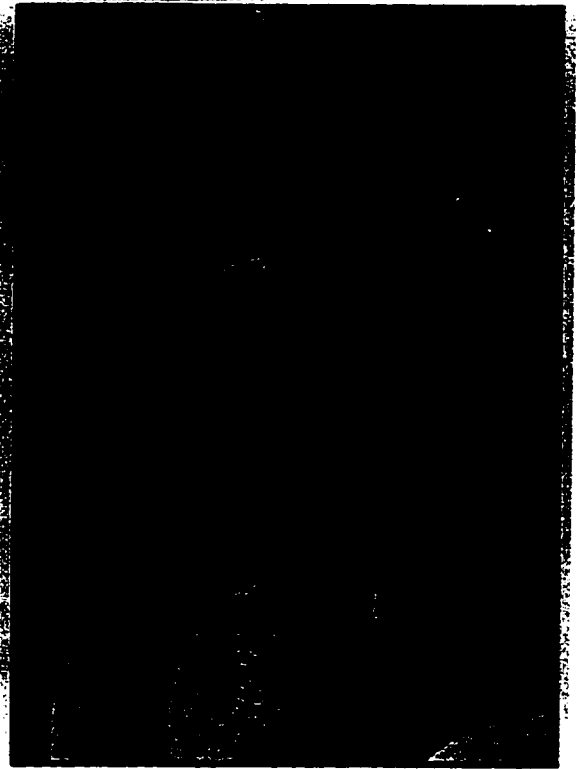
Fig.5. Electron Micrograph of the Pollen-Stigma Interface.

A) Germination of a compatible pollen grain. B) Self-Incompatible interaction. In the self-incompatible interaction, the pollen tube does not invade the papillar cell.

Figure from Nasrallah and Nasrallah (1995), Signalling in Plant Development Conference, Cold Spring harbour NY.



A



B

1.5 Pollen Tube Growth

1.5.1 Structure and composition

The process of pollen tube growth in itself is characterized by intense cytoplasmic streaming, during which the movement of smaller organelles (like secretory vesicles) and ones (including the generative cell and the vegetative nucleus) are precisely coordinated (Cai *et al.*, 1996). The initial phase of pollen tube growth known as pollen germination occurs very rapidly and the rate of tube growth is extremely high for most plants. In maize, for example, pollen germination occurs within 5 minutes of deposition on the silk, and the pollen tube grows through as much as 50 cm of style at rates close to 1 cm/hr (Mascarenhas, 1993).

The nature of pollen tube growth is similar to the growth of root hairs. The root hair develops from a tubular extension of an epidermal cell wall void of cuticle. Similarly, the pollen tube is an extension of the intine void of exine. Both pollen tube and root hair growth are otherwise known as tip growth. In *Prunus* pollen tubes, five distinct cytological zones have been identified: The apical growth zone, the smooth endoplasmic reticulum (ER) zone, the rough ER zone, the vacuolation zone and the deterioration zone (Uwate and Lin, 1980) (Fig.6). The cytoplasm of the pollen tube in the non-growing region behind the tip contains an abundance of mitochondria, dictyosomes (or Golgi bodies), ER and vesicles. The growing tip region, by contrast, contains a large number of vesicles and an absence of other organelles. All of the cytoplasmic structures appear to be aligned parallel to the long axis of the pollen tube except for the tip where structures are more randomly distributed (Fig.6). This cytoplasmic organization is typical of plant cells having cytoplasmic streaming. Cytoplasmic streaming simply defines the process of movement which occurs during shuttling of materials within the cell and between the cell and its environment.

The composition and structure of the pollen tube wall are complex and incompletely characterized. The pollen tube is a double layered structure except for the tip where it is a

single layer. Many sugars, such as, glucose, galactose, arabinose, rhamnose and xylose in order of abundance (% mole) constitute the pollen tube wall with galacturonic acid being the most abundant sugar derivative. In addition, the most abundant polysaccharide is callose which is located specifically in the inner wall layer as determined by immunocytochemistry (Meikle *et al.*, 1991). Callose was also immunolocalized to the outer layer of the non-growing double layered wall (Anderson *et al.*, 1987). Proteins have also been identified in the pollen tube wall, many of which are glycoproteins and hydroxyproline-rich proteins (Mascarenhas, 1993).

1.5.2 *Cytoplasmic Motors and Tip Growth*

The pollen tube structure and growth dynamic are governed by an active cytoskeleton. Both microtubules and microfilaments make up the cytoskeleton which gives cells their characteristic shape and form, and provides attachment points for organelles (Fig. 7). The cytoskeleton is not to be viewed as a rigid, permanent framework of the cell but as a dynamic, changing structure (Lehninger, 1982). Microfilaments, largely consisting of actin and myosin filaments, often in association with one another, contribute to the process of cyclosis, or cytoplasmic streaming. Microtubules, on the other hand, are a complex of α - and β -tubulin, arranged in a helix forming a hollow core, undergo constant assembly from their building blocks and disassembly. They give the cell its shape and structure from which the microfilaments form a loose web network and participate in the contractile system involved in various types of cellular or intracellular movement and shuttling of cell metabolites (Lehninger, 1982).

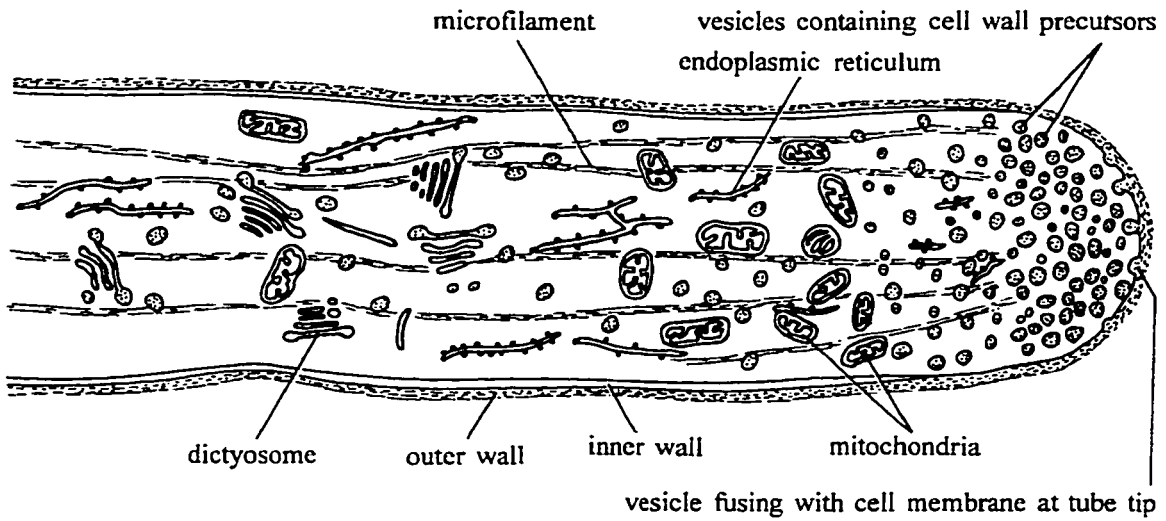
Cytoplasmic streaming is extremely active in growing pollen tubes. Three main types of movement occur within the tube to ensure proper growth (Cai *et al.*, 1996). 1. The elongation of the tube is determined by the continuous fusion of secretory vesicles with the cell membrane at the tip via phospholipid-binding annexins. The vesicle membranes

Fig.6. Schematic Model of the Tip of a Growing Pollen Tube.

Like the root hair, the pollen tube extends via tip growth following the mechanism of cytoplasmic streaming. Shuttling of nutritive components is from tip to back, while components added to the growing tip are shuttled to the apex.

Adapted from Mascarenhas (1993).

Cytoplasmic Streaming →



Nutrients + Chemotropic Molecules ←

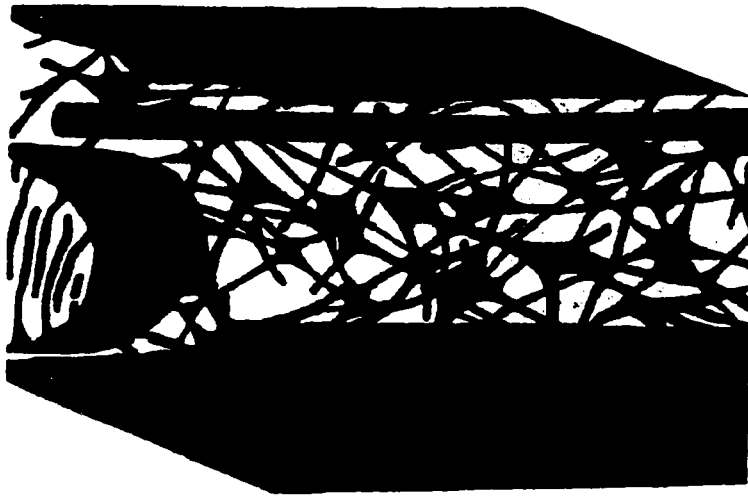
contributing to the tip plasma membrane, and the contents are discharged to the outside where they contribute to the growing tube wall. 2. The pollen tube cytoplasm is constantly translocated during tube elongation. 3. Finally, both the generative cell and vegetative nucleus move within the tube cytoplasm. It is possible that these three types of intracellular movement rely on different kinds of motor activities, or that different regulation of the same mechanism produces different types of organelle motion. Recent immunocytochemical work on *Pinus* pollen tubes has shown that microtubules are important in controlling cellular polarity (Terasaka and Niitsu, 1994) and the movement of the vegetative nucleus (Miyake *et al.*, 1995). The movement of the generative cell and transport of the secretory vesicles to the tip region, on the other hand, appear to be more closely related to a microfilament or actin-myosin based mechanism (Astrom *et al.*, 1995).

This actin-myosin mechanism appears to be governed by a cytosolic free calcium gradient increasing from base to tip (Pierson *et al.*, 1994). This calcium gradient is absent in non-growing pollen tubes and disruption of the gradient inhibits tube growth (Miller *et al.*, 1992). The mechanisms that maintain the calcium gradient are not clearly understood but the recent localization of calcium channel activity in the growing tip could be an important factor (Pierson *et al.*, 1994). Jaffe *et al.* (1975) initially provided evidence that calcium influx is localized at the pollen tube apex. These findings are supported by Malho *et al.* (1995) who reported that pollen tube growth and reorientation by calcium ionophoretic microinjection, electrical fields or photolysis of caged Ca^{2+} may be regulated by voltage-gated calcium channels. The sequence of events noted from these experiments was: 1) an immediate increase in cytosolic free calcium, 2) an inhibition of growth, 3) return to normal levels of cytosolic free calcium, 4) tip swelling, and 5) a recovery of pollen tube growth, usually in a different direction. Recent work suggests that the observed increase in cytosolic free calcium is mediated, at least in part, by an inositol 1, 4, 5 - triphosphate- induced calcium release signal transduction pathway (Franklin-Tong *et al.*, 1996). However, much remains to be elucidated in this area of pollen tube growth. As noted, microtubules appear to also play a role in

Fig.7. Schematic Diagram of the Structure of the Cytoskeleton.

This diagram is based upon observations made with an electron microscope. Rod-like structures represent microtubules and matrix-like structures represent network of microfilaments.

Adapted from Lehninger (1982).



— Microtubule

— Microfilament

motility, for example, in the movement of the vegetative nucleus. Other microtubule-based motor molecules are being identified at the tip of the growing pollen tube. For example, a pollen kinesin homologue has been localized at the pollen tube cortex using an anti-kinesin heavy chain antibody from bovine brain (Cai *et al.*, 1993). Other studies (Cyr and Palevitz., 1995) have described the dynamic properties of cortical microtubules in plant cells. It has been hypothesized that short fragments of microtubules translocate within the cortex to act as primers for the construction of new, differently oriented microtubules. The cortical localization of the pollen kinesin suggests the possibility that the protein could also be important in the re-organization of the microtubular cytoskeleton following tube elongation

1.6 Pollen-Pistil Interactions

The pollen tube has to migrate distances often thousands of times the diameter of the pollen grain to deliver the male gametes to the embryo sac for fertilization. The pollen tube pathway begins with pollen tube penetration of the stigmatic papilla cell presumably through the action of hydrolytic enzymes located at the tip of the pollen tube (Nasrallah *et al.*, 1994). The pollen tube then elongates and enters the stylar transmitting tract within the extracellular matrix (ECM) by tip growth. Pollen tubes never penetrate the protoplasts of cells they encounter before they reach the embryo sac. The lining of this transmitting tissue is abundant in cellular exudates rich in glycoproteins, carbohydrates, invertases and calcium (Cheung, 1996). It has been demonstrated that all these compounds along with mechanical support provided by the style play a vital role in proper pollen tube guidance towards the ovule. Several models postulate that glycoproteins are multi-functional. They have been observed to be incorporated into elongating pollen tubes *in vivo*. In other systems, cell surface glycoproteins are known to be important for many cell-cell recognition processes (Lowe, 1994). These glycoproteins may also provide cell surface adhesion as well as nutrition to the elongating pollen tube. A gradient of increasing calcium concentration has been observed in

the *Antirrhinum* pistil. These gradients along with other chemotropic molecules may play critical roles in pollen tube guidance.

1.7 *Self-Incompatibility (SI) in Brassica*

SI is based on the ability of the pistil to discern the presence of self-pollen and inhibit the germination or subsequent development of self-related, but not genetically unrelated, pollen (Nasrallah and Nasrallah, 1993). Recent studies indicate that SI is achieved by diverse molecular mechanisms in different plant species. In the Brassicaceae family the incompatible pollen-stigma interaction is controlled by a single, highly polymorphic Mendelian locus, the S locus. Inhibition of pollen occurs at the stigmatic surface when the same allele of the S locus is active in the pistil and pollen whether pistil and pollen are derived from the same plant or from different plants that carry the same allele. That is, the pollen phenotype is determined not gametophytically by the haploid genotype of the pollen grain but sporophytically by the diploid genotype of the parent (Fig.8). This model is known as sporophytic SI, in contrast to gametophytic SI common to the Solanaceae family (Fig.8). Recent molecular analyses of the S-locus region in *Brassica* have identified a region of ca. 200 Kb containing several physically linked transcriptional units (estimated at 60 alleles in *B. oleracea*) that co-segregate perfectly with the SI phenotype and that comprise an "S haplotype". Two of these S-locus genes represent a pair of related but not identical genes. One (*SLG*) encoding an S-locus glycoprotein is secreted into the stigmatic wall (Kadasamy et al. 1989) and the other (*SRK*) an S-receptor kinase which phosphorylates serine/threonine residues is situated in the plasma membrane of the stigma. Sequence analysis revealed over 80% amino acid identity between the S-domain of both genes (Nasrallah and Nasrallah, 1993). By phosphorylating intracellular substrates, the *SRK* protein would couple the initial molecular recognition events during pollen "adhesion" at the papillar cell-pollen interface to the signal-transduction chain that leads ultimately to pollen rejection. Because *SLG* and *SRK* are both expressed in the stigma

cells in the absence of pollen, a pollen-borne component possibly a ligand for *SRK* is postulated. *SLG* which is freely diffusible in the cell wall, would be essential, either by acting as an extracellular regulator of ligand access to the signalling receptor or by being an integral part of a functional receptor complex.

In vitro binding experiments clearly demonstrate that members of the pollen-coat peptide family derived from the sporophytic tapetal tissue bind to S-locus specific glycoproteins (*SLGs*) (Doughty *et al.* 1992, 1993) and also to S-locus related (*SLR*) glycoproteins (Hiscock *et al.* in prep). A recently cloned pollen coat protein (*PCP1*) has been found to be pollen-specific and not sporophytic in nature (Stanchev *et al.*, 1996). The peptide family members characterized to date show no haplotype specificity, and it is not clear whether they function in SI. Haplotype specificity and its physical linkage to the S-locus are properties expected for a gene that encodes the pollen determinant of sporophytic SI.

The understanding of self-incompatibility in plants, would also shed some light on other mechanisms which show parallels to SI. These include, the signalling systems found in animals, such as, the major histocompatibility complex (*MHC*) involved in the immune response, as well as, host-pathogen interactions that are also mediated by cell-surface receptors with signal transducing activity. Furthermore, sequences encoded by the S-locus family of genes have been cloned from diverse plants and tissues, such as maize roots, and *Arabidopsis* shoots and roots. This is consistent with the hypothesis that the *Brassica* S-locus genes are derived from ancestral genes having a very basic role in intercellular communication during plant development.

1.8 Fertilization

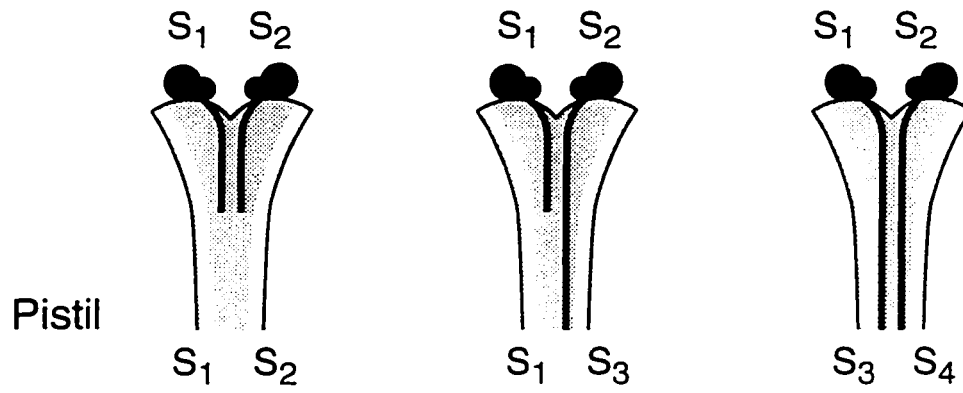
Once in the ovary, the pollen tube turns from a basally-oriented growth direction to gain access to the ovule via the micropyle (Fig. 1). In *Arabidopsis*, this change is triggered by a long-range activity that is governed by the ovule (Hulskamp *et al.*, 1995).

Fig.8. Model Diagram of Sporophytic versus Gametophytic Self-Incompatibility (SI).

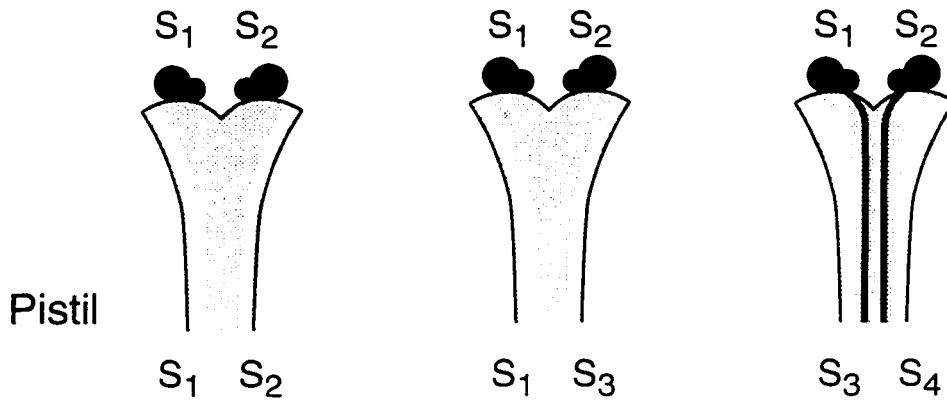
Members of the Solanaceae family (e.g. *Nicotiana*) exhibit sporophytic SI, while members of the Brassicaceae family (e.g. *Brassica*) exhibit gametophytic SI. S1, S2, S3 and S4 represent different alleles.

Figure from Clark (1993).

Gametophytic SI



Sporophytic SI



Hulskamp *et al.* (1995) postulated a model that was supported by previous evidence in pearl millet. This model involves rupturing of one of the synergid cells just prior to pollen tube entrance creating a high local concentration of calcium around the micropyle that is potentially chemotropic in nature (Chaubal *et al.*, 1992a, b). Embryo sac ablation studies have shown significant lack of pollen tube guidance from the transmitting tissue to the ovule (Hulskamp *et al.*, 1995). Therefore, there is clearly a long distance chemotropic signal generated by the embryo sac that directs the pollen tube to receptive ovules. Preuss *et al.* (1993) have discovered a male-sterile mutant in *Arabidopsis*. In this mutant, pollen tubes grow randomly around the ovule, cross the micropyle without entering and, unlike the wild type, do not adhere tightly to the ovule surface. These results suggest that guidance to the micropyle requires two interdependent processes; namely, adhesion of pollen tubes to cell surfaces and a response to chemotropic signals emanating from the micropyle.

Transport of the angiosperm non-motile sperm cells in the pollen tube occurs via ATP-dependant actin-myosin interactions that are found axially aligned in the pollen tube cytoplasm (Heslop-Harrison and Heslop-Harrison, 1989a). The sperm cells are typically connected to one another and physically associated with the vegetative nucleus, and are transported within the pollen tube as a "linked unit" (Russell and Cass, 1981) termed the "male germ unit" (Dumas *et al.*, 1984). The linkage of sperm cells increases the effectiveness of gamete delivery and ensures simultaneous transmission of both sperm cells into the receptive embryo sac (Russell and Cass, 1981). Once discharged from the pollen tube, the sperm cells are freed from their association with the vegetative nucleus and with each other. Consequently, the sperm cells lose their ensheathing vegetative cell membrane, exposing their own plasma membranes within the embryo sac (Russell, 1994). Presumably, actin bands in the embryo sac interact with the sperm cells to transport the gametes to their site of fusion with the egg and central cell for double fertilization (Russell, 1996).

Preferential fertilization, in which one sperm cell has a greater likelihood of fusing with the egg is common in plants but poorly understood. In tests of *in vitro* fusions, 80% of

sperm-egg pairs fused, 2% of sperm-mesophyll protoplast pairs fused, and 18% of two-sperm pairs fused (Faure *et al.*, 1994). Egg cells did not fuse with each other or with mesophyll protoplasts. These results suggest some specificity of gamete cell surfaces. Recently, immunolocalization procedures provided evidence that arabinogalactan protein (AGP) epitopes are membrane components of sperm cells (Southworth and Kwiatkowski, 1996). Although their function in cell-cell recognition has not yet been elucidated these findings represent the first specific epitopes recognized on plant sperm cell surfaces. Preferential fertilization has been demonstrated to occur in *Plumbago*. Here, the two sperm cells of a pair differ greatly from one another in size, shape, and organellar content making it easier to differentiate both members of the pair during fertilization, which is not a general rule in all species. *Brassica* sperm have been shown to be dimorphic in size and organelle content as well (McConchie *et al.*, 1987). In this particular case the smaller, plastid-rich sperm almost always fertilizes the egg cell forming a diploid zygote while the other sperm cell enters the central cell composed of two polar nuclei forming a triploid primary endosperm nucleus (Russell, 1993). The endosperm nucleus divides mitotically forming the endosperm which has a nutritive role. The zygote develops into an embryo, and through mitosis differentiates into the globular, heart, torpedo and cotyledonary morphological stages (Raven, 1987).

