The Plant Cell, Vol. 3, 1073–1084, October 1991 © 1991 American Society of Plant Physiologists

Isolation and Developmental Expression of Bcp1, an Anther-Specific cDNA Clone in *Brassica campestris*

Piyada Theerakulpisut,[1] Huiling Xu, Mohan B. Singh, John M. Pettitt,[2] and R. Bruce Knox[3]

School of Botany, University of Melbourne, Parkville, Victoria 3052, Australia

Differential screening of a mature *Brassica campestris* pollen cDNA library has identified five cDNA clones that represent transcripts expressed exclusively, or at elevated levels, in pollen. We show here that the expression of one of these, clone Bcp1, is tissue specific and temporally regulated. The gene is activated during microspore development, as detected by in situ hybridization. Expression is enhanced at the time of pollen maturation and during pollen germination. In situ hybridization has also shown that Bcp1 is activated in the tapetal cells in early anther development and continues to be expressed until tapetal dissolution. Homologous transcripts are present in pollen of other taxa of Brassicaceae including Arabidopsis, but not in pollen of any other families tested.

INTRODUCTION

Pollen, the male gametophyte of flowering plants, plays a central role in sexual reproduction. This role is in the production and transmission of the sperm cells to the embryo sac for double fertilization. The progenitor of the sperm cells, the generative cell, is present in bicellular pollen at dehiscence. This is a cell within a cell because it is encompassed within the vegetative cell (Knox, 1984). In bicellular pollen such as tomato, division of the generative cell to form two sperm cells takes place after pollination within the confines of the growing pollen tube. In contrast, formation of the sperm cells occurs simultaneously with maturation of the pollen grain in tricellular types, including Brassica.

Pollen grains have a number of unique structural characteristics, including the outer wall, which is made of sporopollenin, the pollen tube wall, which is lined with a layer of callose (Knox, 1984), and the structural organization of the sperm cells in a single transmitting unit, the male germ unit (Knox et al., 1988). Both pollen types are highly specialized to perform the complex functions of pollination and cell-cell recognition that manifest themselves in self-incompatibility reactions with pistil tissues.

Considering the key role in the reproductive cycle that is played by the pollen grain and its unique structure and organization, the question arises as to what type of developmentally expressed genes are involved in the successful production of mature, viable pollen grains. Unique mRNAs as well as mRNAs common to sporophytic tissues are expressed during pollen development (Mascarenhas, 1989), the former comprising only about 10% of the total pollen-expressed transcripts. These are likely to control pollen-specific functions and, consequently, have created a great deal of interest.

Various approaches have been pursued to identify pollen-specific genes that are expressed exclusively or show elevated levels of expression in pollen. The first approach involved the preparation of cDNA libraries from whole anthers and anther-specific clones selected by differential screening with sporophytic RNAs (McCormick et al., 1987; Goldberg, 1988; Nacken et al., 1991). The specificity within the complex tissues of the anther has been addressed by in situ hybridization (Smith et al., 1987; Ursin et al., 1989; Koltunow et al., 1990). Smith et al. (1987) and Koltunow et al. (1990) obtained clones specifically expressed in the tapetal layer of the anther. Ursin et al. (1989) obtained cDNAs expressed in pollen as well as in the endothecial and epidermal layers of the anther wall. In these studies, whole anthers were the source of RNA for the cDNA libraries. Consequently, it is not surprising that most of the clones isolated were not pollen specific, the tapetum and anther wall cells being major components of the anther, and the tapetum is an active secretory tissue (Knox, 1984).

[1] Current address: Department of Biology, Khon Kaen University, Khon Kaen 40002, Thailand.

[2] Current address: Department of Pathology and Immunology, Monash University Medical School, Prahran, Victoria 3181, Australia.

[3] To whom correspondence should be addressed.