1.9 Microspore Embryogenesis

Embryos can also be induced in culture from a variety of sporophytic and gametophytic tissues (reviewed in Raghavan, 1986). 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, 1989) and *B. napus* (Chuong and Beversdorf, 1985; Fan *et al.*, 1988; Keller *et al.*, 1987; Pechan and Keller, 1988; Telmer *et al.*, 1992). Cultured embryos although not identical to zygotic embryos develop through

similar morphological stages and given the appropriate culture conditions will express many of the same genes as zygotic embryos with the same spatial and temporal expression profiles. The primary advantage of this culture system is the large number of embryos at specific developmental stages that can be generated. This allows one to access and study the earliest events of differentiation of the proembryo. Furthermore, the development of embryos in culture can be experimentally manipulated to study the processes involved in development. Microspore or pollen embryogenesis culture systems have bypassed many of the disadvantages encountered by other culture systems. For example, the somatic embryogenesis culture system has a prolonged culture period which often results in an accumulation of phenotypic variants acquired through mutations. Also, because these explants are cultured for extended periods of time, the initial cell types which arise during the initiation of embryogenesis are ill defined. Gametophytic culture systems utilizing anthers and ovaries have the disadvantage of generating embryos from both sporophytic and gametophytic tissues, and therefore generating embryos having different ploidy levels.

Using a highly enriched population of immature pollen at the unicellular stage and regulating culture conditions allows one to direct the developmental pathway of the pollen to either gametogenesis or embryogenesis (Fig.9). In general, pollen or microspore embryogenesis is induced by a single stress treatment of defined duration. In *B. napus*, heat treatment of late unicellular/ early bicellular 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; Pechan and Keller, 1988; Telmer *et al.*, 1992) (Fig.9). The same microspores when cultured in the absence of heat treatment, continue to develop as pollen (Fig.9). In contrast, heat-treated microspores or pollen from either earlier or later stages of development do not exhibit this response. In heat-treated embryogenic microspores the first mitotic division toward microsporic embryo development takes place in the vegetative nucleus and, in

contrast to the first pollen mitosis, is symmetric in nature (Fan *et al.*, 1988; Zaki and Dickinson, 1991). The subsequent mitotic divisions give rise successfully to similar globular, heart, torpedo and cotyledon morphological stages observed in zygotic embryos (Crouch, 1982; Pomeroy *et al.*, 1991). Work by Touraev *et al.* (1995) on tobacco indicated that symmetrical division of the microspore does not necessarily block gametophytic development and, although it might be required, is clearly not sufficient for induction of embryogenesis when using anther extracts. However, they also reported symmetrical division was correlated with an embryogenic-specific pathway when using heat-shock treatment instead of anther extracts.

1.10 *Brassica as a model plant*

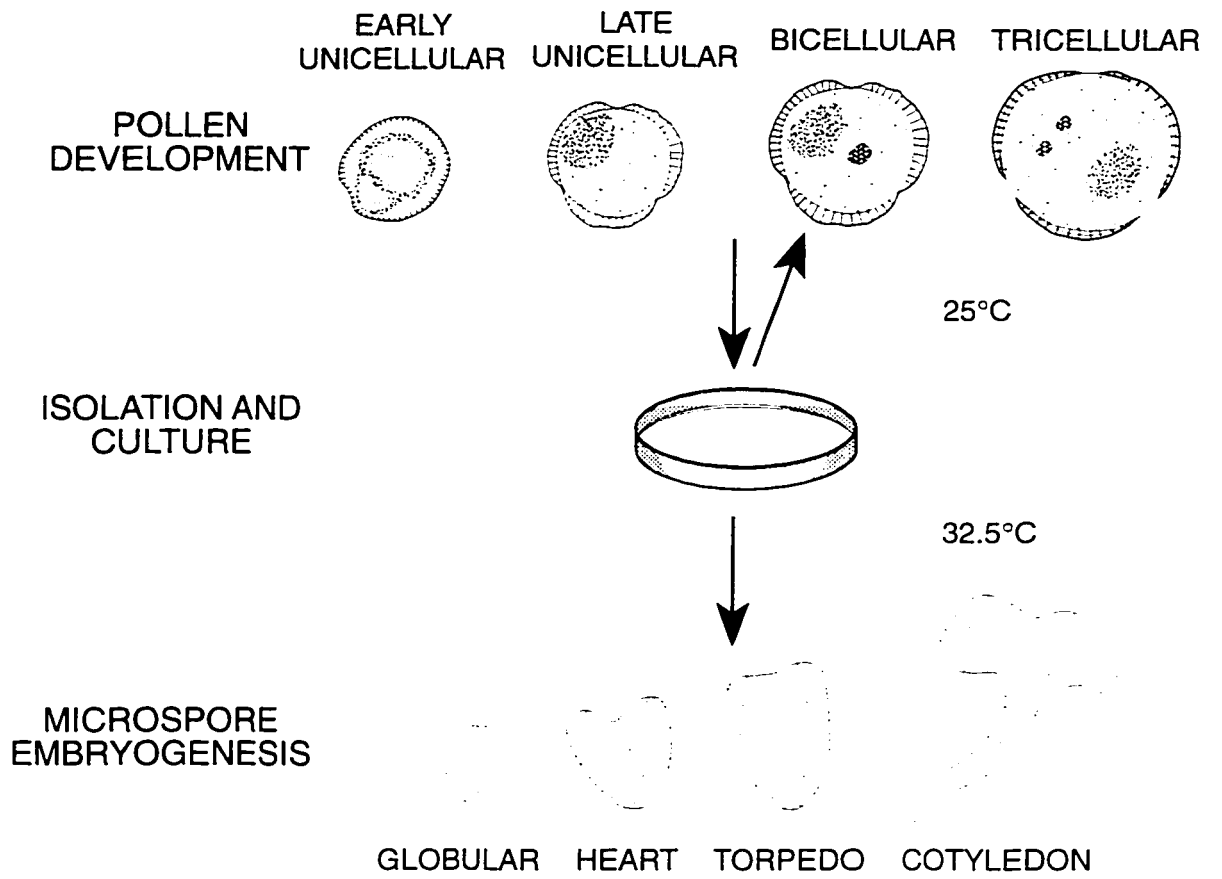
The objective of plant breeding is to improve performance over existing cultivars. Improvements include increased yield, increased adaptability to environmental variation (i.e. temperature, drought, pests) and reduced requirements for fertilizers and herbicides. Traditional plant breeding methods, such as selecting superior genetic combinations from crosses between elite cultivars achieves these objectives. However, the introduction of new variation into existing cultivars is limited to plants that produce fertile offspring when crossed with a particular crop species. Gene cloning and the introduction of novel traits via *Agrobacterium*-mediated transformations (other technologies include protoplast fusions and tissue culture) have greatly increased the range of genetic variation theoretically available to the plant breeder (Lydiate *et al.*, 1993).

The ability of *Brassica* to grow at relatively low temperature makes it suitable for growth in Canadian and Northern European climates, and it is one of the very few oil sources successfully grown in temperate regions. This has led to the development of rapeseed into Canola, the plant that is now one of Canada's major cash crops.

Fig.9. Schematic Illustration of Microsporogenesis and Microspore Embryogenesis in *B. napus*.

When cultured *in vitro* at 25°C, late unicellular and early bicellular microspores continue to develop as pollen. In contrast, when microspores from the same stage of development are cultured *in vitro* at 32.5°C, haploid embryos are induced to form.

Adapted from Boutilier (1992, Ph.D Thesis).



Today, Canola leads all crops in field trials of genetically-modified crops in Canada and has attracted great interest by the plant biotechnology industry worldwide. The six cultivated species of *Brassica* (Fig.10) provide a wide range of crop types (including oilseed, vegetable and fodder crops) that are all closely related. *Brassica. napus*, *B. juncea* and *B. carinata* are all amphidiploid (allotetraploid) species, formed naturally through interspecific hybridizations of the three diploid ancestors (Fig.10). Genetic exchange through sexual crosses is possible across most of this very extensive gene pool; therefore, facilitating the introduction of novel traits obtained through molecular techniques.

1.11 *Pollen as a Model System*

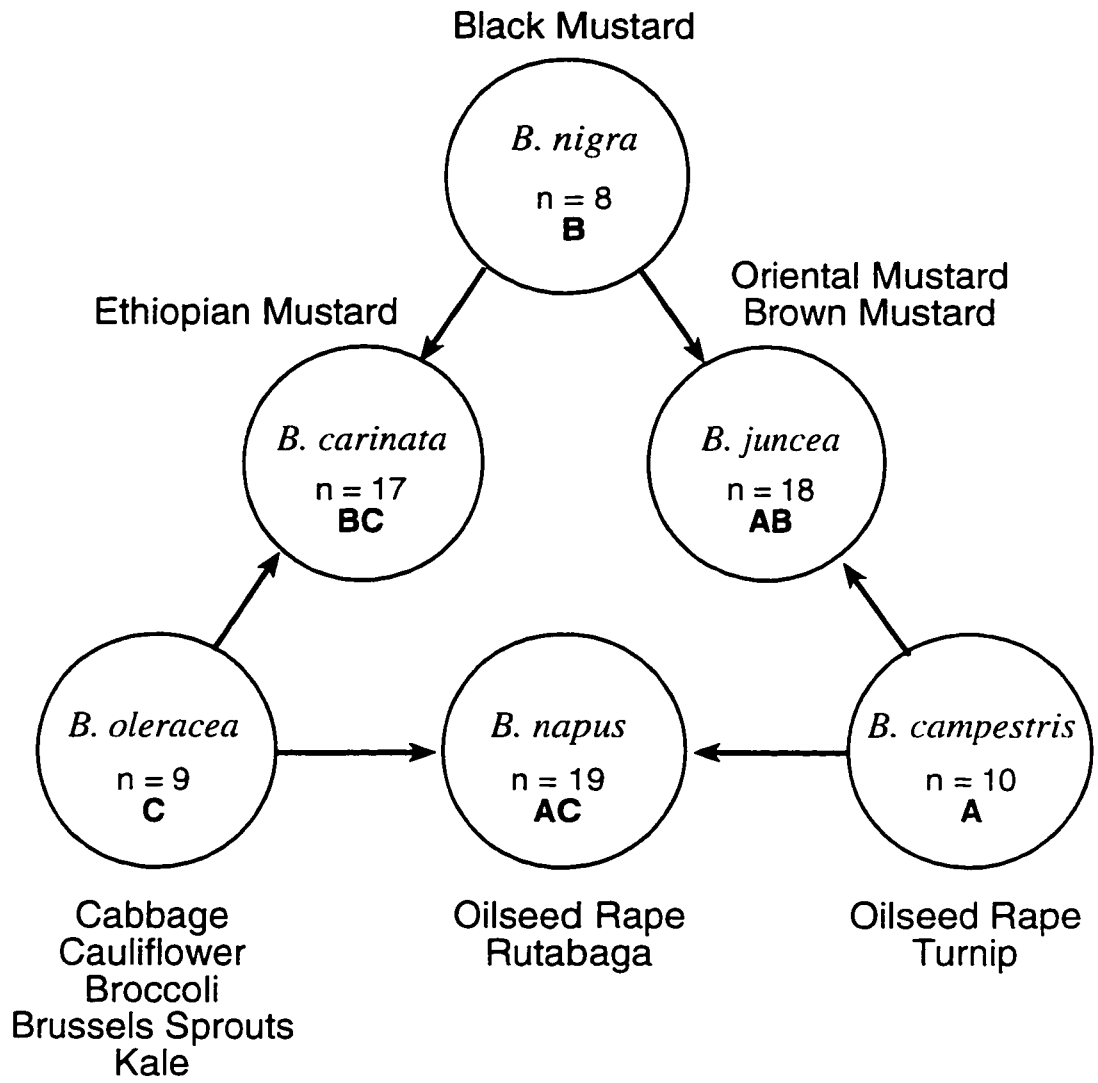
Pollen as a biological system offers the opportunity to answer many of the challenging questions faced in biology today. Many laboratories have benefited from the Self-Incompatibility (SI) response in some plants to explore cell-cell communication and signal transduction mechanisms at the pollen-pistil interface. Other laboratories have cloned genes whose products elicit an allergenic response in humans. The biology of cell fate can be scrutinized by examining the cellular differentiation of a generative and a vegetative cell in maturing pollen leading up to double fertilization. Yet, other groups interested in manipulating breeding systems have generated male-sterility by disrupting key components essential for proper pollen formation.

1.12 *Pollen-Specific Regulatory Elements*

Genes are regulated in part by a number of sequences lying in the 5' and 3' flanking regions of the gene. The DNA sequences referred to as *cis*-acting factors include the proximal promoter and the distal promoter. The proximal promoter dictates where RNA polymerase II

Fig.10. Diagram illustrating the Relationships between Diploid Progenitor Species and Amphidiploid Species. A, B and C are letters assigned to genomes by U (1935). n is the haploid number of chromosomes.

Adapted from U's "Triangle" (1935).



starts transcription while the distal promoter contains other regulatory elements. Some of these elements dictate tissue specificity and developmental regulation and are believed to mediate gene expression through interaction with *trans*-acting factors. It is often assumed that these elements are not species-specific and may be studied in heterologous hosts. Promoters of pollen-specific genes have been studied to a limited extent. These studies have been carried out by constructing promoter-reporter gene fusions and introducing these constructs into the plant via *Agrobacterium*-mediated transformation or microprojectile bombardment of pollen. Reporter genes such as *luc*⁺ and *gusA* provide easily assayable markers to study gene expression. The expression of promoter-reporter gene constructs of pollen-specific genes has been shown, for the most part, to be developmentally regulated and tissue-specific in transgenic plants.

The most detailed analysis of pollen-specific promoters are from the three genes from tomato *LAT52,56,59* (Twell *et al.* 1989,1990,1991). Three distinct regions that regulate developmental expression in pollen were found. Two regions (-492 to -145 and -124 to -86) enhance expression in pollen (Twell *et al.*, 1990), while the regions between -84 and -55 for *LAT52* and -98 and -69 for *LAT59* are sufficient to drive gene expression in pollen alone (Eyal *et al.*, 1995). Eyal *et al.* (1995) show that pollen-specificity is not due to transcriptional repression in tissues other than pollen but rather to a positive element(s) occurring in pollen. No obvious promoter sequences are conserved among pollen promoters (McCormick, 1993). Only limited sequence similarities in *LAT52, 56, 59* proximal promoters were noted. Most notably, the 5 bp sequence ACTGT is common to both *LAT52* and *LAT59* proximal promoters and is absent in *LAT56* (Eyal *et al.*, 1995). Indeed, the recognition of variable *cis* elements has been noted for the maize *opaque2* transcription factor, which binds to completely different sequences in α -zein genes and in a *b-32* albumin gene (Yunes *et al.*, 1994). Further understanding of coordinate expression of pollen promoters requires more knowledge about the *trans*-acting factors which may regulate their expression. Hochstenbach *et al.* (1996) have recently demonstrated using gel mobility shift assays that the proximal

(-103 to -51) pollen-specific promoter NTP303 interacts with a leaf nuclear GT-1 transcription factor. These findings suggest that ubiquitous transcription factors are involved with pollen-specific gene expression *in vivo*.

1.13 Genes Characterized in Microspores and Pollen

Pollen development is associated with the expression of both sporophytic and gametophytic genes. Stinson *et al.* (1987) have shown that approximately 20,000 different kinds of mRNA are present in the mature pollen of *Tradescantia*. Approximately 15% of the total mRNA mass represents 40 different mRNAs that are very abundant, while the major fraction of the mRNA (60%) represents 1,400 different sequences each present in moderate abundance. The least abundant fraction (24%) is made up of 18,000 different mRNAs present in about 100 copies per mature pollen grain. Of all the genes expressed during pollen development approximately 10% of mature pollen mRNAs, represent genes specifically expressed in the male gametophyte (Stinson *et al.* 1987). The vast majority of these pollen-expressed genes have been isolated through differential hybridization of cDNA libraries prepared from mRNA isolated from mature pollen, anthers or whole buds at different developmental stages. Here, the strategy is to clone the most abundant transcripts specifically expressed in the male gametophyte. Alternatively, pollen allergenic proteins such as LolpI in ryegrass (Griffith *et al.* 1991) have been cloned from cDNA expression libraries and have been used to elicit the formation of a specific class of antibody (Ab) IgE in susceptible humans. The differential display approach coupled with RT-PCR has been particularly successful in identifying a number of SI genes, although, the pollen-borne component of SI has yet to be identified.

Transcripts appearing during male gametophyte development can be broadly classified into two groups for convenience (Mascarenhas, 1990). The first set (early genes) is present during microspore development (after tetrad release) reaching a maximal level at the haploid

mitosis and then decreasing until pollen maturity. A few genes which are expressed specifically at the early stages of pollen development are expressed in both developing microspores and in the tapetum (Roberts *et al.*, 1993; Shen and Hsu, 1992; Theerakulpisit *et al.*, 1991), the only exception being the *NTM19* gene, recently cloned in tobacco (Oldenhof *et al.*, 1996) which is specifically expressed in unicellular microspores. The second set (late genes) appears with the primary mitosis and reaches a maximal level at maturity. More than 40 late pollen-specific genes have been isolated and sequenced. This has led to the identification of sequences similar to those of known genes and has provided important clues as to their function (Table 1). For instance, the *NeIF-4A8* gene in tobacco (Brander and Kuhlmeier, 1995) which is expressed in bicellular and tricellular pollen encodes a translation initiation factor that may play a role in mediating translational control during post-mitotic pollen development and perhaps during pollen germination and pollen tube growth.

Pollen-specific genes may be conserved at the DNA level across diverse taxa. For example, the *P2* gene which encodes a putative polygalacturonase in *Oenothera* (Brown and Crouch, 1990) hybridized to transcripts of similar size in both monocot and dicot families. Other genes may be restricted to a single family of plants. The *Bcp1* gene from *B. rapa* L., Syn. *B. campestris* L., (Theerakulpisit *et al.*, 1991) was found to encode transcripts that were only present in members of the Brassicaceae (Syn. Cruciferae) family. Another late pollen-expressed gene encoding a receptor-like kinase (*PRK1*) was restricted to members of the Solanaceae family (Mu *et al.*, 1994). In both cases it was hypothesized that the restricted distribution of the gene was related to the specificity of pollen-stigma interactions.

Direct evidence for the presence of specific transcripts within the vegetative cytoplasm has been provided by RNA *in situ* hybridization for the genes listed in Table 1. To date, no generative cell-specific, nor sperm cell-specific sequence has been cloned. It is possible to speculate that the generative and sperm cells that are highly reduced in size, may only be transcriptionally active for short periods of time i.e, just prior to the second pollen mitosis and during recognition and fusion events leading up to fertilization. Under these circumstances,

Table 1. Summary of Cloned Pollen-Expressed Genes

Species	Gene	Expression Profile/Detection	Putative Function /Location	Reference
<i>Arabidopsis thaliana</i>	A6	(Tet, Uni)Gus, RNase	Callase /Tapetum	(Hird, 1993)
<i>Arabidopsis thaliana</i>	A9	(Tet, Uni)Gus, RNase	/Tapetum	(Paul, 1992)
<i>Brassica campestris</i>	Bcp1	(Tet, Uni)IS	/Tapetum	(Theerakulpisut, 1991)
<i>Brassica napus</i>	BA 42	(Tet, Uni)IS	/Anther	(Shen, 1992)
<i>Brassica napus</i>	BA 112, 58	(Uni)IS	/Tapetum	
<i>Nicotiana tabacum</i>	TA 13,26,29,32,36	(Tet, Uni)	/Tapetum	(Kulturnow, 1990)
<i>Nicotiana tabacum</i>	TA 20, 56	(Tet, Uni, Bi, Tri)IS	/Anther	
<i>Sinapis alba</i>	SaTap35,44	(Tet, Uni)IS	/Tapetum	(Staiger, 1993)
<i>Zea mays</i>	MF 14	(Tet, Uni)	/Tapetum	(Wright, 1993)
<i>Zea mays</i>	18	(Tet, Uni)	/Anther	
<i>Nicotiana tabacum</i>	NTM19	(Uni) IS	/ Mspore	(Oldenhof, 1996)
<i>Brassica napus</i>	Olmb1,3,6	(Uni, Bi)IS	Oleocoin/ Tapetum,tryphine	(Ross, 1996)
<i>Arabidopsis thaliana</i>	Tua1	(Bi, Tri)Gus	Tubulin /Pollen*	(Carpenter, 1992)
<i>Arabidopsis thaliana</i>	Pfn4	(Mat+Germ)Gus	Profilin /Pollen	(Christensen, 1996)
<i>Brassica campestris</i>	Bcp1	(Bi, Tri)Gus, IS	/Pollen *	(Theerakulpisut, 1991)
<i>Brassica napus</i>	BP4	(Bi, Tri)	/Pollen	(Albani, 1990)
<i>Brassica napus</i>	19	(Bi, Tri, Germ)	/Pollen*	(Albani, 1991)
<i>Brassica napus</i>	BP 10	(Bi, Tri, Germ)Gus	/Pollen	(Albani, 1992)
<i>Brassica napus</i>	BA54	(Bi, Tri)	/Pollen	(Shen, 1992)
<i>Brassica napus</i>	73	(Bi, Tri)	/Anther	
<i>Brassica napus</i>	Sta 44-4	(Bi, Tri)IS	/Pollen	(Robert, 1993)
<i>Brassica napus</i>	Bnm1	(Bi, Tri)Gus, IS	/Pollen	(Treacy, 1997)
<i>Brassica oleracea</i>	Pcp1	(Tri) IS	/Pollen	(Stanchev, 1996)
<i>Helianthus annuus</i>	SF1,2,18,19	(Bi, Tri)IS	/Anther	(Evrard, 1991)
<i>Helianthus annuus</i>	SF3	(Bi, Tri)	Zinc finger/Pollen	(Evrard, 1992)
<i>Helianthus annuus</i>	SF17	(Mature)	Leu-Rich/Anther	(Reddy, 1995)
<i>Lycopersicon esculentum</i>	LAT52,56,59	(Bi, Mat, Germ)IS, Gus	PL	(Ursin, 1989)
<i>Nicotiana tabacum</i>	TP10	(Bi, Mat)	PL	(Rogers, 1992)

<i>Nicotiana tabacum</i>	Tac25	(Mature)	Actin	/Pollen	(Thangavelu, 1993)
<i>Nicotiana tabacum</i>	NTPg303	(Bi, Mat, Germ)IS	AOO	/Pollen	(Wetering, 1995)
<i>Nicotiana tabacum</i>	NeIF-4A8	(Bi, Mat, Germ)Gus	Helicase	/Pollen	(Brander, 1995)
<i>Oenothera organensis</i>	P2	(Bi, Mat, Germ)Ab	PG	/Pollen	(Brown, 1990)
<i>Oriza sativa</i>	PS1	(Mature)Gus	Lat52	/Pollen	(Zou, 1994)
<i>Petunia inflata</i>	PRK1	(Bi, Mat, Germ)Gus	Kinase	/Pollen	(Mu, 1994)
<i>Solanum berthaultii</i>	SB401	(Mat, Germ)Ab	GARP	/Pollen	(Liu, 1997)
<i>Zea mays</i>	Zmc13	(Bi, Tri, Germ)IS	/Pollen		(Hanson, 1989)
<i>Zea mays</i>	3C12	(Bi, Tri)IS, Gus	PG	/Pollen	(Rogers, 1991)
<i>Zea mays</i>	W2247	(Bi, Tri)	/Pollen *		(Allen, 1993)
<i>Zea mays</i>	Pex1	(Bi, Tri, Germ) Ab	Extensin/Pollen		(Rubinstein, 1995)
<i>Zea mays</i>	CDPK	(Tri, Germ)Ab	Kinase	/Pollen	(Estruch, 1994)

Asterisks signify expression is not pollen-specific. AAO (ascorbate oxidase), CHS (chalcone synthase), PE (pectin esterase) PL (pectate lyase), PG (polygalacturonase). Mat (mature pollen), IS (RNA In Situ hybridization), Gus (Promoter-Gus characterization), Ab (Antibody produced), GARP (Glutamic acid-rich protein). Tet, tetrad; Uni unicellular microspores; Bi, bicellular pollen; Tri, tricellular pollen; Germ, germinated pollen; blank space, no putative function

the transcripts may be under-represented and therefore cloning is very difficult. Indirect evidence, however, by Zhang *et al.* (1993) demonstrated that intact sperm cells isolated from *Zea mays* pollen synthesize RNA and proteins. Recently, Blomstedt *et al.* (1996) demonstrated by metabolic labelling with [³⁵S]-methionine that the generative cells of *Lilium longiflorum* possess translatable mRNA and functional protein synthesis machinery.

1.14 Functional Analysis of Cloned Pollen-Specific Genes

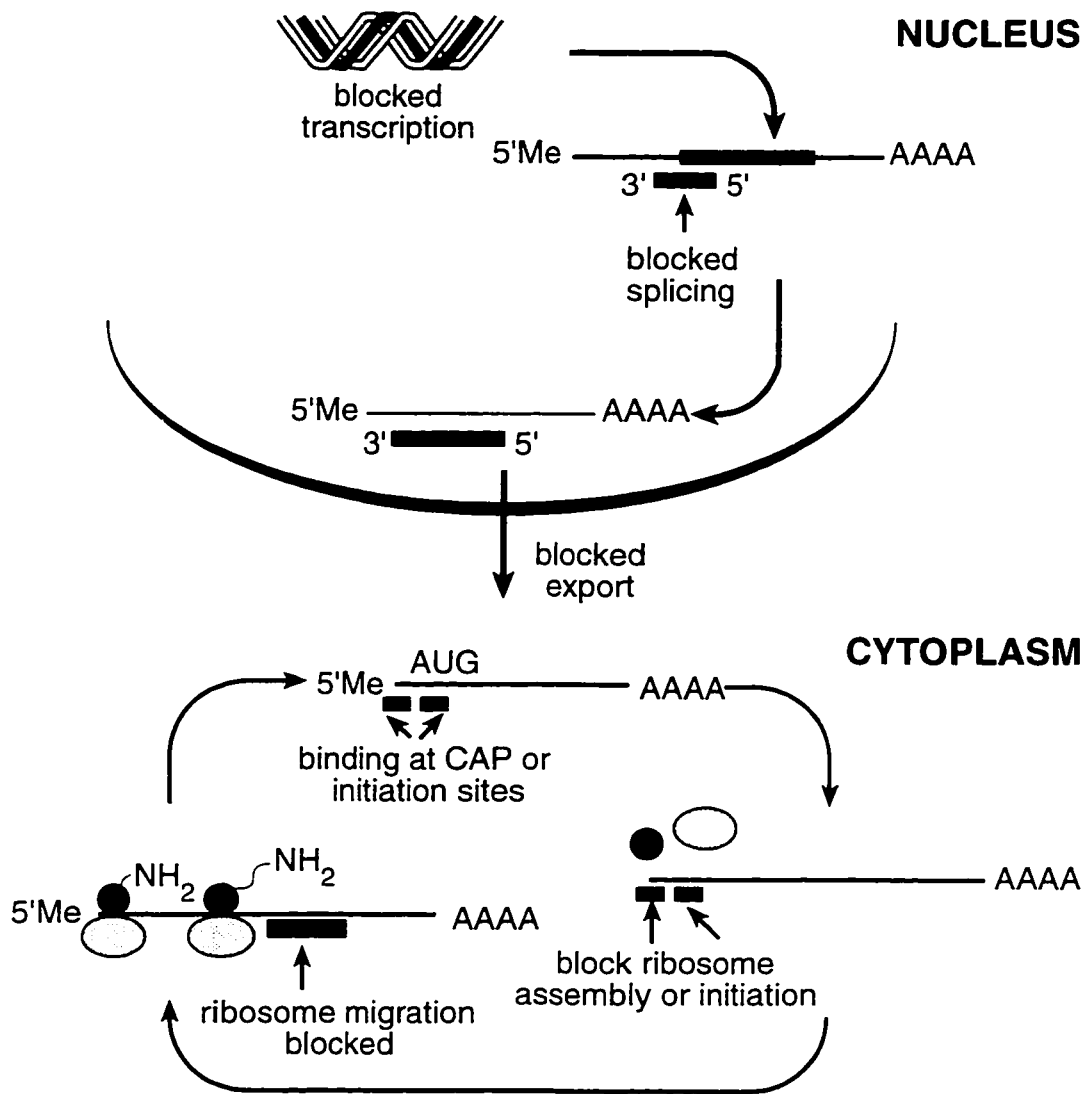
Little is known about the function of the proteins expressed by pollen-specific genes. One method for inferring function is to create mutations by introducing antisense gene constructs into transgenic plants. If a pollen-specific gene plays a central role in development and/or germination, the pollen of these transgenic plants might exhibit an altered phenotype resulting from the inhibition of gene expression.

Antisense RNAs were initially recognised in bacteria as naturally occurring mechanisms for regulation of gene expression (Simons *et al.*, 1983) which subsequently led to the design of artificial antisense control strategies (Green *et al.*, 1986). The simplest antisense model follows the synthesis *in vivo* of complementary RNA from a construct introduced into the plant via *Agrobacterium*-mediated transformations. The rationale for antisense action occurs when this complementary RNA hybridizes to its target RNA and prevents expression. The exact mechanism of repressed gene expression caused by antisense mRNA remains poorly understood (Bourque, 1995). However, from the many reports on this topic, several probable mechanisms operating at several levels *in vivo* have been suggested (Fig.11). At the nuclear level, mRNA processing may be prevented by the sense-antisense duplex. These double-stranded RNA hybrids would also be targets for degradation by RNAses similar to RNase III in *E. coli*. Also, these duplexes may be unable to be exported to the cytoplasm for translation.

At the cytoplasmic level, inhibition of translation may result from transient sense-antisense expression which ultimately leads to the high degree of variability in inhibition levels

Fig.11. Diagram Illustrating Proposed Mechanisms of Antisense Inhibition.

Adapted from Murray and Crockett (1992).



observed in a population transformed with an antisense gene (Bourque, 1995). Variation associated with the site of insertion but not copy number, coined as position effect, is a common theme associated with this approach. In addition, some differences in gene expression within a transformed population have been correlated with differential methylation (Bourque, 1995). Therefore, screening of transgenic plants for down-regulation is essential for a successful study.

1.14.1 *Antisense Phenotypes in Pollen*

The first study to employ an antisense construct to down-regulate a gene specifically expressed in mature pollen involved the *LAT 52* antisense construct introduced into tomato (McCormick *et al.*, 1991). From this experiment, it was demonstrated that the *LAT 52* protein plays a functional role during the pollen germination stage (McCormick *et al.* 1991). More precisely, in a *LAT 52* antisense plant 50% of the pollen tubes extend in a corkscrew manner during *in vivo* germination and 50% do not appear to hydrate normally during *in vitro* germination. In transformants with a single locus insertion(s), only 50% of the pollen grains would be expected to express the antisense copy following Mendelian inheritance. The phenotypes suggest that the *LAT 52* protein may be involved in establishing exine adhesion to facilitate water transfer from the stigma cell. Alternatively, *LAT 52* may be involved in establishing a proper osmoticum required for water transfer to occur. Although an antibody was generated against the *LAT52* protein, recognition of the native protein *in situ* was unsuccessful. Further work will be needed to define the biological role. Furthermore, from these experiments, Muschietti *et al.* (1994) noted no correlation between transgene copy number, *LAT52* downregulation and phenotype. Muschietti *et al.* (1994) explained these observations as being related to the position of insertion of the transgene in the genome

The second functional analysis of an anther-specific gene involves the antisense study of *Bcp1* in *Arabidopsis* (Xu *et al.*, 1995). Although *Bcp1* is a single copy gene in

Arabidopsis, it remains at this time the only gene to be expressed specifically in both diploid tapetal cells and haploid mature pollen. When tapetal expression of this gene, driven by the *Bcp1* promoter, was perturbed, all unicellular microspores died and shrivelled. The same phenotype is observed in 50% of the pollen grains at the late bicellular stage when antisense *Bcp1* is driven by the *LAT52* pollen-specific promoter (Xu *et al.*, 1995). Although *Bcp1* is required for pollen viability, the physiological basis of action of this anther-expressed protein remains to be elucidated. In contrast to *LAT52*, Xu *et al.* (1995) noted a correlation between downregulation of *Bcp1* and transgene copy number. At least two copies of the transgene were required to induce complete male sterility, otherwise, a leaky phenotype was observed.

The only other functional approach involves the recent study by Lee *et al.* (1996). *PRK1*, a pollen-specific receptor-like kinase in *Petunia*, when down-regulated, produced aborted pollen grains. More precisely, 50% of the pollen grains at the bicellular stage were collapsed. The exine was essentially normal, however, its cytoplasm contained only starch-like granules. The highest level of RNA down-regulation in *PRK1* antisense plants showing a mutant phenotype was 50% of wild type. Although Xu *et al.* (1995) were successful in obtaining complete shut-down of the *Bcp1* transcript, Lee *et al.* (1996) revealed that certain transgene inserts co-segregated with the pollen abortion phenotype.

1.14.2 Immunocytochemistry

Antisense methodology has provided gene-specific mutations, however, these lesions are often difficult to interpret. Knowledge of the cellular location of pollen-expressed proteins should help suggest their function, particularly for those genes that have no sequence similarity to known proteins. Many of the pollen-expressed genes that have been characterized encode proteins with N-terminal hydrophobic sequences, some of which follow the consensus of a potential signal peptide. If a "late" pollen-specific protein was secreted

extracellularly during germination and pollen tube growth, antibodies would be expected to label the length of the pollen tube. This could support a role for this protein in structure or in pollen-pistil interactions. If antibodies label the growing tip of the pollen tube, this protein may be associated with cytoskeleton activities, or motor activities, involved in cytoplasmic streaming. Antibodies to the Glutamic Acid Rich Protein (GARP) might be expected to label particular regions of the cytoskeleton at the growing tip of the pollen tube if indeed this protein plays a role as a microtubule associated protein as postulated (Liu *et al.*, 1997).

1.15 Thesis Objective

Emphasis is often placed on the degree of specificity determined for many pollen-expressed genes, in particular because the expression of genes with absolute pollen-specificity strongly implies an exclusive role for the encoded gene product in male gametophyte development (Scott and Stead, 1994).

Identifying the molecular components that control the spatial and temporal patterns accompanying pollen development is an important step in understanding sexual reproduction in plants. Such studies are important because they indicate critical stages in pollen development when new genes become activated (Mascarenhas, 1992). Developing pollen provides a unique system with which to study cell-specific gene regulation and control of cellular differentiation. Of particular interest are those genes and mechanisms responsible for the events accompanying the asymmetric mitotic division of the haploid microspore. An important step in pollen development appears to occur at this time and this step is conserved among all known flowering plants (Twell, 1991). Furthermore, the pattern of proteins synthesized during pollen germination *in vivo* is almost identical with that observed from *in vitro* translation of mRNA isolated from mature pollen (Mascarenhas *et al.*, 1984), and translation is an absolute requirement for pollen germination and/or early pollen tube growth (Mascarenhas, 1975). Taken together, these studies show that large numbers of genes, in

particular "late" pollen-specific genes, are actively transcribed from the haploid genome of the male gametophyte and many of their corresponding transcripts are stored and translated during pollen germination and pollen tube growth (Scott and Stead, 1994). These patterns of mRNA and protein accumulation are derived from two dimensional gel analysis of total *in vitro* translation products (Bedinger and Edgerton, 1990; Schrauwen *et al.*, 1990) and from *in vivo* labelling of all newly synthesized proteins (Mandaron *et al.*, 1990) respectively. It is clear that more work is needed in the area of mRNA and protein accumulation patterns derived exclusively from the haploid genome. In addition to including a functional approach to assigning a biological role to their gene products.

My goal is to provide a detailed characterization of the *Bnm1* (*Brassica napus* microspore 1) gene cloned in our lab, and determine the biological function of the *Bnm1* protein product, BNMI. To date, the majority of pollen-expressed genes have been crudely characterized and functions have only been suggested for a few pollen-specific genes largely based upon relative sequence similarity at the DNA or amino acid level to other genes of known function found in databanks. No pollen-specific gene has been assigned a definite role in the development of the male gametophyte which governs the next generation in sexual plant reproduction. Since *Bnm1* has no significant sequence similarity with other identified sequences in the databank it cannot even be assigned a putative function. However, as demonstrated by Southern blot analysis, *Bnm1* appears to be family-specific and perhaps even tribe-specific. This observation would support the hypothesis that the *Bnm1* gene product has a very precise function and not an important role in the development of the pollen grain.

It is anticipated that these findings will lead to a better understanding of the genetic controls that govern the differentiation of pollen in gametogenesis and the relationship between the developmental programs involved in the induction of microspore embryogenesis and pollen development. This research will provide the frame work and technologies for manipulating pollen development which in turn may provide possible avenues for agricultural

benefits not only in Canola but in other cash crops as well, through the use of the promoter which will serve as a "magic bullet" to target the expression of novel genes.

The characterization and functional analysis will determine how far the lessons can be applied to other systems and therefore, important for a better understanding of fundamental biological processes such as differentiation and cell-cell communication.

CHAPTER TWO

MATERIALS AND METHODS

2.1 *Plant Material and Growth Conditions*

Leaves from *Brassica oleracea* subsp. *oleracea* (3085), *B. oleracea* subsp. *alboglabra* and wild members of the C cytodeme: *B. incana* (8011), *B. cretica* (8006), *B. rupestris* (3025), *B. montana* (8096), and *B. rapa* were obtained from S. Warwick (Agriculture and Agri-Food Canada, Ottawa). Leaves from species of the tribe Brassiceae: *Eruca vesicaria* subsp. *sativa*, and from six other tribe representatives of the Brassicaceae family: Tribe Hesperideae: *Cheiranthus cheirii* L., tribe Matthioleae: *Matthiola longipetala* subsp. *bicornis*, tribe Arabideae: *Nasturdium officinale*, *Arabis caucasica*, tribe Lepideae: *Iberis amara*, tribe Sisymbrieae: *Cleome hasslerana*, tribe Alysseae: *Berteroa incana* were collected from plants grown from seed (purchased from "The Flowery Branch" GA) in growth cabinets at 20°C/15°C (day/night) with a 16 hr photoperiod. *Raphanus sativus* L. (tribe Brassiceae) was grown from seed (Dominion Seed) as outlined above. DNAs from black spruce, barley, maize and *Arabidopsis thaliana* were a gift from Dr. Robert Rutledge (Natural Resources Canada, Chalk River), Adele MacKay and Dr. Linda Harris (Agriculture and Agri-Food, Canada, Ottawa) and Dr. Bertrand Lemieux (York University, Toronto) respectively. Sunflower and tobacco tissues were obtained from the ornamental gardens (Agriculture and Agri-Food Canada, Ottawa).

Brassica napus L. cv Topas plants were preconditioned for microspore embryogenesis (Keller *et al.* 1987) in a growth cabinet at 10°C/5°C (day/night) with a 16 hr photoperiod (400 $\mu\text{E}/\text{m}^2/\text{s}$) provided by VHO cool white fluorescent lamps (165W, Sylvania) and incandescent bulbs (40 W, Duro-test). Microspores and pollen were isolated by disrupting flower buds with a mortar in a 10% sucrose solution. The suspension was filtered through a 44 μm nylon

mesh to remove sporophytic tissue, and cells were washed twice in 10% sucrose. Once harvested, microspores were immediately used for total RNA extraction.

For microspore culture, flower buds, 3.0 to 5.0 mm in length were collected by Nancy Long (Agriculture and Agri-Food Canada, Ottawa) and surface sterilized for 15 min in 7% (w/v) calcium hypochlorite. Buds were then rinsed 3 times, for 10 min each in sterile water. Sterile buds were then transferred to a 50 ml beaker containing 5ml B5 medium (Gamborg *et al.*, 1968) without hormones and 13% (w/v) sucrose (B5-13 medium). Microspores were isolated as outlined above but washed in B5-13 medium instead of 10% sucrose. After centrifugation the microspores were resuspended in 1/2 NLN-13 (1/2 NLN salts plus 13% sucrose; Keller *et al.*, 1987) at a density of 50, 000 to 100, 000 microspores/ml. Ten ml of microspore suspension was added per Petri dish (100 x 15 mm).

Transgenic *Brassica napus* L. cv Westar plants harboring the *Bnm1* promoter fused to the GUS reporter gene were preconditioned as above as well as grown in a cabinet at 20°C/15°C (day/night). Microspores and pollen were isolated and harvested as outlined above and immediately used for GUS histochemical and fluorogenic analysis. Each experiment was carried out a minimum of three times for three different plants.

2.2 Analysis of Developmental Stages of Pollen

The pollen developmental stages were determined by staining with 25 µg/ml DAPI (4',6-diamidino-2-phenylindole) in ddH₂O (Eady *et al.* 1994) and fluorescence microscopy. Petal length was used as an indicator of pollen development for unicellular, bicellular, and tricellular pollen grains. 1.5-2.5 mm for unicellular, 4.0-5.5 mm for bicellular and > 6.5 mm for tricellular pollen. Bud length of less than 2 mm proved to be a reliable marker for the tetrad stage.

2.3 Isolation of Plant genomic DNA

Plant genomic DNA was isolated from leaves following a protocol from Hattori *et al.* (1987). Approximately 3-5 g of leaf tissue were frozen and ground to powder in liquid nitrogen with mortar and pestle. Five to ten ml of extraction buffer (0.1 M Tris-HCl pH 8, 0.05 M EDTA and 1% SDS) were added and homogenized to a paste. Further liquid nitrogen was added and the extract was ground again to powder. More extraction buffer (10-15 ml) was added followed by a phenol/chloroform/iso-amyl alcohol extraction (10 ml chloroform:iso-amyl alcohol (24:1) and 10 ml phenol to 20 ml green slurry). Following centrifugation at 6000 rpm for 6 minutes at 4°C, the aqueous phase (top supernatant) was removed and slurry was re-extracted. Both aqueous phases were combined and precipitated with 0.5 volumes of 7.5 M ammonium acetate (NH₄Ac) pH 7.0 (or 0.1 volumes sodium acetate (NaOAc) pH 5.5,) and 2.5 volumes ethanol. The precipitate was resuspended in 9.5 ml TE (10 mM Tris-HCl, 1mM EDTA pH 7.6) to which 9.5 g of CsCl and 0.25 ml of 10 mg/ml ethidium bromide were added. The samples were then transferred to a Quick seal tube (Beckman Instruments Inc., Toronto, ON) and centrifuged 18-20 hrs at 60, 000 rpm in a Ti 70 rotor at 4°C. The DNA band was collected under UV light and extracted with 10 mM Tris-HCl (pH 7.5) saturated butanol or isopropanol to remove ethidium bromide. Two volumes of ddH₂O and two volumes of 95% ethanol were added to precipitate the DNA. The DNA was collected immediately or after overnight precipitation at -20°C. The precipitated DNA was then washed in 70% ethanol, air dried briefly, and resuspended in TE for use or storage at 4°C.

2.4 DNA Electrophoresis

DNA restriction fragments were resuspended in 1x loading buffer (6x = 15% (w/v) Ficoll, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 0.2 M EDTA) and

separated by electrophoresis through agarose gels in TAE buffer. The concentration of the gels varied from 0.7- 1.0 % depending on the size of the fragment. DNA molecular weight markers were lambda *Hind* III restriction fragments. DNA was generally visualized by staining the gels in a 1 µg/ml solution of ethidium bromide. Gels were destained in water for 10 min. Alternatively, 1 µl of ethidium bromide (100 µg/ml) was added to the sample to be loaded on the gel.

2.5 Isolation of Plasmid DNA

Small scale plasmid preparations (minipreps) were performed using the Magic Miniprep or Wizard Miniprep kits from Promega (Madison, WI).

2.6 Isolation of DNA fragments

DNA restriction fragments were isolated from TAE (0.04M Tris-acetate, 0.001M EDTA, pH 8.0) agarose gels using the GENECLEAN (Bio 101 Inc., La Jolla, CA) or ELU-QUICK (Mandel Scientific Company Ltd., Guelph, ON) kits according to the recommendations of the manufacturers.

2.7 Competent Bacterial Cells and Transformation

New competent cells were prepared using the calcium chloride method as described by Hanahan (1985). Alternatively, Library Efficiency DH5α Competent Cells (Gibco BRL, Burlington) were used. Bacterial transformation was carried out following the recommendations of the manufacturer (Gibco BRL, Burlington) for both types of preparations.