The second approach to the isolation of pollen-specific clones has involved use of isolated microspores or pollen as the source of RNA (Stinson et al., 1987; Brown and Crouch, 1988). cDNA libraries were made from mature pollen and pollen-specific clones isolated by differential screening against sporophytic tissues. A variation on this theme was the use of a genomic library and screening with microspore RNA as well as sporophytic tissues of *Brassica napus* to isolate microspore-specific clones (Albani et al., 1990, 1991).

A third approach involved the use of specific monoclonal antibodies to defined pollen-specific antigens or specific IgE antibodies from allergic humans to select clones encoding allergenic proteins of grass (Griffith et al., 1991; Silvanovich et al., 1991; Singh et al., 1991) and ragweed pollen (Rafnar et al., 1991). The grass pollen cDNA clones proved to be pollen specific by RNA gel blot analysis.

For a number of reasons, we have selected *B. campestris* as an experimental system to identify pollen-specific genes. It is easily cultivated in the greenhouse, and four generations can be obtained per year with the rapid cycling cultivars. Brassica is closely related to the model plant Arabidopsis; its breeding system is also well known, and the molecular biology of pollen-stigma interactions has been well characterized (Nasrallah, 1989). We have studied the structural biology and physiology of the pollen, characterized the male germ unit (McConchie et al., 1985, 1987), and identified a mutant plant defective in expression of β-galactosidase in the pollen (Singh and Knox, 1984, 1985).

In this paper, we report the isolation of five cDNA clones from a mature pollen cDNA library that are pollen specific. One clone, designated Bcp1, has been characterized and sequenced. In situ hybridization shows that Bcp1 is expressed in mature pollen grains but also in developing microspores and tapetal cells. This represents a new class of transcript that is not represented in earlier studies.

Table 1. Results of the Hybridization Experiment Designed To Evaluate the Pollen Specificity of Clones Bcp1 to Bcp10

	Hybridization with Labeled cDNA from				
Clone	Pollen	Leaves	Seedlings	Flowers[a]	Fruits
Bcp1	+	–	–	–	–
Bcp2	+	–	–	–	–
Bcp3	+	–	–	–	–
Bcp4	+	–	–	–	–
Bcp5	+	–	–	–	–
Bcp6	+	+	+	+	+
Bcp7	+	–	–	+	–
Bcp8	+	–	+	+	+
Bcp9	+	+	+	+	+
Bcp10	+	+	+	–	–

[a] Minus anthers.

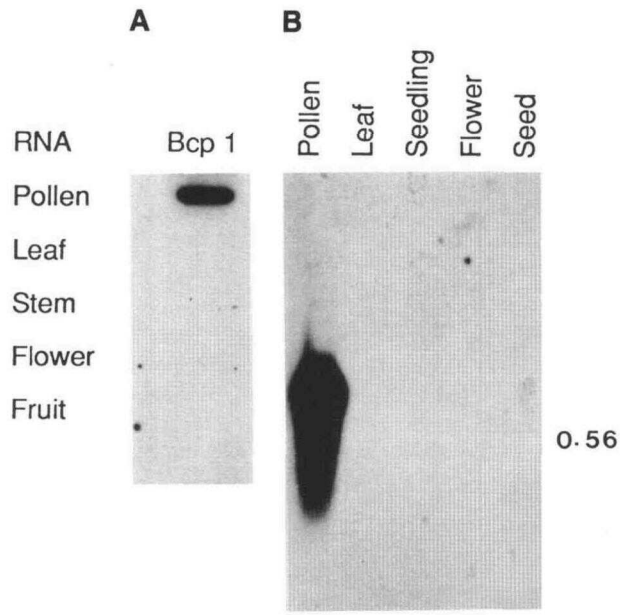

Figure 1. RNA Hybridization To Demonstrate Specificity of Bcp1 mRNA.

Total RNA isolated from the indicated tissues of *B. campestris* was hybridized with a radiolabeled Bcp1 cDNA probe as outlined in Methods.

(A) Hybridization of Bcp1 with slot blots of total RNA (5 μg per slot).

(B) Hybridization of Bcp1 with RNAs from different tissues fractionated on a denaturing agarose gel and then transferred to a nitrocellulose membrane (20 μg per lane).

The length of the RNA marker is indicated in kilobases at right.

RESULTS

Isolation of cDNA Clones Complementary to mRNAs Predominantly Expressed in Pollen

A differential screening procedure has been used to identify and isolate clones representing genes with preferential expression in mature pollen relative to expression in various sporophytic organs. The initial high density screening resulted in isolation of 200 to 250 plaques that showed substantially stronger hybridization to the pollen cDNA probes than to leaf cDNA probes. The putative pollen-abundant clones were plated at low density for secondary screening to isolate single plaques. Replicate filters from secondary screening were hybridized with leaf and pollen probes. A total of 46 clones showing detectable hybridization with the pollen probes and nondetectable hybridization with the leaf probes were isolated.

Ten individual clones were randomly chosen for further screening, which involved hybridization with ^{32}P-labeled cDNA synthesized from pollen, leaves, seedlings, flowers

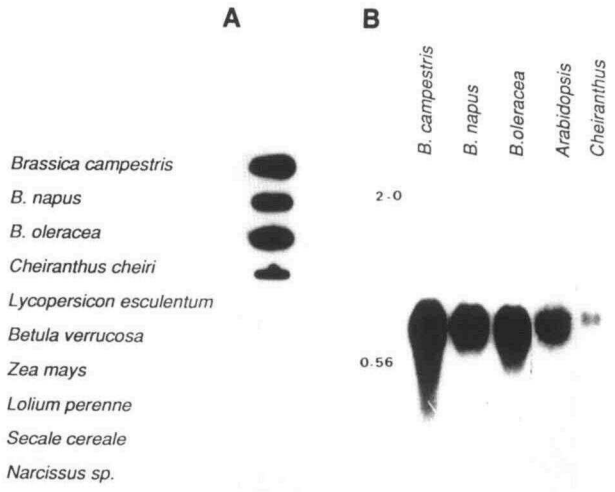

Figure 2. Representation of RNAs Homologous to Bcp1 in Other Plant Species.

Total RNA was isolated from mature pollen of indicated plant species and hybridized with radiolabeled Bcp1 probe.
(A) Hybridization of Bcp1 with RNAs from 10 plant species. Five micrograms of total RNA was used for each slot.
(B) Hybridization of Bcp1 with RNAs from five members of the family Brassicaceae that were fractionated on a denaturing agarose gel and transferred to a nitrocellulose membrane (20 μg per lane).
The lengths of the RNA markers are indicated in kilobases at left.

without anthers, and fruits. Five clones (1 to 5) showed no hybridization with any of the four cDNA probes from sporophytic tissues in the overexposed autoradiograms. Another five clones (6 to 10) showed hybridization with the cDNA probes from different sporophytic tissues as well as pollen. The results of plaque hybridization experiments are summarized in Table 1, which shows the specificity of expression of the 10 clones in different plant tissues. Cross-hybridization studies showed that all these clones are likely to represent unique sequences. Clone Bcp1 was chosen for detailed analysis because of its strict pollen specificity and the abundance of mRNA corresponding to this clone in mature pollen. The tissue specificity of Bcp1 gene expression is shown in Figure 1, using RNA blotting and RNA gel blot analysis. Labeled Bcp1 showed no hybridization to RNA from sporophytic tissues. A transcript of approximately 700 nucleotides from pollen showed strong hybridization to labeled Bcp1.

RNAs Homologous to Bcp1 Are Expressed in Other Genera of Brassicaceae

Figure 2A shows that transcripts hybridizing with Bcp1 cDNA were detected only in pollen of other species of *Brassica* and other genera in the family Brassicaceae that were tested. The transcripts detected in pollen of the related genus *Cheiranthus* showed a lower level of expression. Little or no expression was detected using high-stringency washing in pollen of the other families of flowering plants tested. Similar results were obtained with both fresh and stored pollen samples. For Arabidopsis, RNA gel blot analysis was carried out with flower bud RNA because of difficulty in isolating enough RNA from pollen only. Figure 2B shows that Arabidopsis flower RNA gave a transcript similar in size to those in the four species of the genus *Brassica*.