2.8 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 0.5-1.0 g of tissue was ground in liquid nitrogen using a pre-chilled mortar and pestle. The ground powder was transferred to a 50 ml Falcon tube and resuspended in at least 3 volumes of ice cold extraction buffer (4M guanidine isothiocyanate, 25 mM sodium citrate, 1 mM EDTA, pH 8, with 100 μ l 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% Sarkosyl was added to the homogenate at 1 ml/ 10 ml of sample volume and the mixture was homogenized for another minute. The homogenized sample was centrifuged at 7000 rpm for 15 min at 4°C. The supernatant was loaded onto 2 ml of a CsCl cushion (5.7 M CsCl dissolved in 0.1 M EDTA, pH 7.0) in a polyallomer tube and centrifuged at 30, 000 rpm for 14-24 hrs at 18°C in a SW 40 Ti rotor. Following centrifugation the RNA pellet was resuspended in 500 μ l guanidine buffer (7.5 M guanidine hydrochloride buffered with 0.025 volume of 1 M Sodium citrate pH 7 and 5 mM dithiothreitol). Heating briefly at 65°C helped dissolve the pellet. The RNA was precipitated from the guanidine solution by adding 0.025 volumes of 1 M acetic acid and 0.5 volumes of ethanol for a minimum of 4 hrs at -20°C. The precipitate was centrifuged for 15 min at 4°C, rinsed in 70% ethanol, air dried and resuspended in 200 μ l ddH₂O. The resuspended RNA was precipitated overnight at -20°C with 0.1 volumes 3M NaOAc (pH 5.0) and 2.5 volumes ethanol for later use.

2.9 RNA Electrophoresis

RNA was separated by electrophoresis through 1% agarose gels containing formaldehyde (Lehrach *et al.*, 1977) and 1x MOPS buffer pH 7.0 (20mM 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 approximately 1 hr in numerous changes of ddH₂O to remove the formaldehyde.

2.10 Southern and Northern Blot Analysis

Plant genomic DNA was isolated from leaves following a protocol from Hattori *et al.* (1987). The DNA was digested at 37°C overnight with 10 units of *EcoRI* per µg of DNA, using the appropriate buffer supplied by the manufacturer (BRL). Southern blot analysis was carried out on DNA samples electrophoresed through 0.8% agarose gels in TAE buffer. The gels were treated with two 30 min washes in 2 volumes of 0.5 M NaOH, 1.5M NaCl and neutralized with two 30 min washes in 2 volumes 0.5M Tris, 1.5M NaCl, pH 7.6 (Rutledge *et al.* 1991). The DNA was transferred overnight onto Biotrans membranes (ICN) using 20X SSC as blotting buffer (3M sodium chloride, 0.3M sodium citrate, pH 7.0), and fixed to the membranes by U.V.-irradiation (2min U.V. Oven. Hoefer). The filters were prehybridized for 4 hrs at 65°C in hybridization buffer (100 µg/ml salmon sperm, 5x Denharts, 5X SSC (0.75 M sodium chloride, 0.075 M sodium citrate and 0.1% SDS). Following prehybridization, buffer was removed and new hybridization buffer was added including the probe labelled to 10⁶ cpm/ml DNA with [α -³²P]- dCTP using random priming (BRL). Hybridizations were carried out overnight. The following day, the filters were washed twice in 2X SSC, 0.1% SDS (30 min each at room temperature), then twice in 0.1X SSC, 0.1% SDS (30 min each at room temperature). As indicated in the Results section, the temperature of the final washes was 42°C (low) or 65°C (high), depending on the degree of stringency desired.

Northern blot analysis was carried out on RNA samples electrophoresed through 1% agarose gels containing formaldehyde (Lehrach *et al.* 1977) followed by transfer to Biotrans membrane (ICN). The filter was hybridized overnight at 42°C in hybridization buffer (100 µg/ml salmon sperm DNA, 50% deionized formamide, 5x SSPE (0.9 M NaCl, 50 mM

NaPO₄, 5 mM EDTA) , 5x Denhart's solution (0.1% Ficoll (type 400, Pharmacia) 0.1% polyvinylpyrrolidone and 0.1% bovine serum albumin) and 0.2% SDS (sodium dodecyl sulphate) according to the manufacturers protocol. The final washes were carried out at 50°C in 0.1X SSC, 0.1% SDS for 30 min. Northern blot experiments were reproduced to confirm results obtained after hybridizations.

2.11 Probe Synthesis

Restriction fragments isolated from agarose gels were radiolabelled with [α -³²P] dCTP using the Random Primers DNA Labelling kit (Gibco BRL, Burlington, ON). Probes were synthesized following the recommendations of the manufacturer. Unincorporated nucleotides were removed using Sephadex G-50 Nick Columns (Pharmacia, Montreal, PQ). Radioactivity of probes was measured using a scintillation counter. One million cpm of probe per ml of hybridization buffer was denatured by heating at 95°C for 5 min and quick cooled on ice for hybridizations.

2.12 In Situ Hybridization

mRNA *in situ* hybridization was performed by K. Boutilier on 10 mm sections of *B. napus* cv. Topas flower buds essentially as described in Cox and Goldberg (Cox and Goldberg, 1988). [α -³⁵S]dCTP-labeled sense and antisense RNA probes were transcribed from the *pBnm1* cDNA , using SP6 and T7 RNA polymerase, respectively. Full-length sense and antisense RNA probes were hydrolysed in alkali to a length of 300 nt and hybridized to sections at a concentration of 100 ng/ml. Hybridization was carried out at 42°C in 50% formamide. The final wash conditions were 0.1X SSPE at 55°C. Each experiment was reproduced to confirm spatial and temporal expression profiles. Sections were photographed with a Zeiss Axiophot microscope using dark-field optics.

2.13 cDNA Library Construction and Screening

The construction of the *Brassica napus* cDNA library has been previously described (Boutilier *et al.* 1994). In brief, *B. napus* cv Topas microspores ranging from late unicellular to early bicellular stages were cultured for four days at 32.5°C to induce embryogenesis (Keller *et al.* 1987). Total RNA was isolated from the microspores using a guanidinium isothiocyanate procedure as described by Ouellet *et al.* (1992) from which poly (A)+ RNA was isolated by oligo (dT)-cellulose chromatography as described in Maniatis *et al.* (1982). Double-stranded cDNA was synthesized using the Riboclone cDNA Synthesis System (Promega Corp.; Madison, WI) and ligated into LambdaGEM-4 (Promega Corp.).

The cDNA library was screened using the corresponding radiolabelled cDNA to isolate highly expressed microspore-specific mRNAs. The probe was produced as follows: Poly (A)+ RNA was isolated from heat denatured total mRNA from microspore induced to undergo embryogenesis (miRNA) using Hybond poly (T)+ paper. Approximately 25-50 ng of poly (A)+ mRNA were used for the synthesis of labelled cDNA using an oligo dT primer. After this initial screen, the probe was stripped and the filter was hybridized to a cDNA probe synthesized using random primers to identify and exclude potential ribosomal RNA contaminants.

2.14 Genomic Library Construction and Screening

A *Brassica napus* genomic library was constructed and screened by Eric Barbour and Chris Baszczynski at Pioneer Hi-Bred Production Ltd (Mississauga, Ontario) as described by Nantel *et al.* (1991). Briefly, *B. napus* cv Westar genomic DNA was partially digested with *Sau* 3A (Maniatis *et al.* 1982) and the DNA fragments (average insert size about 8 kilobases) were ligated into the partially gap-filled *Sal* I site of the plasmid pTZ18R (Pharmacia). The

library was collected in 42 fractions, DNA isolated from each fraction was digested with *EcoRI* and the *EcoRI* digests were examined by Southern blot analysis using the *Bnm1* cDNA probe. Approximately 10,000 colonies from each positively hybridizing fraction were screened on Hybond-N filters (Amersham Corp.; Arlington Heights, IL) using a [α - 32 P]- dCTP cDNA probe labelled by the random primers method.

2.14.1 Amplification, Subcloning and Sequencing of Genomic Fragments

The 5' region of each positive clone, which included the region upstream of the translation initiation codon plus a small segment of the 5' coding region was PCR amplified (Perkin Elmer Cetus) by Eric Barbour and Chris Baszczynski at Pioneer Hi-Bred Production Ltd, (Mississauga, Ontario) using modified versions of the universal or reverse sequencing primers in combination with antisense primers homologous to regions at the 5' end of the cDNA. Primers were designed to anneal at 65°C in 1X PCR buffer containing 2.5 mM MgCl₂. PCR fragments were digested with *EcoRI* and subcloned into the *EcoRI-HincII* site of pTZ18R (Pharmacia). Ligation reactions were transformed into the *E. coli* strain DH5 α MCR-. Subcloned double-stranded PCR fragments were sequenced using a Sequenase kit (USB) based on the dideoxy-mediated chain termination protocol (Sanger and Coulson, 1975).

2.15 DNA Sequencing

Sequencing was carried out using the dideoxy chain termination method of Sanger *et al.* (1977) using the T7 Sequencing kit (Pharmacia Biotech). Internal synthetic primers were synthesized by J. Hattori (Agriculture and Agri-Food Canada, Ottawa) using the Gene Assembler Plus machine (Pharmacia Biotech). Sequencing reactions were separated on 6%

polyacrylamide denaturing gels using 0.5 to 1X TBE (1X TBE: 89 mM Trizma base, 2.5 mM EDTA, 89 mM boric acid, pH 8.0) as the running buffer. Gels were dried under vacuum (80°C, 45 min) on Whatman 3MM filter paper and exposed to Kodak X-Omatic AR film at room temperature.

DNA sequence information was analysed using IBI Pustell sequence analysis software (New Haven, CT), and Motif Search (DNASIS, ver. 2.1). Computer searches performed to compare nucleotide and amino acid similarities were carried out with sequences contained in GenBank and EMBL databases.

2.16 *GUS Reporter -Gene Vector Construction*

A *SalI* site was created at the 5' end of the promoter with a mutagenic primer for ease of cloning into both pPHI1741 and pBlueScriptIIISK+ (Stratagene). pPHI1741 differs from pBI101.1 (Jefferson, 1987) by having the CaMV 35S 5' and 3' regulatory sequences driving the *nptII* selectable marker gene rather than the nopaline synthase sequences. A second mutagenic PCR primer was designed to create an *NcoI* site at the ATG start codon. Amplification conditions were identical to those outlined previously (section 2.14.1). Once the amplified promoter fragment was verified by sequencing, it was digested with *NcoI* and *SalI*, ligated to an *NcoI-EcoRI* fragment containing a GUS reporter gene and the 3' untranslated region (terminator) from a potato proteinase inhibitor (PINII) gene. This construct was cloned into the *SalI-EcoRI* sites of both pBlueScriptIIISK+ and DP1741 to produce the vectors pPHI5476 and pPHI5477 respectively, by Eric Barbour and Chris Baszczynski at Pioneer Hi-Bred Production Ltd (Mississauga, Ontario). The binary vector, pPHI5477 was transformed into *B. napus* c.v. Westar via *Agrobacterium*-mediated transformation of cotyledonary petioles as described by Arnoldo *et al.* (1992). The transformations were carried out by Pioneer Hi-Bred Production Ltd (Mississauga, Ontario). Transgenic plants were evaluated for expression of GUS in a variety of *B. napus* tissues.

2.17 Antisense Vector Construction

A 579 bp *SalI*-*DraI* fragment from the *pBnm1* cDNA clone containing the full open reading frame (ORF) was ligated into the *SalI*-*EcoRV* site of pGEM-5Z to place the *Bnm1* coding region into antisense orientation. An *NcoI*-*SalI* fragment was then generated from this clone and ligated into the *NcoI*-*SalI* site of the SGM-3 cloning vector (Sylvia McHugh, personal reference) which harboured the napin promoter and the nopaline synthase (NOS) terminator. To substitute the GUS reporter gene and PINII terminator of the binary vector pPHI5477, a *HindIII*-*EcoRI* fragment containing the cDNA in antisense orientation and NOS terminator was generated from the SGM-3 clone and ligated into the *HindIII*-*EcoRI* sites of the binary vector pPHI5477 to produce the final antisense vector. This binary vector was sent to M. Arnoldo at Pioneer Hi-Bred Production Ltd (Mississauga, Ontario) for transformation into *B. napus* c.v. Westar as outlined above.

2.18 β -Glucuronidase Assays

GUS histochemical and fluorogenic analyses were performed as described by Jefferson (1987).

2.18.1 GUS Histochemical Analysis

Fresh pollen was collected as above (section 2.1) and resuspended in 10 ml GUS fixative buffer (0.1 M NaPO₄ pH 7.0, 0.1 % formaldehyde, 0.1% triton x-100, 0.1% β -mercaptoethanol). The samples were centrifuged immediately and resuspended in 5ml fresh GUS fixative buffer. The samples were put under vacuum for 15 min to promote infiltration then centrifuged to remove fixative. Samples were resuspended in GUS wash buffer #1 (0.1M NaPO₄, pH 7.0, 0.1% β -mercaptoethanol), centrifuged and resuspended in GUS wash buffer

#2 (0.05M NaPO₄, pH 7.0) in order to eliminate β-mercaptoethanol. For histochemical assays X-Gluc (0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide) was used as the substrate, and 20% (v/v) methanol was added to the assay buffer (100 mM NaPO₄, pH 7.0, 10 mM EDTA, pH 7, 0.5 mM K₃ [Fe (CN) 6], 0.5 mM K₄ [Fe (CN) 6] , (X-gluc: Clontech Laboratories Inc., Palo Alto, Ca)) to eliminate background activity in pollen (Kosugi *et al.*, 1990). Incubations were carried out at 37°C overnight. For tetrad and unicellular microspores both intact (and fixed) along with fractionated (unfixed) tissues were assayed to ensure access to substrate. Eleven plants were screened for GUS staining from which three plants were selected on the basis of their strong staining. All histochemical and fluorogenic assays were performed in triplicate on three individual plants, except for flower organ samples (sepals, petals, anthers and pistils) which were pooled for all three plants.

2.18.2 Fluorogenic Analysis

Tissue samples were collected in liquid nitrogen and stored at -80°C. Samples were then placed on ice one at a time and ground until homogenous in 3 volumes of ice cold extraction buffer (50 mM NaPO₄, pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, pH 8.0, 0.1% sarkosyl and 0.1% Triton x-100) using a hand-held homogenizer (Omni International, Waterbury, CT). The cellular debris was removed by centrifugation at 8000 rpm (4°C, 10 min). The supernatant was transferred to a fresh chilled tube, frozen in liquid nitrogen and stored at -80°C until used.

Frozen tissue extracts to be assayed were thawed on ice and then vortexed. 50 μl of tissue extract were added to 500 μl of prewarmed assay buffer (extraction buffer containing 1mM 4-methyl umbelliferyl-β-D-glucuronide [MUG; Sigma, St-Louis, MO]) and incubated in a 37°C water bath. 100 μl aliquots were removed at various time points (from 5-120 min) and added to 1.9 ml of stop buffer (0.2 mM Na₂CO₃). Fluorescence was measured using a Hitachi F2000 fluorometer calibrated with known methylumbelliferone (MU) standards. The

excitation and emission wavelengths were set at 365 and 455 nm respectively. GUS activity is expressed in nmol MU produced per min per mg protein (nmol/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 (BSA) as a standard. These experiments were reproduced to confirm results obtained.

2.19 Overexpression of *Bnm1* in *Escherichia coli* and antibody production

A GST fusion protein expression system (Pharmacia) was used to produce antigens for antibody production. The entire translated portion of the cDNA (182 amino acids) was used. For the *GST-Bnm1* construct, the 576 bp *EcoRI-DraI* fragment from the cDNA *pBnm1* was ligated into the *EcoRI-SmaI* sites of pGEX-4T-3. The construct was transformed in the bacterial host strain JM101. The induction and purification of the fusion protein was performed according to the manufacturer's recommendations. The fusion protein was further purified by SDS-PAGE. The protein was eluted from excised gel fragments from SDS-PAGE gels and used to immunize 2 mice at Queen's University by K. Cole. Pre-immune serum showed no cross-reactivity to the GST fusion protein by Western blot prior to injection. Approximately 20 µg protein was used per immunization for two boosts. Blood serum (1:5000 dilution) was characterized directly by Western blot analysis for antibody specificity. Titer was low and a 1:500 dilution was carried out for subsequent experiments, except for immunolocalization experiments where a 1:100 dilution was used.

2.20 Protein extraction

Pollen from different developmental stages (Tetrad, Unicellular, Bicellular, Tricellular and Germinating pollen) from wild-type plants was collected, as outlined (section 2.1), and resuspended in protein extraction buffer (0.0625M Tris buffer pH 6.8; 2% SDS; 10%

glycerol; 0.1M DTT) and frozen in liquid nitrogen. Samples were then disrupted in a microfuge tube with a glass homogenizer until thawed. Samples were then boiled for 5 min, and centrifuged in a microfuge at 14 000 rpm for 5 min to remove cellular debris and aliquoted immediately in loading buffer and stored at -80°C for subsequent experiments. Protein concentration was determined using the Bio-Rad protein reagent using bovine serum albumin as a standard. Protein extracted from somatic tissues were extracted and stored as outlined, however, SDS was reduced to 0.5% in extraction buffer.

2.21 Western Blot Analysis

Western blot analysis was carried out by separation of protein through a 4% stacking and 12% separating SDS-PAGE denaturing gel (Laemmli, 1970) and blotted to PVDF membranes (Immobilon-P, Millipore) by electro-transfer (Bio-Rad). The membranes were blocked with 1% blocking reagent (Boehringer Mannheim), incubated with 1:500 dilutions of Anti-BNM1 antisera and 1:1000 dilutions of Anti-Hsp70 (StressGen) control monoclonal antibody. Detection of hybridization of primary antibodies was carried out using the POD chemiluminescent secondary antibody (Boehringer Mannheim). Film was developed following manufacturers recommendations (Kodak). Molecular weight rainbow markers (Helix) were applied in all gels. Experiments were reproduced on a minimum of three separately transformed plants to confirm results obtained.

2.22 Immunolocalization

Fresh wild-type pollen was germinated (section 2.24) in a petri dish (35x15) and then prepared following a protocol from Simmonds *et al.* (unpublished data). Germinated pollen grains were fixed in 6% paraformaldehyde; 10% NP-40; 20% DMSO; 1µg/ml leupeptin pH 8.0 for 45 min at room temperature. The cells were washed in B1 buffer (25 mM NaPO₄;

0.1M Mannitol; 10% DMSO (Dimethyl sulfoxide) and 0.5 mM PMSF (phenylmethylsulfonyl fluoride)) pH 8.0 and resuspended in 500 µl of digestion buffer (0.1M Mannitol; 25 mM NaPO₄; 5 mM EGTA (Ethylene glycol-*bis*(β-aminoethylether)-N,N,N',N'-tetraacetic acid); 1 mM MgSO₄; 0.5 mM PMSF; 1µg/ml leupeptin and 1% glucuronidase (Sigma)) pH 8.0 for 15 min. After washing in B1 buffer, cells were rinsed twice in PBS and then incubated with a 1:100 dilution of the primary Anti-BNM1 antibody for 60 mins. After three PBS (Phosphate buffered saline, pH 7.0) washes, cells were incubated with a 1:200 dilution of the secondary Anti-mouse FITC (Sigma) conjugate antibody for 30 mins in complete darkness. After three PBS washes, cells were viewed with a fluorescence microscope. Samples incubated with only the secondary antibody were included as controls.

2.23 Pollen Viability Assays

Viability of transgenic pollen compared with wild-type pollen was evaluated using the fluorescein diacetate (FDA) method developed by Heslop-Harrison (1970). This method is designed to show the viability of pollen cells based on integrity of cell membranes. Fresh pollen from dehisced anthers was collected by dipping the anther into a 10⁻⁶ M FDA solution in 0.5 M sucrose for 10 mins, in a 10 well drop culture plate. Stained pollen was observed directly with a fluorescence microscope. Over 100 pollen grains were observed for each experiment.

2.24 In vitro Pollen Germination

The ability to germinate was evaluated *in vitro* for both transgenic and wild-type pollen. Fresh pollen from dehisced anthers was collected as above in Robert's Medium (Roberts *et al.*, 1983), standardized for *Brassica oleracea* and incubated under humid conditions for 75 min. The medium contained: 10 mg/l Boric Acid; 362 mg/l Calcium

Chloride; 100 mg/l Potassium Nitrate; 100 mg/l Tris in 20% sucrose. The pollen grain was considered germinated when the length of its tube was more than the diameter of the pollen grain (Shivanna and Rangaswamy, 1992). Duplicate experiments were carried out observing over 100 pollen grains each time.

2.25 *In Vivo* Pollen Germination

Staining was adapted from Kho and Baer (1968) and Muschiatti *et al.* (1994). Pistils were collected 24 hrs post-pollination and cut longitudinally in two equivalent pieces while immersed in a 3:1 ethanol:acetic acid fixative. Pistils remained in fixative for a total of 1 hr at room temperature. Pistils were then softened in 5M NaOH overnight. Following washing several times with distilled water, samples were incubated with aniline blue (Fisher) (0.1%, dissolved in 0.1M K_3PO_4), for 3 hrs in complete darkness. The stained pistils (aniline being callose specific) were placed in a drop of glycerol on a microscope slide, carefully squashed under a cover slip and observed with a fluorescence microscope. Four separate flowers per plant were analysed each time to confirm results obtained.

CHAPTER THREE

RESULTS

3.1 Cloning and Sequence Analysis of *Bnm1*

Our original objective was to isolate the most highly-expressed mRNAs in a cDNA library prepared from *Brassica napus* microspores and pollen induced to undergo embryogenesis in culture (Boutilier *et al.*, 1994). The library was screened using [α - 32 P]-labeled, cDNA from embryogenic pollen. Clones, other than napin and rRNA clones which hybridized most strongly, were selected by Isabelle Prud'homme at Agriculture and Agri-Food Canada (Ottawa). Two of these clones contained 800 bp inserts and were found to be identical upon further analysis. The cDNA insert of one of them, *pBnm1*, was used as a probe for the screening of a *B. napus* genomic library by Eric Barbour and Chris Baszczyński at Pioneer Hi-Bred Production Ltd. (Mississauga, Ont.). Five positive clones were identified of which one, *pBnm1-8*, was found to correspond to the cDNA clone *pBnm1* (Fig.12).

The complete nucleotide sequence of the putative coding region of *pBnm1-8* as well as 817 bp 5' to the translation initiation codon (ATG) and 250 bp 3' to the translational stop codon (TGA) is accessible through Genbank [accession number U86642] (Fig.13). A single intron of 362 bp is located 183 bp downstream from the translational start with splice sites that follow the GT/AG rule (Brown, 1986). The coding sequence of the cDNA was identical to that of the genomic clone.

A detailed analysis of the promoter sequence with sequences contained in the Eukaryotic Promoter Database (Genbank) and Motif Search (DNASIS, ver. 2.1) has not shown any significant similarity to known regulatory regions of other pollen-specific genes. Interestingly, the sequence ACTGT noted to have the strongest gain-of-function effect on expression in

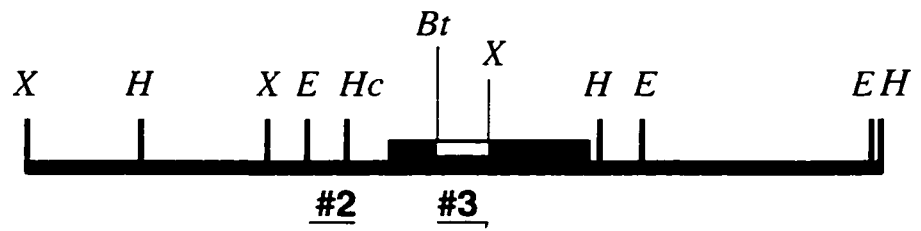
Fig.12. Structure of *Bnm1*.

A graphical representation of p*Bnm1*-8 genomic and p*Bnm1* cDNA sequences indicating the translation start site (ATG) and the TGA stop codon, the intron position (white box) and the relevant endonuclease sites. Probes #1, #2 and #3 used for Northern and Southern blot analysis are indicated. Asterisks define adaptors used for cDNA cloning. X, *Xba*I; H, *Hind*III; E, *Eco*RI; Hc, *Hinc*II; Bt, *Bst*XI; S, *Sal*I; D, *Dra*I.

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pBnm1-8

1 Kb



pBnm1

0.2 Kb

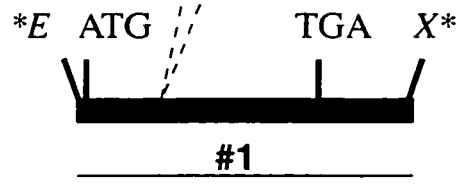


Fig.13. Nucleotide Sequence of p*Bnm*1-8 Genomic DNA and the Predicted Amino Acid Sequence.

The sequence of the intron is in italics. The predicted cleavage site is indicated by the arrow and the predicted protein kinase phosphorylation sites are indicated with asterisks. The translational start and stop codons are in bold. Potential TATA box and polyadenylation signal are underlined. Two gain-of-function regulatory elements are bold underlined.

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- 817 GCACCGCATC AAAGTGATGC GGAAGGAGTA ATTATTCTGT AAATTTAAAT
- 767 ATTTAGTCTT ACATTGTTCA AATTTTTATG TTTTATATTA TTTTATTTTT
- 717 TGATTTTGAC GATTTAAGTA TATTTGAATT TTTTAAAGAA AAATACATAA
- 667 TTAAAATGGG TACCCGAAC TCGAATCTGAA TCGAACCCAC AATGATTCTGA
- 617 ACAAATTC AACCAAAATT TATAAATATT CCAATACGAT TGAATTTTCT
- 567 AATATAAGAA TCAGAAATCT GAATAGATTA ACTGAATTCA AACAGGTATA
- 517 AGAGTGTCCA CCCCACAAA TCCCTTAGTA CAATATATAG TTATAAATAA
- 467 TTCAATAAAC TATTTTATTA TGCACAGCGC GGACTACTAC TAGTATAGTA
- 417 TAATGTATGT CAAATAAAC TTCAGTGAAA TGTGTTTATA TTAGATTAGA
- 367 CCACTTCTTT TCTATGATCA CCAAGGACCT CAACACTTGT CACGACATAG
- 317 CTCAATTTTC TAAACAAAGA AAGAAGATCA CAGACGATTT TTTTCGTTGAC
- 267 TAAATCTATA CAAACCACAT ACTATTTAGA TAGGTTCTCC AAATTTAGCA
- 217 AATATAAGCA AACTTTTAGT AAAAACCTTA CGCATTTTAC ATCAATTTCT
- 167 AATATAGTAG TTTCCAAGAA TATCAAACGT CCCTGACCAA CCCTAGGTG
- 117 TACTTGTATA TATACCCACC CACAACTAA AAGCAAATCA ACATACAGAA
- 67 AACTGAATAA CAACCGGAAG AAAAAAGAGA AAAAAATAAA TAAAACAAAA
- 17 CTTCCGACGC AAAGAAA **ATG** GCA ACG TTC TCA GTT CTG TCT ACC
a1 M A T F S V L S T
+ 28 TTC GCC GCG GCG GCA ATT ACG TTG CAA CTA CTC CTA GTT CCA
a10 F A A A A I T » L Q L L L V P
+ 70 GCT TCA GCC TCT CCT CAC ATG AAA TAC ATT GAC GCT ATC TGC
a24 A S A S P H M K Y I D A I C
+ 112 GAT CGC TCC CAC GAC CAA GAT TAC TGC GTT AAA ACA TTG ACC
a38 D R S H D Q D Y C V K T L T
+ 154 ACC AAC CCC CCT ACA GCT GCT CCC ATT GGC CTG G TACTCA
a52 T N P P T A A P I G L
+ 194 TCTTTAAACC **ACTGTCCTTT** TGTTCGCTT AAATCACAGA AGAAATTTAC
+ 244 GTTTGAATTA TGGTTTATTC AGTTTATTG GCAGTCCGGT AATATGTAAT
+ 294 CCGAAAATCT TCTAACATTA GTCGAAAAAC ATTTTAAACA GACAATCCGA
+ 344 CAATGTGATA CTTTTTCCA **CACTGTAGCA** TCTAGTGTGT TTATACCGCA
+ 394 GCTGGCCGGA TTAGCTAGCT GCATATATAT TAAAAAATAA TCATGTTTAC
+ 444 TTAATATGTT TCAAAAATAC AACTGCATAT GCTTTACGTG TGAAAGAGCT
+ 494 TAAACGAGAA TGATCATTAG TATTAATACT AATAAAATCT CTTTATTATC
+ 544 TCTAG AAT CCA CTG GCC GAG GTG ATG GCG CTC ACC ATA GCC
a63 N P L A E V M A L T I A
+ 585 CAC GCC GAG AAG ACA GCG GCT TTC GTG GCT GAG ACG GGT AAG
a75 H A E K T A A F V A E T* G K
+ 627 GCT GAT CAA ACG TTT ACT GAG TAC CAC AAG GCC TAC TTA GCC
a89 A D Q T F T E Y H K A Y L A A
+ 669 GTG GTG GCT GAT CTC AAG AGC GCA AAC CTG AAG CTC AAG CAA
a103 V V A D L K S A N L K L K Q
+ 711 TCC CCT GAC ACT GCT CAC TAC GAC GTT AGG TCT TCG ACC GAC
a117 S P D T A H Y D V R S S T D
+ 753 CAG ATG AAG CGC GTG GAG GGA TTA GTT GCC AGC AAA AAT GAC
a131 Q M K R V E G L V A S K N D
+ 795 CAG GCT TCA ACT ACT CTT AAG GAA ATG ACG GTG CAG ATG GAG
a145 Q A S T T* L K E M T V Q M E
+ 837 AAA CTT CTT GAT CTT GCA GCT AGT GCC GCC GAT GCT GTG GAC
a159 K L L L A A S A A D A V D
+ 879 GAC GAT GAT GAG AAC ATC CAC CGT CGC GTC **TGA** TT
a173 D D D E N I H R R V -
+ 914 TTAAACCGGT CCGGTTTCGT TTTTTTGTGT TCACAATACA AAATATAATA
+ 964 AATAAATGAA TATACATATA CACACACACA AATGTGTTGT GATAAACTAG
+1014 TAATTAAGTT TTTGAAATAT TTGCAGAACT AATGTTGTCA ATATTTTTGG
+1064 CATATATAAA GAGTCTGCTG TATTATCTTT TTATAAAACT AAATATAAAT
+1114 CTGATTTGTA TCAATTGTTG GACAACCCAA AAGCGCCAAG ACATCACCTG
+1164 GTACAAACAT ATTGACTTTT GTAAGCTTAT CGATACCGTC GACCTCGA

pollen (Eyal *et al.*, 1995) was not found in the *Bnm1* promoter sequence but was found twice within the 362 bp intron.

Computer analysis (IBI) of the cDNA sequence shows that *Bnm1* encodes a putative 182 amino acid protein (BNM1) with a molecular weight of 19.7 kDa and a predicted pI of 5.14. The predicted protein sequence contains 2 potential protein kinase phosphorylation sites at amino acids 86 and 149. The N-terminus of the hydropathy profile of BNM1 contains a particularly hydrophobic amino-terminal region which displays the features of a transit peptide when this profile is constructed following the method of Kyte and Doolittle (1982). There is one predicted signal sequence cleavage site in this region that conforms to the -3, -1 rule (von Heijne, 1983). This potential cleavage site is between amino acid 16 (Thr) and 17 (Leu) (Fig. 14). The predicted molecular weight of BNM1, if cleaved, would be 17.9 kDa.

Computer searches (BLAST) were performed comparing the nucleotide and deduced amino acid sequence of the *Bnm1* gene with sequences contained in Genbank and EMBL databases. No significant similarities were found between the *Bnm1* deduced protein sequence and previously published protein sequences. However, high similarity (85% identity over 402 bases of ORF) was found with two *Arabidopsis* partial EST cDNA clones obtained from a flower bud cDNA library (Genbank accession numbers F15396 and Z26006) when comparing with the *Bnm1* cDNA sequence (Fig. 15).

3.2 *Bnm1* Origin, Gene Family and Distribution

Southern blot analysis of *EcoRI*-digested DNA was employed to examine the gene family composition of *Bnm1* and its distribution among diverse plant species. *EcoRI* does not cut within the transcribed region or intron of the cloned *B. napus* cv Topas gene. Under conditions of low stringency, *Bnm1* appears to be related to a small family of genes in all 10 of the representative species examined in the Brassiceae tribe (Fig. 16B and Fig. 17B). Similar genes were present in three members of seven other tribes of the Brassicaceae family at low

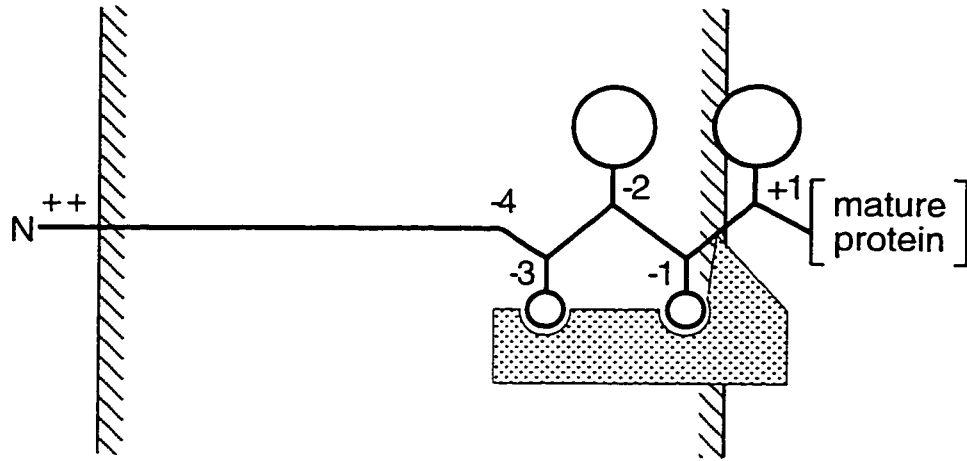
Fig.14. Predicted Cleavage Site of BNM1 Transit Peptide.

A) A graphical representation of -3, -1 model proposed by von Heijne (1983). Cleavage occurs between amino acid -1 and amino acid +1.

B) Number of residues grouped by von Heijne (1983) into different physical-chemical characters. I (aromatic residues) : Phe, His, Trp, Tyr; II (charged residues): Asp, Glu, Lys, Arg; III (large polar residues): Asn, Gln; IV (hydrophobic residues): Phe, Ile, Leu, Met, Val; V (small neutral residues): Ala, Cys, Ser, Thr; G: Gly; P: Pro. Consensus is based on 78 eukaryotic transit peptides. BNM1 residues are in bold. Positions -3 and -1 are underlined.

Adapted from von Heijne (1983).

A)



B)

Group	Number of residues in position							
	-6	-5	-4	-3	-2	-1	+1	+2
I	11	6	7	0	21	0	6	7
II	0	6	7	0	9	0	25	15
III	0	6	1	0	13	1	14	11
IV	42	7	19	22	29	0	13	16
V	27	27	22	52	9	57	18	9
G	0	7	21	1	3	18	2	4
P	7	16	6	0	0	0	0	11
Consensus:	IV	V	V	V	IV	V	II	IV
BNM1:	V	V	V	V	IV	V	IV	III

Fig.15. Comparison of the Nucleotide Sequence of the *Bnm1* cDNA and *Arabidopsis* ESTs (Expression Sequence Tags).

A) Alignment of the *Bnm1* cDNA with the ATTS 213 partial cDNA clone (1996) shows an 84.5% match between both sequences.

B) Alignment of the *Bnm1* cDNA with the ATTS 1283 partial cDNA clone (1994) shows a 77.1% match between both sequences.

Two dots are used to indicate sequence identity, while the letter x indicates a different nucleotide at that site. A dash line indicates where a gap was inserted to obtain the best possible alignment. Both *Bnm1* translational start (ATG) and stop (TGA) codons are in bold caps.

A)

```

          10      20      30      40      50
BNTMICDA  AAAAACTTCCGACGCAAAGA-AAATGGCAACGTTCTCAGTTCTGTCTACCTTCGCCGCG
           :::::::::::x:::: :::::::::::x::x::::::::::x::::::::::x:::
ATS213    AAAAACTTCCGACGCTAAGAGAAATGGCAACGTCCTCTGTCTGTCCACCTTCGCTGCG
20         30      40      50      60      70
60         70      80      90     100     110
BNTMICDA  GCGGCAATTACGTTGCAACT--ACTCCTAGTTCAGCTTCAGCCTCTCCTCACATGAAAT
           :::::::::::x::::xx::x::::::::::x::x:::::::::::
ATS213    GCGGCAATTACGTTGCAACTAATCTTATCTCCTGCTTCAGCTTCCCCTCACATGAAAT
80         90     100     110     120     130
120        130     140     150     160     170
BNTMICDA  ACA-TTGACGCTATC-TGCG--ATCGCTCCCACGACCAAGATTACTGCGTTAAACATTG
           ::: ::::::::::x::: :::: ::x:::::x:::::~x:~x::::::::::~x:~
ATS213    ACATTTGACGCCATCTTGCGAATTCGTTCCCATGACCAAGCTTCTTGCGTTAAACAAAG
140       150     160     170     180     190

          180
BNTMICDA  ACCACCAACCCCC
           :x:::::x:::::
ATS213    ACTACCAATCCCC
200       210
```

B)

```

          380      390      400      410      420      430
BNTMICDA  CCCTGACACTGCTCACTACGACGTTAGGTCTTCGACCGACCAGATGAAGCGCGTGGAGGG
           ::x:::::x::::x::::::::::x::x:::::x:~x::x::x::::x::::x:x
ATT1283   CCCCACACGGCTAACTACGACGTCAGGGCTTCCAGTGACAACATGAGGCGCGTGAATGA
          10      20      30      40      50      60
          440      450      460      470      480      490
BNTMICDA  ATTAGTTGCCAGCAAAAATGACCAGGCTTCAACTACTCTTAAGGAAATGACGGTGCAGAT
           ::x:::~x:~x::x:x:::::x::::::::::x::x::x::::::::::x:::::
ATT1283   ATTAGTTGGGAAAAAACTGACAAGGCTTCAACCACGCTCAAGGAAATGACAGTGCAGAT
          70      80      90     100     110     120
          500      510      520      530      540      550
BNTMICDA  GGAGAACTTCTTGATCTTGACGCTAGTGCCGCGGATGCTGTGGACGACGATGATGAGAA
           ::::::::::x::::::::::x:x::::::::::x::x::x:::::x:::::
ATT1283   GGAGAACTCCTTGATCTTGACGCTGGAGCCGCGGATGCCGTTGATGACGACGATGAGAA
          130     140     150     160     170     180
          560      570      580      590      600
BNTMICDA  CATCCACCGTCGCGTC---TGAATTTAAACCGGTCGGGTTTCGTTTTTTTGTGTTTCA
           ::x:x::x:::::~x::x:::::~x:~x::::::::::~x:~x:::::~x:~
ATT1283   CATGCGCCTTCGCGTCTAATGGATGTTAAACCGAGCGGGTTTCGTTTTTTTGGTTTATA
          190     200     210     220     230     240
          610      620      630      640      650      660
BNTMICDA  ATACAAAATATAATAAATAAATGAATATACATATA-CACACACACAAATGTGTTGTGATA
           ::::::::::xxxx::x::::::::::xx::x:~x::x:x::x::::x::x::
ATT1283   ATACAAATGGAAATAAGTAAATGAATATGTATACATCACATAGA-AACTGTGTTATGGTA
          250     260     270     280     290
          670      680      690      700      710      720
BNTMICDA  AACTAGTAATTAAGTTTTTGAAT-ATTTGCAGAACTAATG---TTGTCAATATTTTTGG
           :x::::::::::x::::::::::~x:~x::x:~x::x::x::x::x::x::x::
ATT1283   AATTAGTAATTCAGTTTTTGAATAATGTGGTTAAGTTTTGCATCTCTTACTAATTTT-G
300       310     320     330     340     350

          730
BNTMICDA  CATATATAA
           x::x::~x
ATT1283   TATCTATCT
360
```

stringency (Fig. 16C and 17B); however, they are not always present in all members of a given tribe. For example, under low stringency hybridizing bands were present in *Arabidopsis thaliana* (Fig.16C) a member of the tribe Sisymbriaceae but not in *Cleome hasslerana* (Fig.17B) of the same tribe. The hybridizing bands in *A. thaliana* may represent the *Arabidopsis* EST cDNA clones which have significant sequence similarity to *Bnm1*. In genomes of more distantly-related species such as tobacco, sunflower, maize, barley and spruce (Fig.16C) bands hybridizing to *Bnm1* were not detected. Under conditions of high stringency wash (62°C), prominent bands remained present only in members of the Brassicaceae tribe (Fig.16A, 17A).

Brassica napus (AACC) is an amphidiploid derived from the interspecific hybridization of the *B. oleracea* (CC) genome and the *B. rapa* (AA) genome (Fig.10). To determine the genomic origins of *Bnm1*, *EcoRI* digests of two *B. napus* cultivars were compared with those of three *B. oleracea* cultivars and three *B. rapa* cultivars (Fig. 16A). Hybridizing bands of ca. 12 kb are found in each of the *B. oleracea* cultivars (Lanes 5-7); whereas, different bands of ca. 2 kb are found in each of the *B. rapa* cultivars (Lanes 10-12). Both bands were present in each of the *B. napus* cultivars examined (Lanes 8 and 9). This is consistent with the amphidiploid nature of *B. napus* (AACC). In order to distinguish which of the 2 bands represents the *Bnm1* gene cloned in this study, gene-specific probes for *Bnm1* were derived from the 5'UTR and intron sequences of p*Bnm1*-8 (Fig. 12). These non-coding sequences have lower evolutionary constraints compared to coding regions and are expected to have an increased number of mutations. As shown in Fig. 18, probe#3 (intron sequence) hybridized more strongly to the ca. 2 kb fragment than the other ca. 12 kb fragment, suggesting that the *Bnm1* gene cloned in this study is probably derived from the A genome. This data was supported by sequence analysis of p*Bnm1*-8 which also revealed a 2 kb *EcoRI* fragment.

3.3 Patterns of *Bnm1* mRNA Accumulation

To determine the spatial pattern of *Bnm1* mRNA accumulation, Northern blot analyses were performed with total RNA extracted from a variety of tissues using the p*Bnm1* insert as a probe by Isabelle Prud'homme and Jiro Hattori. These studies revealed that the *Bnm1* cDNA hybridized to a single RNA band of about 0.8 kb in developing flower buds, flowers and anthers, but did not hybridize to RNA isolated from roots, stems, leaves, pistils and developing seeds (Fig.19). Pollen developing in tissue culture was also examined. Samples cultured at 24°C, for 4 days to allow continued pollen development (Fan *et al.*, 1988) contained high levels of *Bnm1* mRNA whereas samples cultured at 32.5°C, for 4 days to block pollen development and initiate pollen embryogenesis (Fan *et al.*, 1988; Keller *et al.*, 1987) had significant but reduced levels of *Bnm1* mRNA. Expression could not be detected in the pollen-derived embryos that subsequently developed in culture (Fig.20).

In order to characterize the temporal expression pattern of *Bnm1* in microsporogenesis and pollen development in plants, samples at specific stages of development were isolated using petal length as a marker for the stage of development (Fig.21). Northern blot analysis showed that *Bnm1* mRNA accumulated first in bicellular pollen, reached higher levels in tricellular pollen (Fig.22) but failed to detect *Bnm1* mRNA at tetrad and unicellular stages (Fig.22). This pattern of expression is consistent with that of a "late" pollen-specific gene. However, in order to define pollen-specificity of *Bnm1* expression within the anther, *in situ* hybridization of whole buds containing a range of developmental stages from pre-meiotic pollen mother cells (data not shown) to mature tricellular pollen was undertaken by K. Boutilier (Agriculture and Agri-Food Canada, Ottawa). These analyses confirmed both the pollen specificity of *Bnm1* (Fig.23) and its accumulation strictly in bicellular and tricellular stages of development as determined by subsequent nuclear staining of tissue sections from the same sample (data not shown).

Fig.16. Southern Blot Analysis of *Bnm1* in Diverse Plant Species.

Ten μ g of genomic DNA were digested with *Eco*RI and fractionated by gel electrophoresis. Hybridization was performed using the 32 P-labeled probe #1. After hybridization, the filters were washed first at low stringency temperature wash (42°C, panels **b** and **c**), followed by a high stringency temperature wash (62°C, panel **a**). The size of lambda *Hind*III fragments (in kb) are shown left of **c**. Panels **a** and **b**: (1) *B. incana*, (2) *B. cretica*, (3) *B. rupestris*, (4) *B. montana*, (5) *B. alboglabra*, (6) *B. oleraceae* cv Oleraceae, (7) *B. oleraceae* cv Alboglabra, (8) *B. napus* cv Topas, (9) *B. napus* cv Westar, (10) *B. campestris* cv Tobin, (11) *B. rapa*, (12) *B. campestris* cv Candle. Panel **c**. (1) Black spruce, (2) *Arabidopsis*, (3) Sunflower, (4) Tobacco, (5) Barley, (6) Maize, (7) *B. oleraceae*.

Tribe representatives recommended by Suzanne Warwick (Agriculture and Agri-Food Canada).