Bcp1 RNA Accumulates in Both Developing Pollen and Tapetum

Figure 3A shows that the mRNA complementary to Bcp1 was first detected in trace levels in 3-mm buds. This is the early tricellular period when the generative cell mitosis that produces the sperm cells has occurred (Singh et al., 1985). High levels of expression of Bcp1 RNA were detected in anthers from 5- and 6-mm buds and in mature pollen.

Figure 3B shows that the mRNA transcripts corresponding to Bcp1 were detectable after 5 hr of in vitro culture.

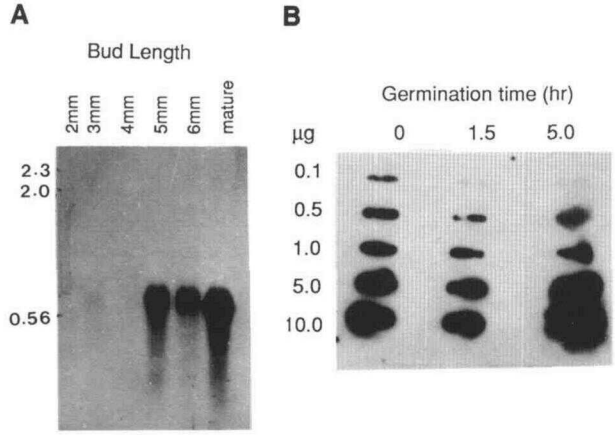

Figure 3. Accumulation of Transcripts Corresponding to Bcp1 during Different Anther Development and Pollen Germination Stages in *B. campestris*.

Total RNA was isolated from anthers and/or pollen at six stages of development, either slot blotted onto a nitrocellulose membrane or fractionated on denaturing agarose gels, and transferred to nitrocellulose membrane. Slot blots and gel blots were hybridized with a radiolabeled Bcp1 probe as outlined in Methods.
(A) Hybridization of Bcp1 with anther/pollen transcripts in gel blots (20 μg of total RNA per lane).
(B) Hybridization of Bcp1 with different quantities of total RNA isolated from mature pollen and after in vitro germination for 1.5 and 5 hr.
The lengths of the RNA markers are indicated in kilobases at left.

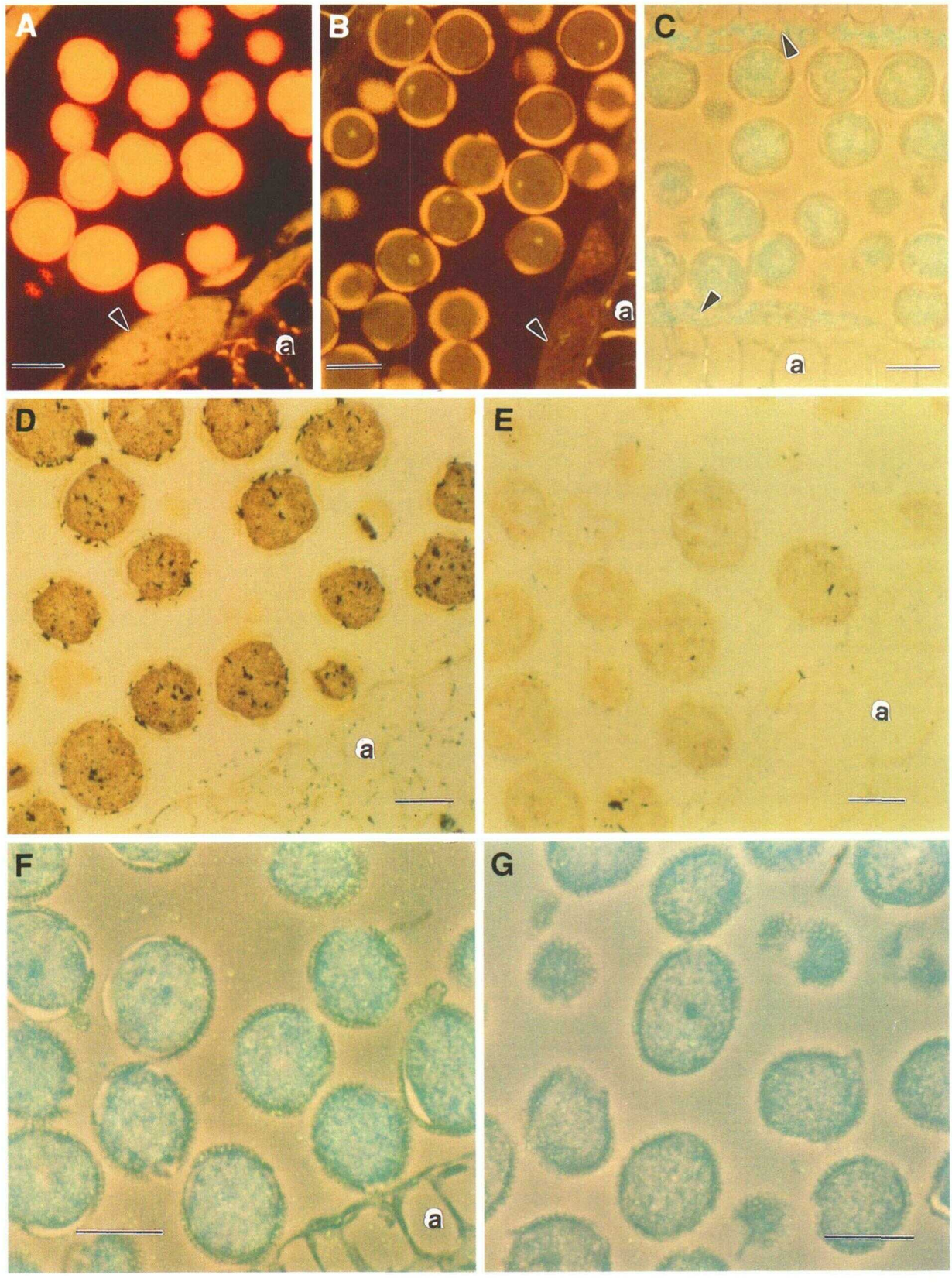

Figure 4. Localization of Bcp1 mRNA during Anther Development in *B. campestris* by in Situ Hybridization of Tangential Sections.

At this stage, the pollen tubes had grown to a length of approximately five to six times the diameter of the pollen grains, and more than 80% of the grains had germinated.

In situ hybridization allowed a precise cellular localization of gene expression in the anther. The specificity of the hybridizations was first determined. Figures 4A and 4B show that treatment of pollen sections with RNase successfully removed the RNA detectable by acridine orange fluorescence. The two different nonradioactive reporter systems used were digoxygenin and biotin. Figures 4D and 4F show that in situ hybridization with both systems gave a strong signal in test sections of anthers containing near mature pollen. Figures 4E and 4G show that only a weak background signal was obtained after treatment of sections with RNase.

The digoxygenin-labeled cDNA hybridized strongly to developing pollen in >5-mm buds, as shown in Figures 5D, 5E, and 5F. Low levels of hybridization were detected in microspores or pollen in smaller buds at earlier developmental stages (Figures 5A, 5B, and 5C). However, in 3- and 4-mm buds, the tapetal cells showed a positive signal with the alkaline phosphatase reporter molecules (Figures 5B and 5C). Figure 5B shows that in 3-mm buds the tapetal signal, when detected with the alkaline phosphatase probe, was so intense that precipitation of the cytochemical reaction product occurred. Figures 4D and 5F show that in mature or near-mature pollen the signal was also intense enough to give a general positive stain and a dark granular deposit on the sections.