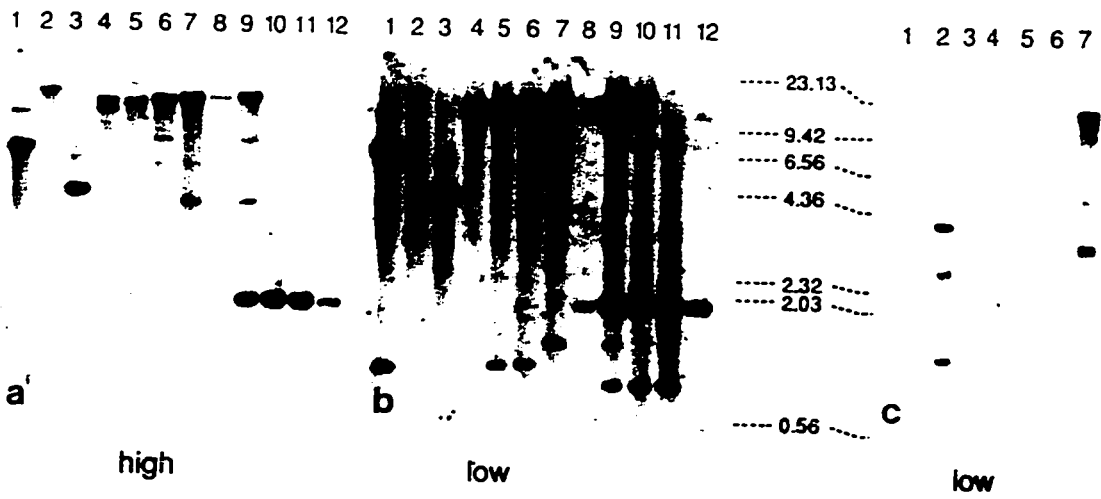
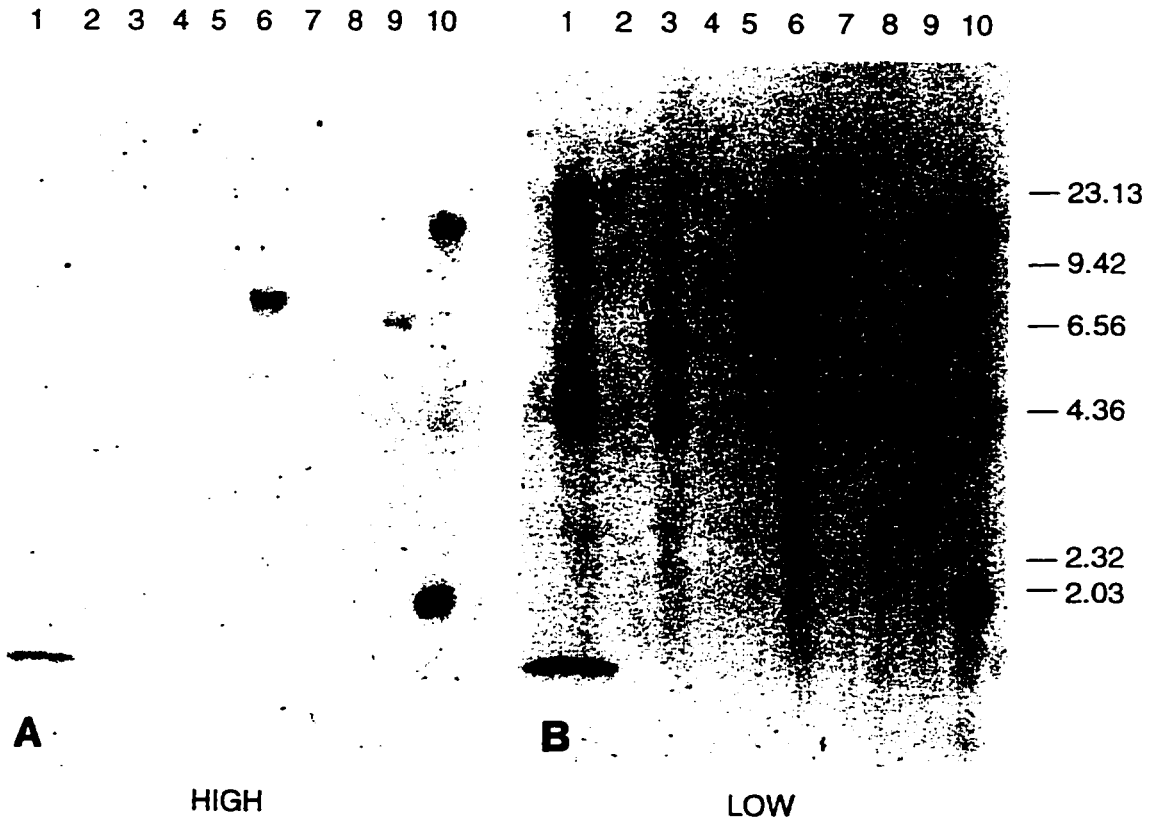


Fig.17. Southern Blot Analysis of *EcoRI* digested DNA from a Number of Diverse Brassicaceae Tribes using the Radiolabeled *Bnm1* cDNA.

Same procedures as outlined in Figure 16. (1) *Nasturdium officinale* (tribe Arabideae), (2) *Iberis amara* (tribe Lepideae), (3) *Matthiola longipetala* (tribe Matthioleae), (4) *Cleome hasslerana* (tribe Sisymbrieae), (5) *Berteroa incana* (tribe Alysseae), (6) *Raphanus sativa* (tribe Brassiceae), (7) *Arabis caucasica* (tribe Arabideae), (8) *Cheiranthus cheirii* (tribe Hesperideae), (9) *Eruca sativa* (tribe Brassiceae), (10) *B. napus* cv topas (tribe Brassiceae).



Hybridization with other *B. napus* pollen-specific cDNAs *Bp4*, *Bp10*, *Bp19* (Albani *et al.*, 1990; Albani *et al.*, 1991; Albani *et al.*, 1992) served as controls for the developmental expression pattern. For example *Bp10* and *Bp19* were only expressed at bicellular and tricellular stages (Fig.22) as previously reported for *B. napus* cv Westar (Albani *et al.*, 1991; Albani *et al.*, 1992); however, *Bp4* mRNA was not detected in unicellular microspores as previously described (Albani *et al.*, 1990). Polyubiquitin mRNAs, which was used as an internal control, were detected in all stages tested, with higher levels of expression in bicellular pollen (Fig.22). Ribosomal RNA bands were included to show the quantity of sample loaded.

3.4 Patterns of BNMI Protein Accumulation and Immunolocalization

In order to analyse the *Bnm1* gene product, BNMI, it was necessary to obtain an antibody that would detect the BNMI protein. Laboratories studying pollen allergens by immunoscreening cDNA expression libraries have used the GST fusion system to preserve protein antigenicity (Griffith *et al.*, 1991). Therefore, a GST-BNMI fusion protein expressed in *E. coli*, (Section 2.19) and purified by SDS-PAGE was used to immunize two mice.

The polyclonal antibodies cross-reacted only with the native GST protein and the BNMI protein as detected by single bands of 26 and 20 kDa as expected. No cross-reactivity was detected in extracts of *E. coli* or from a protein sample obtained from *Brassica* seed (K. Cole, personal communication).

Fig.18. Southern Blot Analysis of *Bnm1* in Diverse *Brassica* Species

(as outlined in Figure 16).

Hybridization was performed using the α - ^{32}P -labeled probe #3 (probe#2 produced same results). After hybridization, the filter was washed at high stringency temperature (65°C). The size of lambda *Hind*III fragments (in kb) are shown to the right.

1 2 3 4 5 6 7 8 9 10 11 12

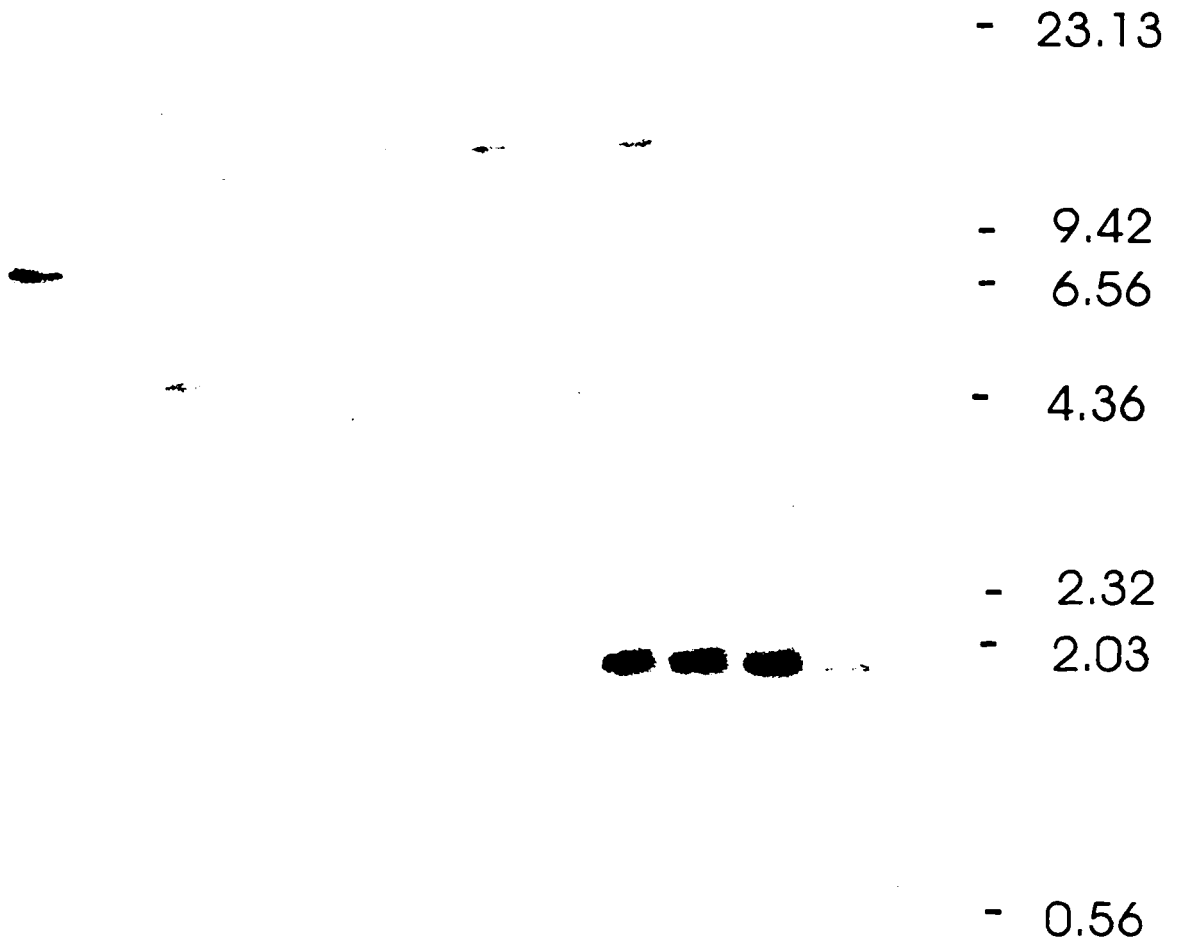
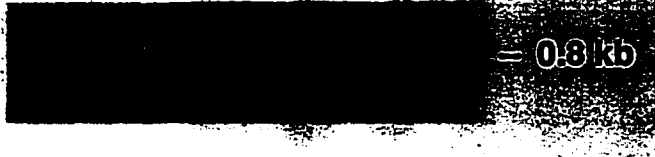


Fig.19. Northern Blot Analysis of *Bnm1* mRNA Accumulation in Various Organs of *B. napus*.

Five micrograms of total RNA were hybridized with the radiolabeled *Bnm1* cDNA as outlined in Materials and Methods. r, root; s, stem; l, leaf; b, bud; f, flower; p, pistil; a, anther, and sd, seed.

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r s l b f p a sd



- 08 kb

Fig.20. Northern Blot Analysis of *Bnm1* mRNA Accumulation in Isolated Pollen Cultured at 24°C or 32.5°C for 4 days and in Pollen-Derived Embryos Collected at Specific Stages of Development.

g, globular; h, heart; t, torpedo; c, cotyledon stage at specific weeks in culture.

32.5° 24° g h t c3 c4 c5

└ | | | | | |

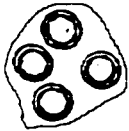


-0.8 kb

Fig.21. Staging of Pollen Development from Nuclear Cytology Observations.

The pollen developmental stages were determined by using the nuclear stain DAPI (4, 6-diamidino-2-phenylindole) and correlating with petal length. Bud length proved to be a reliable marker for the tetrad stage.

Tetrad



<2mm

|--bud--|

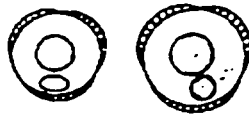
Unicellular



1.5-2.5mm

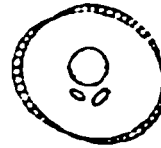
|-----petal length-----|

Bicellular



4.0-5.5mm

Tricellular



>6.5mm

Fig.22. Northern Blot Analysis of mRNA Accumulation in Isolated Pollen at Specific Developmental Stages in *B. napus*.

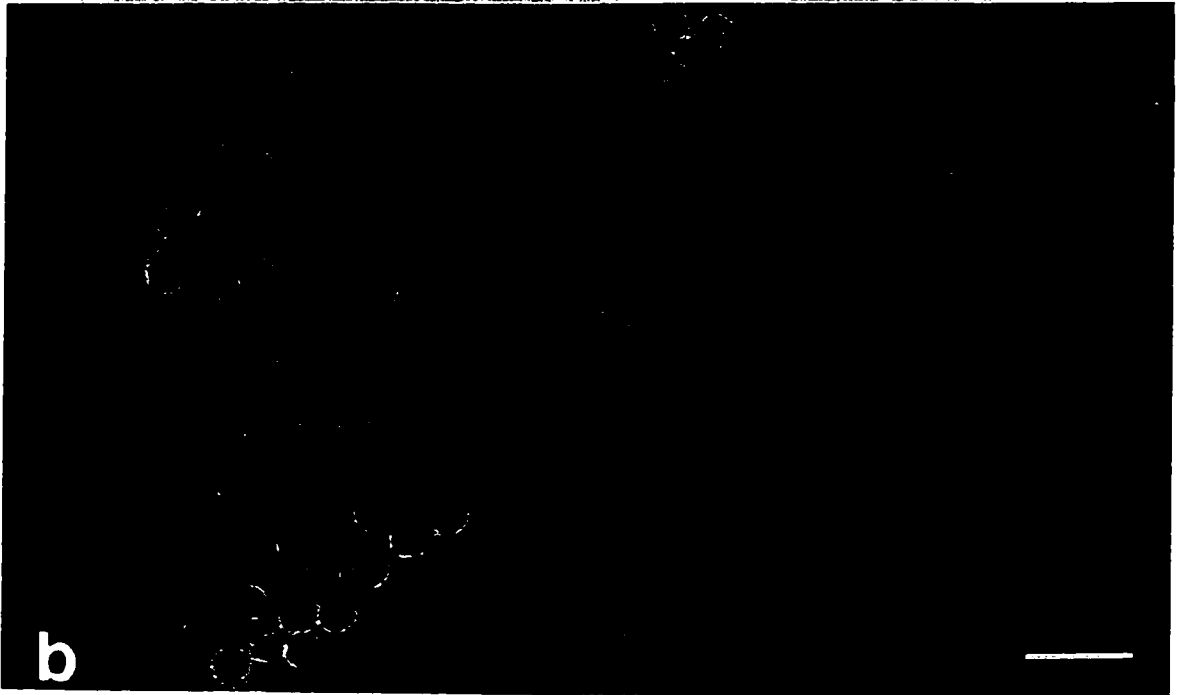
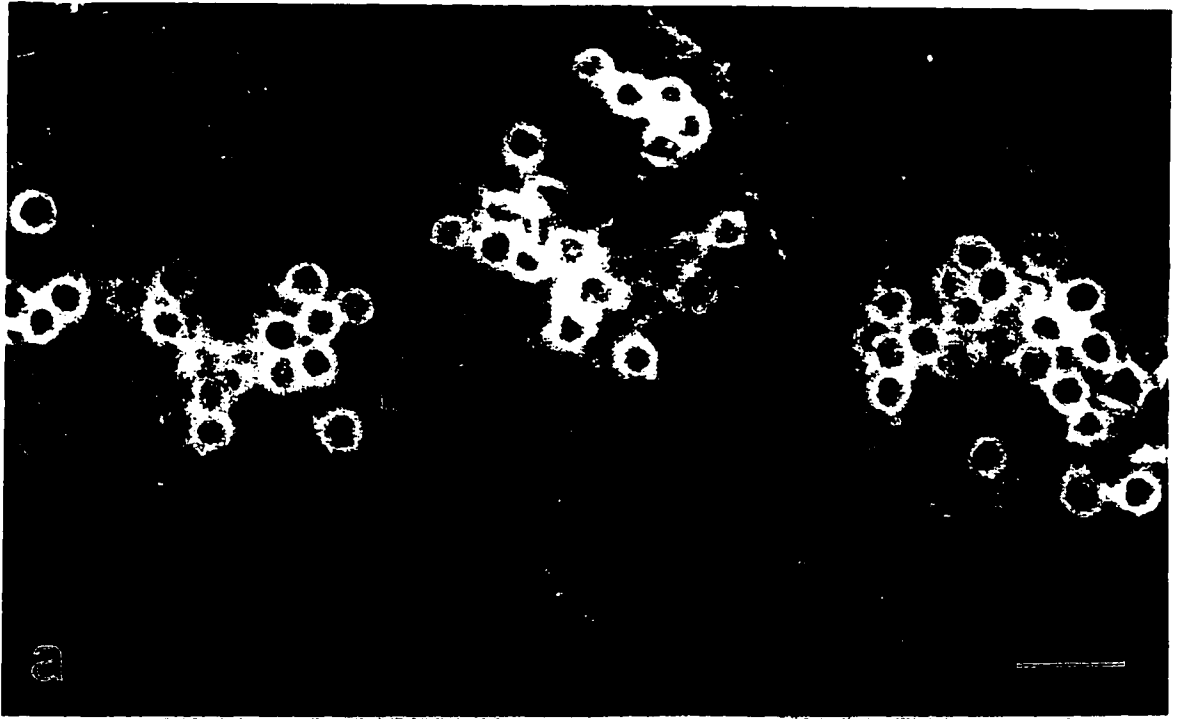
Seven micrograms of total RNA were isolated from pollen at specific stages of development and hybridized with the radiolabeled *Bnm1* cDNA. 1, unicellular microspores; 2, bicellular pollen; 3, tricellular pollen. *Bp4*, *Bp10*, *Bp19*, are *B. napus* pollen-specific cDNA probes. UBQ4 is the *Arabidopsis* ubiquitin probe. The ethidium bromide-stained rRNA bands are shown for each lane. Size of transcripts based on size markers (BRL) are indicated to the right.



Fig.23. Localization of *Bnm1* Expression in *Brassica napus* Flower Buds.

mRNA *in situ* hybridization of flower buds containing mature pollen grains using *Bnm1* antisense (a) and sense (b) RNA probes. Bar= 80 μ m.

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To determine whether *Bnm1* transcripts were stored and translated during pollen germination, as proposed for late pollen-specific genes by Mascarenhas *et al.* (1984), protein samples of pollen at specific stages of development including germinating pollen were collected. Western blots of total proteins separated by size on SDS-PAGE gels detected a single band of ca. 20 kDa in tricellular and germinating pollen (Fig.24A). The bacterial GST protein (26 kDa) was included as a positive control. No band was detected in other developmental stages, including; tetrad, unicellular microspores and bicellular pollen. Protein samples extracted from somatic tissues, leaf and stem, serving as negative controls, were not recognized by the anti-BNM1 primary antibody but, as shown, shadow bands did appear in the stem, leaf and marker samples after longer exposure. This was in contrast to the anti-heat-shock70 protein (Hsp70) primary antibody which was used as an internal control and showed detectable levels of Hsp 70 protein in all stages tested with higher levels in the tetrad/unicellular sample (Fig.24B). Figure 24C reveals the SDS-PAGE gel, stained for protein content, which was loaded with the same samples and amount of protein and run simultaneously as the gel in Fig. 24B.

Considering the *Bnm1* RNA transcripts appear in bicellular pollen and accumulate to higher levels in tricellular pollen and the BNM1 protein only appears in tricellular pollen, perhaps the *Bnm1* transcripts are stored and translated prior to germination.

To determine if BNM1 harbours a functional signal peptide, the intracellular location of BNM1 *in situ* was examined. Unfortunately, the BNM1 primary antibody did not recognize and/or the FITC-secondary antibody did not provide the resolution required, to identify the native configuration of the BNM1 protein *in situ* (data not shown).

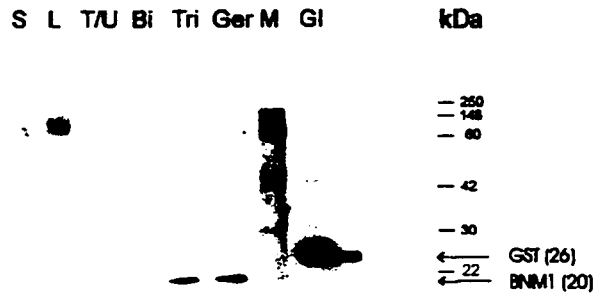
3.5 Activity of the *Bnm1* Promoter in Transgenic Plants

An 817 bp promoter fragment was generated from the genomic clone p*Bnm1*-8 by PCR. This fragment was ligated to the *gus* A protein coding region and 3'UTR of the potato

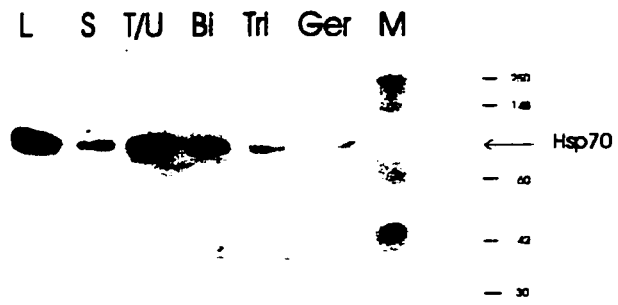
Fig.24. Western Blot Analysis of Protein Accumulation in Isolated Pollen at Specific Developmental Stages.

Twenty micrograms of total protein were isolated from pollen and from leaf and stem tissues of *B. napus*. A) Blot was probed with antibody generated from GST-BNM1 fusion protein. B) Blot probed with internal control Hsp70 antibody. C) Coomassie stained SDS-PAGE gel loaded with the same samples and the same amount of protein as in B). S, stem; L, leaf; T/U, tetrad and unicellular microspores; Bi, bicellular pollen; Tri, tricellular pollen; Ger, germinating pollen; M, size marker; GI, bacterial GST. Size markers in kDa are indicated to the right.