Figure 6A shows that the mRNAs from 2-mm buds gave a positive signal in both the tapetum and vacuolate microspores, using the biotin-labeled cDNA. No specific signal was observed in the anther wall tissues. A strong signal was obtained in tricellular pollen (4-mm bud, Figure 4C; 6-mm bud, Figure 6B). This signal appeared to be confined to the cytoplasm of the vegetative cell.

Nucleotide Sequence of cDNA Bcp1

The nucleotide sequence and putative open reading frame of Bcp1 are shown in Figure 7. The results of the primer extension reaction indicated that Bcp1 contains a full-length copy of the mRNA (H. Xu, unpublished data). The longest open reading frame identified showed the predicted 119-amino acid sequence, which is equivalent to 12 kD. The protein contained 16% alanine.

Figure 8 shows that the results of a plot of the average hydrophobicity of Bcp1, using the values and algorithm of Kyte and Doolittle (1982), revealed an approximately 28-amino acid hydrophobic region at the N terminus.

Identification of Bcp1-Encoded Protein

To identify the protein encoded by Bcp1, antibodies were raised against two synthetic peptides according to the deduced amino acid sequence. Figure 9 shows that a 12-kD protein band was detected by both polyclonal antisera in the mature pollen extract. This polypeptide was also detected but at a reduced level in the germinated pollen extract. This 12-kD band, corresponding to Bcp1, appears to represent a major pollen protein, as judged by the intensity of the corresponding band stained with Coomassie Brilliant Blue R 250 shown in Figure 9. No protein gel blot signals were detected in other sporophytic tissues (data not shown).

DISCUSSION

The expression of Bcp1 is tissue specific and temporally regulated during anther development in *B. campestris*. The gene is activated during microspore development, and expression is enhanced at the time of pollen maturation and remains high in germinating pollen. Expression of Bcp1 during development conforms to the pattern of the late-expressed pollen genes (Mascarenhas, 1990). Albani et al. (1990, 1991) have recently detected early expressed genes in *B. napus*; however, the expression of the genes decreases significantly during pollen maturation.

A unique feature of Bcp1 gene expression is its occurrence in the haploid pollen grains and in the diploid tapetal

Figure 4. (continued).

(A) and **(B)** Fluorescent detection of RNA in 4-mm buds using the fluorochrome acridine orange.
(A) Test section showing orange-red fluorescence of RNA and green fluorescence of DNA.
(B) Control after treatment with RNase, showing the absence of detectable RNA fluorescence in the section. The tapetum is indicated by arrowheads; fluorescence microscopy with blue excitation.
(C) to **(G)** In situ hybridization.
(C) Section of a 4-mm bud after hybridization in which a biotin reporter molecule was detected using a streptavidin-colloidal gold/silver system, showing tapetal (arrowheads) and pollen hybridization signals using combined epipolarization and phase contrast optics.
(D) Section of 6-mm bud after hybridization with digoxygenin-labeled cDNA probe and hybridization signal detected with anti-digoxygenin antibodies conjugated with alkaline phosphatase (bright-field optics).
(E) Sequential section to **(D)** pretreated with RNase before hybridization; shows markedly reduced intensity of staining.
(F) Section of the anther from a 6-mm bud using a biotin reporter molecule as was done in **(C)**.
(G) Sequential section to **(F)** pretreated with RNase.
Bars = 20 μm; a, anther wall.

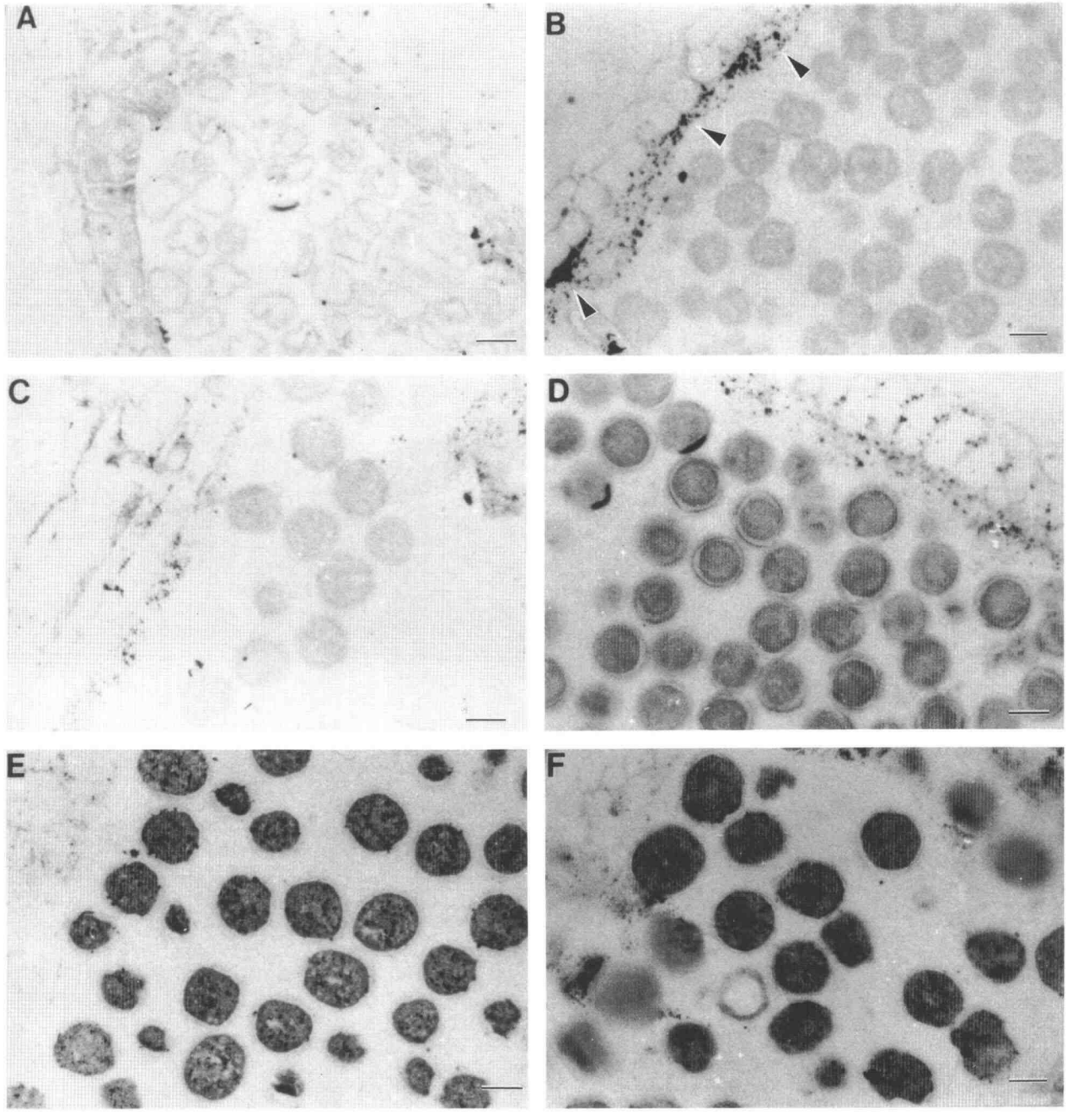

Figure 5. Localization of Bcp1 mRNA during Anther Development in *B. campestris* by in Situ Hybridization of Tangential Sections Using a Digoxygenin Reporter Molecule and an Alkaline Phosphatase Detection System

(A) Section of a 2-mm bud (unicellular microspore stage) showing a faint signal on tapetal cells.
(B) Section of a 2.5-mm bud (bicellular pollen stage). Arrowheads show intense signal resulting in precipitation of the reaction product on tapetal cells and faint signal on pollen grains.
(C) Section of a 4-mm bud (early tricellular pollen stage).
(D) Section of a 5-mm bud (midtricellular stage).
(E) Section of a 6-mm bud (late tricellular pollen stage).
(F) Section of a mature anther.
Bars = 20 μm.