A)



B)



C)



proteinase inhibitor (*pinII*) gene (Section 2.16) (Fig.25) by Eric Barbour and Chris Baszczyński at Pioneer Hi-Bred Production Ltd. (Mississauga, Ont.). The construct was introduced into *B. napus* cv. Westar by *Agrobacterium*-mediated transformation by M. Arnoldo at Pioneer Hi-Bred Production Ltd. (Mississauga, Ont.). Three transgenic plants (T1, T2, T3) and two wild type (WT) non-transformed plants (W1, W2) were examined. As shown in Figures 26A and 26B, GUS specific activities in seedlings, leaf, stem, root, seed, sepals, petals and pistils were similar to background activities in both the transgenic and WT plants; whereas, a 500-fold increase in GUS specific activity was found in anthers of the transgenic plants as compared to untransformed plants. Although the *Bnm1* intron containing two “gain of function” sequences ACTGT was not included in this GUS construct, GUS specific activities in bicellular and tricellular pollen were over 200 fold higher in transgenic plants relative to untransformed plants. GUS activities in tetrad and unicellular microspores from transgenic plants were also higher than WT control samples (Fig.26C). However, histochemical analysis did not reveal GUS staining in tetrad and unicellular stages (Fig.27). Pollen development in tissue culture was also examined. Samples cultured at 24°C, for 4 days to allow continued pollen development showed GUS positive staining in bicellular and tricellular pollen. Whereas samples cultured at 32.5°C, for 4 days to block pollen development and initiate pollen embryogenesis showed no GUS staining in those cells having undergone the first symmetric embryogenic-specific division (K. Boutilier, personal communication). Therefore, only bicellular and tricellular cells committed to the pollen pathway stained positively for GUS.

In this study, GUS was used as a marker to assess activity of the *Bnm1* promoter. This GUS fusion lacks the *Bnm1* open reading frame and therefore any information regarding translational control that may be contained within this open reading frame. Taken together, one can speculate that the developmental programs involved in the induction of microspore embryogenesis and pollen development are mediated transcriptionally.

3.6 Down-regulation of *Bnm1* Transcript and Protein in Antisense Transgenic Plants

In order to investigate whether the BNM1 protein is important in pollen germination and/or pollen tube growth, antisense transgenic plants were generated. An 817 bp fragment of the *Bnm1* promoter was fused to the complete cDNA in reverse orientation and the 3'UTR of the potato proteinase inhibitor (*pinII*) gene (Fig.28). The construct was introduced into *B. napus* cv. Westar by *Agrobacterium*-mediated transformations at Pioneer Hi-Bred Production Ltd. (Mississauga, Ont.) by M. Arnoldo. Twenty one primary transformants carrying the *Bnm1* antisense construct were generated. All these plants contracted Blackleg disease but were self-pollinated to produce seed. Seeds obtained were sterilized before planting. Over 250 plants were grown from these seeds and no gross abnormality in pollen structure was detected at the light microscope level from over 140 plants screened.

To screen for down-regulation of the BNM1 protein with a more manageable number of antisense plants, protein extracted from mature pollen of 58 antisense plants was analysed and levels were compared with those of wild-type plants by Western blot. Protein concentration was determined using the Bio-Rad protein reagent using bovine serum albumin as a standard. Equal amounts of protein were loaded in each well. Anti-Hsp-70 primary antibodies served as a control for equal loading. The anti-BNM1 polyclonal antibody detected lower amounts of BNM1 protein in plants #1 and #11 (data not shown). However, detection levels in untransformed plants were also low.

The anti-BNM1 primary antibody proved to be very unstable in hybridization buffer and stocks became depleted. To extend screen for inhibition of the *Bnm1* gene, mRNA levels in mature pollen from wild-type and antisense transformants were analyzed. Total RNA extracted from mature pollen of 14 plants was analyzed by Northern blot hybridization (Fig.29). Plant #26 shows a near complete shut-down of the endogenous *Bnm1* transcript, while plants #7, 11, 17, and 20 showed a significant decrease in *Bnm1* transcript.

3.7 Pollen Phenotypes

Pollen from plants #7, 11, 17 and 20 with clearly reduced RNA levels was examined for a potential phenotypic lesion. Pollen size, shape and structure showed no obvious differences from pollen of wild-type plants at the light microscope level (data not shown).

Viability of transgenic pollen versus wild-type pollen was evaluated using the Fluorescein diacetate (FDA) method. Fluorescein diacetate is a substrate for esterases which cleaves the non-fluorescent FDA to release fluorescein (Heslop-Harrison, 1970). This method reveals the integrity of cell membranes by retaining fluorescein within the cells. No difference in staining between transgenic and wild-type plants was observed (Fig.31A and 31B) suggesting that *Bnm1* is not critical to the viability of the pollen grain.

The ability to germinate and grow *in vitro* was examined. No obvious differences were observed with transgenic pollen with reduced *Bnm1* mRNA when compared to wild-type pollen (Fig.30C and 30D). In later experiments some *in vitro* assays showed significant reduction in germination rates but the same observation was made with wild-type pollen. To investigate physiological processes specific to pollen-pistil interactions, germination was examined *in vivo*. Initially, 4 flowers from each plant #7, #17 and #42 were self-pollinated by hand, and growth of pollen tubes in the styles was analyzed 24 hrs later and compared to wild-type self-pollinations using a procedure adapted from Muschietti *et al.* (1994). Plant #42 had *Bnm1* expression levels equal to wild-type and served as a transgenic control. Plants #7 and #17, had reduced *Bnm1* mRNA levels and showed a reduced rate of pollen tube growth compared to wild-type pollen and pollen from plant #42 (Fig.31). Transgenic pollen was also used to pollinate wild-type stigmas and wild-type pollen was used to pollinate stigmas of transgenic plants whose self pollen appeared to be restrained. These analyses suggested that *Bnm1* may not play an important role in pollen tube growth, because wild-type pollen also had restrained pollen tube growth down the style of plant #17 (Fig.32). Furthermore, pollen from plant #17 germinated and pollen tubes migrated down the style of wild-type stigmas as

Fig.25. Restriction Map of the GUS Construct

This construct harbours the *Bnm1* promoter driving the *gus* A gene- *pinII* terminator cassette inserted into the *SaII* and *EcoRI* sites of the binary vector DP1741. This vector also contains the CaMV 35S promoter driving the *nptII* gene used as an antibiotic resistance marker for selecting transformed plant material. The left and right T-DNA borders are labeled LB and RB respectively. *pin II*, potato proteinase inhibitor II; CT, CaMV 35S terminator.

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Fig.26. GUS Specific Activity in Transgenic *Brassica napus* Plants (T1, T2, T3) and Wild-Type Plants (W1, W2) Using Fluorometric Analysis.

A) GUS specific activity in sporophytic and fruit tissues of individual plants.

B) GUS specific activity in floral organs pooled from transgenic (t) or WT plants (wt).

C) GUS specific activity in isolated pollen stages: Tetrad (tetra), unicellular (uni), bicellular (bi), tricellular (tri). Bars represent standard errors. Note the different scales between A, B, and C.

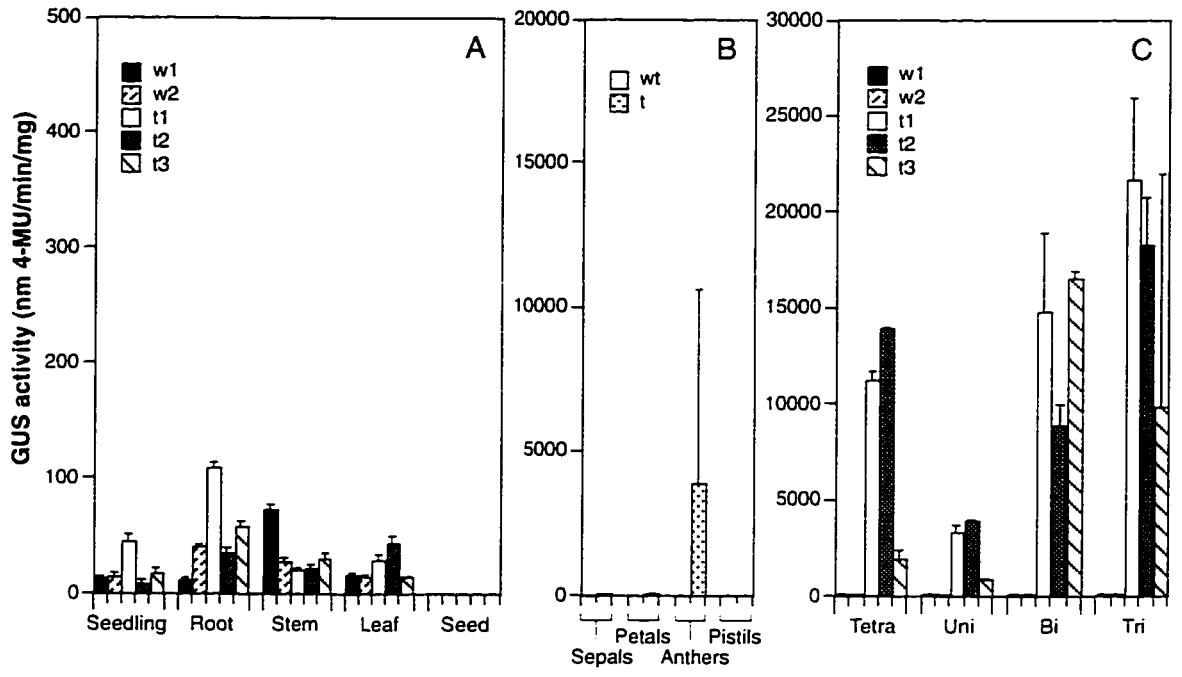


Fig.27. Histochemical Localization of GUS Activity in Developing Pollen of Transgenic *Brassica napus* cv. Westar Plants.

These transgenic plants harbour the *Bnm1* promoter-GUS fusion (e-h). Tetrad stage (e), unicellular stage (f), bicellular stage (g), tricellular stage (h). Pollen from the same developmental stages was isolated from *B. napus* cv. Westar Wild-Type plants (a-d) and assayed in the same manner as described in Materials and Methods.

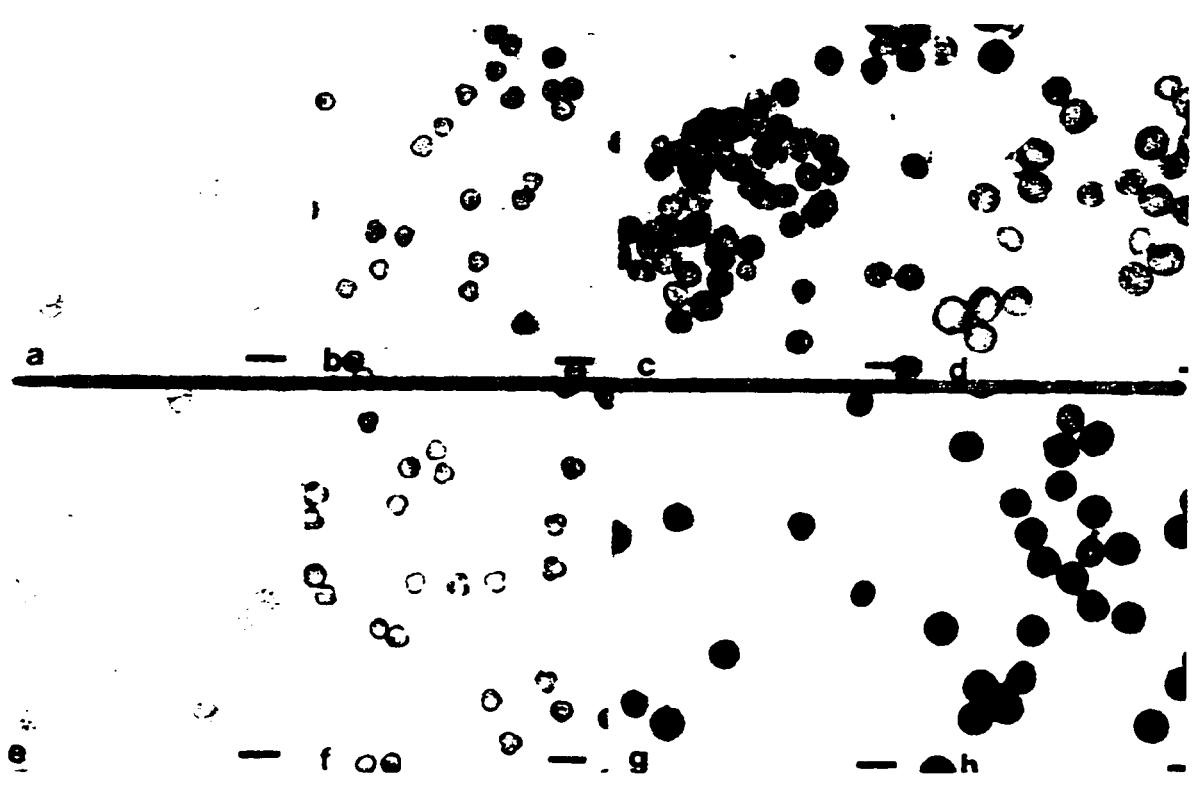


Fig.28. Restriction Map of the Antisense Construct.

This construct harbours the *Bnm1* promoter driving the complete *Bnm1* cDNA in reverse orientation and *nos* terminator. The construct was generated from the DP5477 binary vector (Fig.25) by substituting the *gus* A coding region for the *Bnm1* coding region in reverse orientation. This construct also contains the CaMV 35S promoter driving the *nptII* gene used as an antibiotic resistance marker for selecting transformed plant material. The left and right T-DNA borders are labelled LB and RB, respectively. CT, CaMV 35S terminator; *nos*, nopaline synthase.

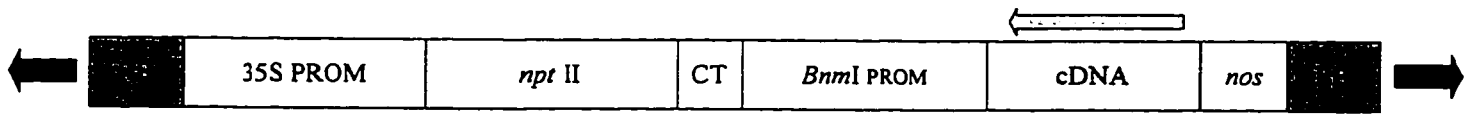


Fig.29. Northern Blot Analysis of *Bnm1* mRNA Levels in Pollen of *Bnm1* Antisense Plants.

Seven micrograms of total RNA were isolated from tricellular pollen and hybridized with the radiolabeled *Bnm1* cDNA. Five, seven and ten micrograms of total RNA isolated from wild-type pollen was loaded respectively to serve as controls. Blot was washed at 60°C and exposed for 4 days to detect low *Bnm1* RNA levels. Plants #7, #11, #17, #20 and #26 are clearly down-regulated for *Bnm1* at the mRNA level.

M 49 42 26 23 20 17 12 11 9 8 7 5 3

WT



- 0.8 kb

successfully as on wild-type self-pollinations (Fig.32). Unlike *in-vitro* pollen germinations, *in vivo* assays proved difficult to make accurate quantitative measurements of the observations, including the number of pollen grains germinated and the number of pollen tubes successfully migrating to ovules in downregulated plants versus wild-type plants.

Fig.30. Viability and *In Vitro* Germination Assays of *Bnm1* Antisense Plants.

A) FDA stain for viability of wild-type control pollen.

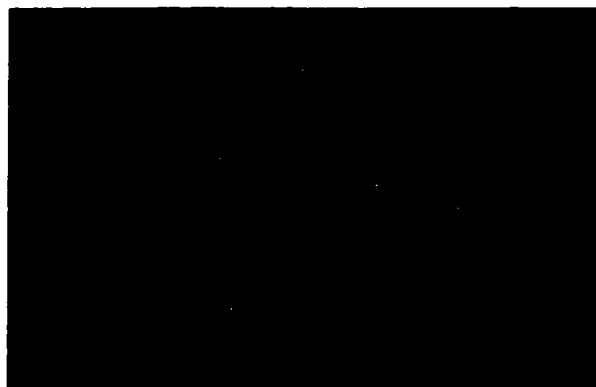
B) FDA stain for viability of *Bnm1* antisense pollen.

C) *In vitro* germination of wild-type pollen.

D) *In vitro* germination of *Bnm1* antisense pollen.

Antisense plants #4, 6, 8, 9, 11, 17, and 42 produced the same results.

Wild-Type



A)



C)

Antisense



B)



D)

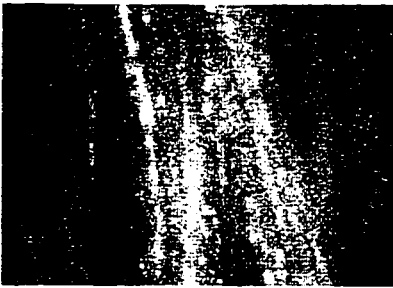
Fig.31. *In Vivo* Pollen Germination Assays of *Bnm1* Antisense Plants. (Self-Pollination)

- A) Self-pollination of wild-type pollen on stigma. (100x) magnification
- B) Self-pollination of pollen from antisense plant #7 on stigma. (40x)
- C) Self-pollination of pollen from antisense plant #17 on stigma. (100x)
- D) Growth of wild-type pollen tubes down style. (400x)
- E) Restricted growth of antisense pollen down style. (400x)
- F) Restricted growth of antisense pollen down style. (400x)

Wild-Type



A)



D)

Anti-7

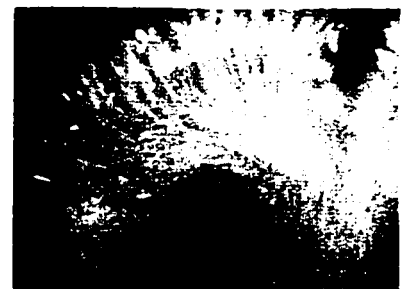


B)



E)

Anti-17



C)



F)

Fig.32. *In Vivo* Pollen Germination Assays of *Bnm1* Antisense Plants.

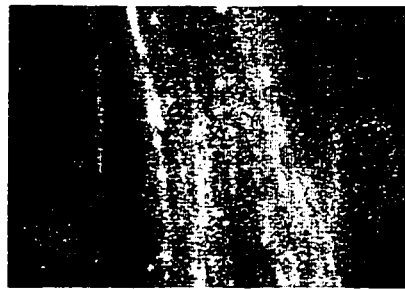
(Cross-Pollination).

- A) and D) Same control germination assay of wild-type pollen as Fig.32. (100x and 400x magnification respectively).
- B) Pollen from antisense plant # 17 germinating on stigma of antisense plant #12. (100x)
- C) Wild-type pollen germinating on stigma from antisense plant #17. (100x)
- E) Growth of pollen from antisense plant #17 down style of antisense plant #12. (400x)
- F) Reduced growth of wild-type pollen down style of antisense plant # 17. (400x)

Wild-Type

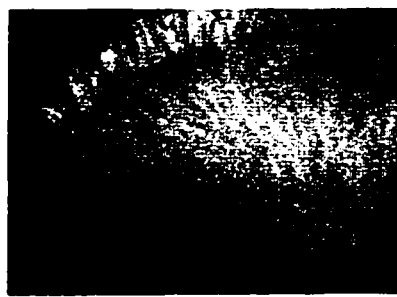


A)



D)

17/ 12

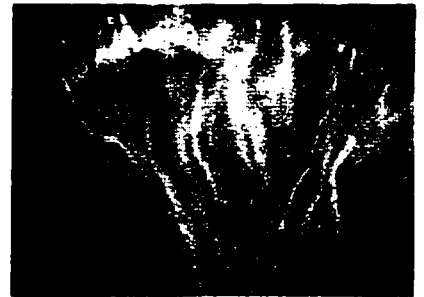


B)

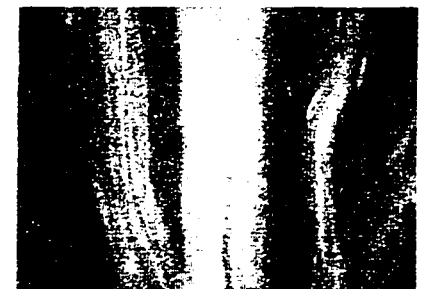


E)

WT/ 17



C)



F)

CHAPTER FOUR

DISCUSSION

4.1 Characterization of Pollen-Specific Genes

Over the past 10 years, there has been a significant increase in the number of laboratories investigating genes expressed in the male gametophyte. The male gametophyte plays a vital role in sexual plant reproduction. It is the site of production and the vehicle by which the male gametes are transported to the embryo sac to participate in fertilization, necessary for generating seeds that will produce the next plant generation. The accessibility of the male gametophyte for direct cytological and molecular analysis, provides an excellent system to explore gene regulation, cellular differentiation, cell-cell communication and the signalling processes which occur during reproduction in plants. Furthermore, the understanding of these mechanisms have and will continue to provide practical avenues towards increasing crop productivity.

The first step towards a better understanding of these mechanisms, involves identifying the molecular components accompanying development. Studies on temporal gene expression patterns are important because they indicate critical stages in pollen development when new genes are activated (Mascarenhas, 1992). Several laboratories have isolated and analyzed clones from cDNA libraries prepared from poly (A) RNA from flower buds at different developmental stages and from various plant species. Initially, a simple pattern for the expression profiles of pollen-expressed genes emerged. It was suggested from the early literature that two sets of genes were sequentially activated. The "early" genes, activated between meiosis and the first pollen mitosis, and the "late" genes, activated between the first pollen mitosis and anthesis (Mascarenhas, 1990a).

More recent data indicate that the temporal expression profiles are more complex. All but four cloned pollen-specific genes are expressed after the first pollen mitosis (Table 1) but their transcripts accumulate to high levels at pollen maturation where some of these transcripts are stored and may not be translated until pollen germination (Mascarenhas, 1993). These genes are presumably needed for rapid pollen germination and initial pollen tube growth. However, two pollen-specific genes are expressed only after the *second* pollen mitosis. These genes include *CDPK*, a calcium-dependent calmodulin-independent protein kinase in maize (Estruch *et al.*, 1994), and *Pcp1*, a pollen coat protein in *Brassica* (Stanchev *et al.*, 1996). *Pcp1* is thought to be involved with SI, while *CDPK* is thought to be involved with the trigger of cytoskeletal organization, required for germination and pollen tube growth. Why these genes are not expressed earlier and stored in a similar fashion to the other "late" genes is unknown. The most simple explanation may be that in the majority (70%) of angiosperm species, the haploid microspore undergoes a *single* asymmetrical mitotic division to produce a bicellular pollen grain at anthesis. Examples being the Solanaceous species; *Nicotiana*, *Lycopersicum*, *Petunia* and *Solanum*. In all other species (30%) which includes the Cruciferae, the generative cell undergoes a second mitotic division prior to anthesis (Brewbaker, 1967). For characterizations of pollen-specific genes in species whose pollen is shed at the bicellular stage, there is no cytological marker to define the later stages of pollen maturation. Eady *et al.* (1994) noted that despite considerable divergence in floral morphology and pollen size, microspore and pollen development occur over similar time periods, and major cytological processes, such as vacuolation and the accumulation of metabolic reserves, are similar between the two pollen types. Therefore, perhaps a better measuring stick of pollen development should be in relation to days (time) before anthesis. Furthermore, Eady *et al.* (1995) demonstrated that although division asymmetry at the first pollen mitosis is essential for proper differentiation of the generative cell, activation of vegetative cell-specific transcription and functional maturation may be uncoupled from cytokinesis. Perhaps transcription of genes expressed *after* this first microspore mitosis are

uncoupled from cytokinesis of the generative cell in the same manner. Therefore, the use of time before anthesis would put both pollen types on a similar scale for timing of expression. Moreover, very few pollen-specific genes have been cloned and characterized. Approximately 50 out of a potential 2000 genes (2.5%) exclusive to the male gametophyte have been analysed rigorously. It remains difficult at this time to provide a more complete profile of temporal expression patterns of pollen-specific genes and what they may signify.

Although the expression of genes with pollen-specificity strongly implies an exclusive role for the encoded gene product in the development of the male gametophyte (Scott and Stead, 1994), it is important to assess the data on their spatial expression profiles more rigorously. So far, it has been found that all of the presumed "early" gametophyte-specific genes are also expressed in the anther tissues with the majority being specific to the secretory tapetal cells. These results were obtained using RNA *in situ* hybridizations. For example, the I3 gene (now renamed OlnB1) in *B. napus* initially characterized as a pollen-specific oleosin (Roberts *et al.*, 1993) was found by RNA *in situ* hybridizations to be expressed exclusively in the tapetal cells (Ross and Murphy, 1996). The only microspore-specific clone, NTM19, from tobacco (Oldenhof *et al.*, 1996) was isolated from a cDNA library prepared from immature microspores (free of sporophytic tissues) and characterized with RNA *in situ* hybridization.

The *Bnm1* gene was isolated on the basis of its high abundance from a library prepared from RNA isolated from microspores and pollen induced to undergo embryogenesis. Northern blot analyses and RNA *in situ* hybridization indicated that *Bnm1* expression was both tissue-specific and developmentally regulated. *Bnm1* mRNA accumulated in bicellular and tricellular pollen collected from plants and from pollen developing in tissue culture. Heat treatments that block pollen development (Fan *et al.*, 1988) also reduced *Bnm1* mRNA levels. This is consistent with immediate changes in gene expression which occur when developmental programs are re-directed. The developmental pattern of *Bnm1* expression suggests that it is a late pollen-specific gene.

Of the relatively few pollen genes characterized fewer have been analysed for their level of conservation or divergence across taxa. The distribution of pollen-specific genes in other genomes as assessed by either Southern, Northern or Western blot analysis would add another dimension to our understanding of their potential function in reproduction.

Bnm1 appears to be conserved at the DNA level and restricted to the Brassicaceae family where it resides as a single gene or small multigene gene family. Southern blot analysis suggests that the *Bnm1* clone isolated in this study is probably derived from the A genome (Fig. 18). It is highly conserved among members of the Brassicaceae tribe where it was found in all 10 species examined (Fig. 16). *Bnm1* is not related to sequences with known function; however, it is similar to two *Arabidopsis* partial EST cDNA clones (85% identity over 402 bases of ORF) obtained from a flower bud cDNA library (Genbank accession numbers F15396 and Z26006). Bands that may correspond to this gene were detected on Southern blots of *A. thaliana* genomic DNA but not in a related member of the Sisymbriaceae tribe, *C. hasslerana* suggesting that either the *Bnm1* sequence has diverged considerably at the nucleotide level outside of the Brassicaceae tribe or that it may have disappeared, in some cases (Fig. 17). Although other pollen-specific genes have been reported, few have been shown to be family specific. Exceptions are the Solanaceae gene *PRK1*, which encodes a receptor-like kinase (Mu *et al.*, 1994) and the Cruciferous gene, *Bcp1*, essential for male fertility (Xu *et al.*, 1995).

Another approach for assessing pollen-specificity involves analysing the promoter in transgenic plants. Although this method is indirect, it remains a highly sensitive approach towards studying the regulatory elements governing expression of individual genes separate from the other members of the gene family (Jefferson, 1987). This proved to be a successful approach for the pollen-specific translation initiation factor *eIF-4A* gene in tobacco (Brander and Kuhlmeier, 1995). *eIF-4A* belongs to a large superfamily of proteins which have a number of amino acid sequence motifs in common. Owttrim *et al.* (1994) had previously

shown by characterization of cDNA clones that at least 10 different eIF-4A genes are expressed in tobacco leaves.

The use of the *gus* A reporter gene showed that the *Bnm1* promoter activity associated with the 817 bp fragment followed a similar pattern of tissue-specificity and developmental regulation in transgenic *B. napus* plants. A significant difference however, was the presence of GUS fluorogenic activity in tetrad and unicellular microspores. This activity was not detected histochemically. GUS histochemical staining of tetrads and unicellular microspores appears to be relatively insensitive under light microscope observation compared with the fluorogenic measurements of specific activity even when the cells were fractionated prior to incubation with X-gluc (data not shown). Artefactual staining of bicellular and tricellular WT pollen was not observed when tetrads and unicellular microspores from transgenic plants were mixed together prior to incubation with X-gluc (data not shown). It was thought that perhaps the cleaved GUS substrate, if any, could not bind to the immature microspore cell wall. Staining was not observed in any other anther tissues of the transgenic plants indicating that GUS activity must be low or absent in these tissues. At this time, we conclude that either the promoter fragment used in this study may not reflect the final temporal expression pattern of the endogenous *Bnm1* gene, or that the endogenous *Bnm1* transcript may be less stable than the *gusA* transcript.

In tissue culture, heat treatments that block pollen development, did not trigger promoter activity. These observations come from histochemical analysis only. Fluorometric analysis, mRNA stability analysis and run-on transcription analysis are required before confirming that regulation of the *Bnm1* gene, committed to the pollen pathway, is at the level of transcription.

The activation of the *Bnm1* promoter fragment at the tetrad stage of microsporogenesis now becomes a valuable agricultural tool. The spatial and temporal expression profiles dictated by the 817 bp *Bnm1* promoter fragment have led to a patent on the microspore and pollen-specific promoter. Mariani *et al.* (1990, 1992) have developed a

genetically engineered male fertility control system in tobacco which relies on a tapetal cell-specific promoter used to disrupt tapetal cells essential to pollen development. This system involves the use of both RNase/RNase-inhibitor genes driven by the TA29 tapetal cell specific promoter. In short, the RNase driven gene leads to pollenless male-sterile plants, while the RNase-inhibitor gene driven by the same promoter acts as a dominant suppressor when crossed with the RNase male sterile plants restoring fertility. The progeny harbour both constructs and are male fertile. The *Bnm1* promoter has the potential to develop a similar system in *Brassica*. For example, this system has the advantage of manipulating *B. campestris*, another cash crop within the same family. Besides being an important commercial cultivar, *B. campestris* is more resistant to frost and drought when compared with *B. napus* and is therefore better suited to the Southern Ontario environment as a winter variety. Although self-incompatible, it is easily crossed with *B. napus* and can therefore, benefit from any value-added biotechnology.

4.2 Functional Analysis

The role of *Bnm1* in pollen development, germination or species recognition is not presently known despite the efforts of this thesis. However, considering the highly conserved pattern of pollen development across diverse families (McCormick, 1993) and the restricted occurrence of *Bnm1* within the Brassicaceae family, it seems unlikely that *Bnm1* plays a fundamental role in the development of pollen. Given the possibility that *Bnm1* codes for an abundant protein that may be secreted in mature pollen raises speculation that it may play a role in pollen morphology and species recognition.

One direct way of determining the biological role of pollen-specific genes is by creating mutations using antisense constructs of these genes driven by a promoter fragment that dictates the correct spatial and temporal expression profiles of that gene in transgenic plants. An example of this approach comes from pTOM13 isolated from a cDNA library from

clones expressed during tomato fruit ripening. Transgenic tomato plants harboring the pTOM13 antisense construct showed that ethylene synthesis was strongly inhibited, suggesting the protein is involved in ethylene biosynthesis (Bouzayen *et al.*, 1992).

Antisense technology for assessing function of pollen-specific genes has only been carried out on 3 different occasions. The observed phenotype in *LAT52* antisense plants, was abnormal hydration *in vitro*, and the production of corkscrew pollen tubes unable to achieve fertilization *in vivo* (Muschietti *et al.*, 1994). Muschietti *et al.* (1994) speculated that although the *LAT52* protein has similarity to several Kunitz trypsin inhibitors (a proteinase inhibitor) *LAT52* may be more related to a family of peptides that negatively regulate a Na⁺, K⁺-ATPase. This speculation comes from work by Araki *et al.* (1989) who reported this negative regulatory effect from a family of porcine peptides having high similarity to proteinase inhibitors but not showing inhibitory activity.

Although an antibody was generated against the *LAT52* protein, the temporal expression profile of the *LAT52* protein in relation to the *LAT52* transcript was not undertaken. Furthermore, the *LAT52* antisera did not recognize the native configuration of the *LAT52* protein and attempts to immunolocalize *in situ* were unsuccessful.

Xu *et al.* (1995) reported that *Bcp1* pollen from antisense transgenic plants show a 1:1 segregation of viable/ aborted pollen. These aborted pollen grains were shriveled and void of cytoplasm. *Bcp1* has no sequence similarity to proteins of known function. It was concluded, therefore, that *Bcp1* is essential for male fertility, however, the physiological basis of the lesion remains to be elucidated. No antibodies have been generated against the *Bcp1* protein, therefore, no information regarding temporal expression profiles or localization of the gene product *in situ* are available.

Lee *et al.* (1996) reported similar findings when pollen from *PRK1* antisense plants was analysed. *PRK1* encodes a receptor-like kinase and obviously, a signal transduction mechanism mediating post-meiotic development was proposed. Although antisera were

developed during the characterization of *PRK1* (Mu *et al.*, 1994), no attempt was made to define the temporal expression profile nor localization *in situ* of the PRK1 protein.

To address the biological function of *Bnm1*, antisense RNA methodology to inhibit the synthesis of BNMI in transgenic plants was carried out. However, before screening transgenic plants for reduced levels of *Bnm1*, the temporal expression profile of the *Bnm1* protein, BNMI, was investigated. It was thought that if *Bnm1* is post-transcriptionally regulated, the BNMI protein may be involved in pollen germination and or pollen tube growth processes, the next developmental step of the male gametophyte program. This experiment would determine "when" the BNMI protein is synthesized and hopefully allow to us pinpoint at which developmental stage to look at for potential lesions.

In order to characterize the *Bnm1* gene product, antisera were raised against a GST-BNMI fusion protein expressed in *Escherichia coli*. The polyclonal antibody detected a single band of ca 20 kDa in total protein extracts of tricellular and germinating pollen. The apparent molecular mass of this BNMI candidate is consistent with the predicted size from the sequence of the open reading frame. Previous computer analysis of the putative BNMI protein predicted a signal sequence with a potential cleavage site between amino acid 16 (Thr) and 17 (Leu). SDS-PAGE gels did not provide the resolution required to define whether the BNMI peptide is cleaved at its potential site. It is, therefore, not clear at this time if the BNMI peptide is post-translationally modified by removal of the signal peptide or any other modifications, such as, phosphorylation.

However, *Bnm1* appears to be post-transcriptionally regulated. Although *Bnm1* mRNA is first detected at the bicellular stage of pollen development, the BNMI protein is first detected at the tricellular stage. No BNMI protein was detected in any other developmental stage, including tetrad, unicellular microspores and bicellular pollen or in sporophytic tissues, stem and leaf. These findings now provide evidence of a "late" pollen-specific gene being post-transcriptionally regulated. It also provides insight towards which developmental process BNMI is involved, i.e since the structure and morphology of the

tricellular pollen grain is established at this point in development, it seems reasonable to expect that the BNM1 protein is involved in the early stages of germination and/or pollen tube growth.

To screen for downregulation of *Bnm1* in antisense transgenic plants, mRNA and protein levels in pollen from wild-type and antisense transformants were compared. Two plants #1 and #11 appear to be downregulated at the protein level (data not shown), while five plants #7, #11, #17, #20 and #26 are drastically downregulated at the RNA level. These plants were further analysed for a potential developmental lesion.

Direct observations of pollen structure and evaluations of the integrity of membranes by FDA staining along with the time of appearance of the BNM1 protein suggest that BNM1 must not be involved with the development of pollen and/or overall structural morphology but, rather, with germination, pollen tube growth and fertilization. Although observations from *in vivo* germinations of plants downregulated for *Bnm1* were not conclusive, there does appear to be a reduced level (although not quantified) of germination and pollen tube growth down the style *in vivo*.

4.3 Conclusions

Throughout my research I have characterized a new pollen-specific gene, *Bnm1*, and demonstrated different levels of developmental and tissue-specific controls that accompany the commitment to pollen development. These findings have contributed to a patent for the *Bnm1* promoter (US #5633438, issued 05/27/97), and have provided practical applications towards the production of male sterility in transgenic plants for hybrid seed production.

In addition to the characterization of *Bnm1*, the temporal expression profile of a previously characterized pollen-expressed gene, *Bp4* (Albani *et al.*, 1990) has been re-defined (Treacy *et al.*, 1997).

The work also reveals a new example of a "late" pollen-specific transcript that is translated *prior* to germination unlike those described by Mascarenhas *et al.* (1984). Those experiments carried out on *in vitro* translation of total mRNA from mature and germinating pollen as well as from total *in vivo* proteins from mature and germinating pollen as described by Bedinger and Edgerton (1990), Schrauwen *et al.* (1990) and Mandaron *et al.* (1990) may simply mask the post-transcriptional regulation patterns of the 10% of transcripts which are exclusive to pollen-specific genes.

Considering the highly conserved pattern of conservation of *Bnm1* from Southern blot analysis it is likely that *Bnm1* may be involved with pollen-pistil interactions as was suggested for the other two family-specific, pollen-specific genes *PRK1* and *Bcp1* (Table 1). Antisense experiments carried out for *PRK1* and *Bcp1* generated a phenotypic lesion at mid and late bicellular stages of development, respectively. This would suggest that the gene product of both of these genes is required for proper pollen maturation of bicellular pollen independent of species recognition events. The BNM1 protein, on the other hand, is only synthesized at the tricellular stage of development and is also present in germinating pollen. This is consistent with the expression profile of a gene involved in pollen-pistil interactions and/or pollen germination and pollen tube growth and not with the development and maturation of the pollen grain.

Since no obvious lesions affecting cell viability occur at the tricellular stage of pollen development in antisense transgenic plants, future functional analysis of *Bnm1* should target germination, the next developmental process in species recognition.

4.4 Is *Bnm1* Involved in Pollen-Pistil Interactions?

In retrospect, *Bnm1* has no sequence similarity to putative enzymes such as, polygalacturonase or pectate lyases. It is probably not a likely candidate for the enzymatic activity required to penetrate the papillae cell of the stigma. Although BNM1 encodes a

putative signal peptide, it has no consensus sequence to suggest secretion to a subcellular organelle. If BNM1 is secreted extracellularly, and considering the BNM1 protein has 2 putative phosphorylation sites (Fig. 13), it may be involved with cell-cell communication which take place at the pollen-papillar cell interface. Here, BNM1 may act as an SI ligand necessary to trigger a compatible pollination, or as a protein involved with the establishment of a zone of adhesion required for proper hydration and germination. A major structural hallmark of members of the SI gene family, including pollen coat peptides shown to bind stigmatically expressed S-locus proteins, is a cysteine-rich domain consisting of 10 to 12 cysteines arrayed in a precise order (Nasrallah and Nasrallah, 1993). The BNM1 protein only has two cysteines in its polypeptide sequence. Furthermore, the characteristic structure of the principal proteins involved with pollen adhesion on the stigma surface and pollen tubes adhering to the transmitting tissue down the style is the heavy glycosylation of these members (Doughty et al., 1992, 1993). Computer analysis of the BNM1 protein does not identify any potential glycosylation sites. Although BNM1 may not be a good candidate for being directly involved with SI and/or cell adhesion, it should not be ignored as an intermediate player. It remains unclear at this time how many *other* proteins may be involved with these processes (Dickinson, 1995).

Alternatively, if BNM1 is not secreted and remains in the cytoplasm. It may be involved with; motor activity, cytoplasmic streaming, interacting with specific style-specific proteins which enter the pollen tube and are thought to play a role in nutrition of the growing pollen tube or BNM1 may play a role in guiding the pollen tube towards the ovary (Lind *et al.*, 1996).

4.5 Future Studies

Perhaps future characterizations of pollen-specific genes from model plants shedding pollen at the bicellular stage of development at anthesis should include a more specific marker

of maturity to better define the exact temporal expression profiles in relation to tricellular pollen species. For example, Liu *et al.* (1997) used cytology to define the appearance of SB401 transcripts *after* the mid-bicellular stage of development.

Further functional analysis of BNM1 should include more *in vitro* germination assays for transgenic antisense plants. Although these experiments were carried out, the *in vitro* assays became unreliable due the senescing state of the plants at that time. These assays would offer a quantitative approach over *in vivo* germination assays. Also, these assays would offer the opportunity to define the level of germination inhibition when compared to wild-type and the possibility of rescuing this lesion by supplementation of the media. Once the plants showing the strongest phenotype have been identified they can be further studied at the *in vivo* level. This offers the opportunity to cross-pollinate and define the lesion as legitimate and not an artifact of tissue culture.

In vitro germination assays may not produce a visible phenotype. *In vitro* germination media supply only compounds such as sucrose and calcium which are only sufficient to permit germination in a standardized *osmoticum*. *In vivo* germination, however, involves many more complex molecules and proteins required for proper hydration, incompatibility interactions, penetration and growth through the papillar cells of the stigma before reaching the style. All of these processes are thought to be under the control of many gene products including pollen-specific gene products and can, therefore, be overlooked in *in vitro* assays. *In vivo* germination, therefore may provide a visual phenotype, although difficult to quantify.

This leads to the importance of the putative signal peptide at the N-terminus of BNM1 and immunolocalization. N-terminal amino acid sequencing of the BNM1 protein would define if the mature protein is cleaved and provide valuable information towards the property of BNM1 entering the secretory pathway.

Attempts to immunolocalize the BNM1 protein *in situ* were unsuccessful. Perhaps inoculating a few mice (not just two) with proper folding of the BNM1 protein obtained through dialysis should be considered for future production of polyclonal antibodies. Location

of BNMI in germinating pollen may give more insight as to function. Immunofluorescence is a quick method to assess location and may give clues towards BNMI being secreted as well. Immunolocalization of *in vivo* germinations at different time periods coupled with germination analysis of down-regulated antisense plants would be ideal and may define the biological role of *Bnm1* in sexual plant reproduction.

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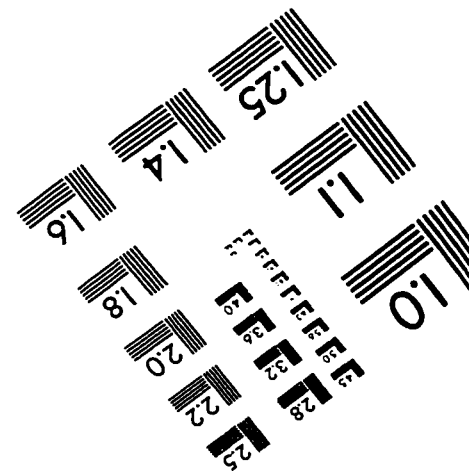
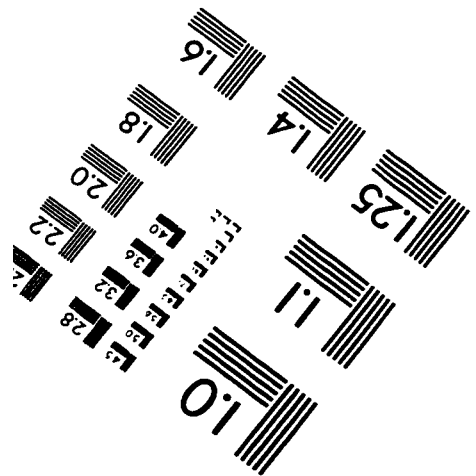
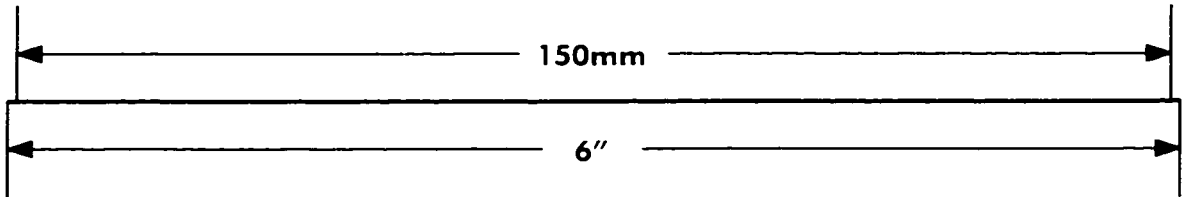
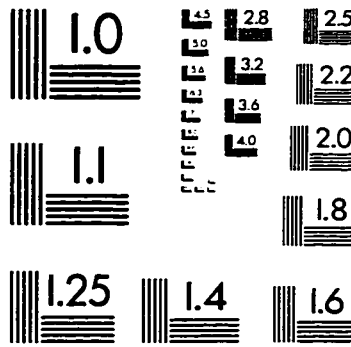
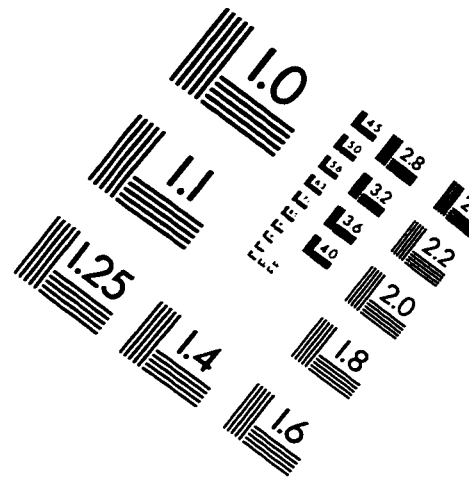
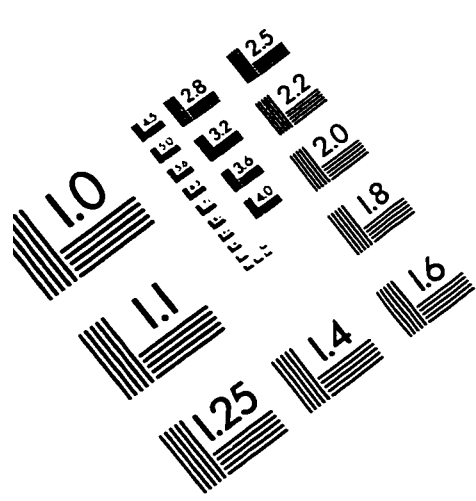
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IMAGE EVALUATION TEST TARGET (QA-3)



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