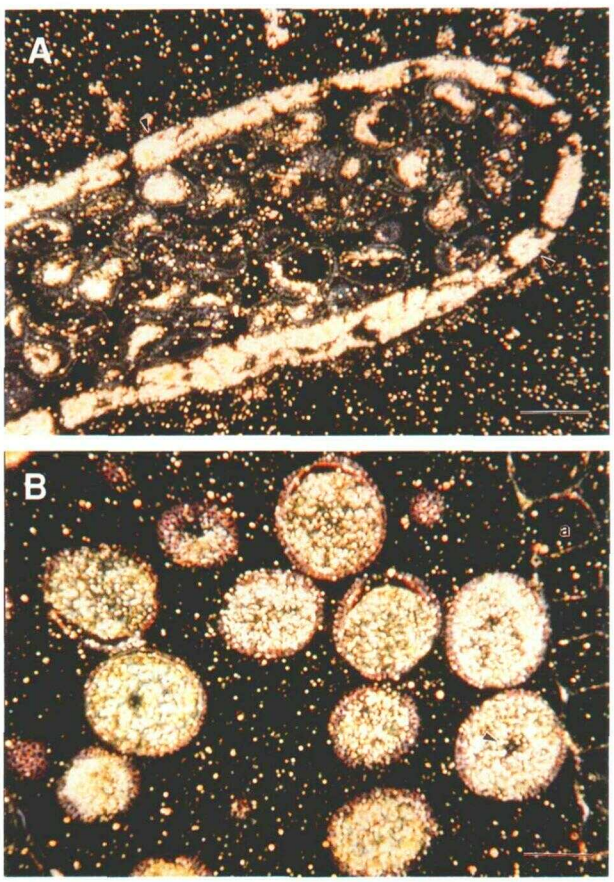

Figure 6. Localization of Bcp1 mRNA during Anther Development in *B. campestris* by in Situ Hybridization of Tangential Sections.

Biotin reporter molecules were detected using a streptavidin-gold/silver-enhanced system and viewed with dark-field optics to enhance resolution. Scattered gold particles that are not over the sectioned material represent background signal.
(A) Section from a 2-mm bud (vacuolate microspores) showing hybridization signal in a layer of tapetal cells (arrowheads) and in the microspore cytoplasm.
(B) Section from a 6-mm bud (near-mature pollen) treated as was done in **(A)** showing a signal in the pollen cytoplasm but not in the sperm cells (arrowheads) or anther wall cells (a). The tapetum has degenerated.
Bars = 20 μm.

cells. In situ hybridization has shown that Bcp1 is activated in the tapetal cells in early anther development and that expression continues until tapetal dissolution. The expression of Bcp1 in the tapetum is of considerable interest. During the microspore stage, the tapetum is physiologically very active. In *B. campestris* at this stage, the tapetum is parietal and is responsible for the secretion into the anther cavity of proteins (Heslop-Harrison et al., 1975) and lipids (Evans et al., 1990) that become part of the outer wall of the pollen grain. These include the 1,3-β-glucanase needed for dissolution of the callose special wall that surrounds the tetrad of microspores (Stieglitz and Stern, 1973), subsequent nutrition of the microspores, and secretion of wall polymers (Knox, 1984).

The pattern of Bcp1 expression in anther cells is similar to the previously reported cytochemical reactivity of esterase in anthers of *B. oleracea* (Vithanage and Knox, 1976). Esterase shows activity in both the early microspores and tapetum. One previous report of a gene expressed in both pollen and anther walls is that of anther-specific clones of tomato (Ursin et al., 1989). LAT52 mRNA was expressed in these tissues but not in tapetal cells. In the case of Bcp1, it is not known whether the transcripts expressed in tapetal cells and microspores represent expression of the same or closely related genes.

The molecular mass of the putative polypeptide encoded by the open reading frame of the Bcp1 mRNA is approximately 12 kD. There is a strongly hydrophobic region near the N terminus. This may indicate the presence of a signal peptide, and the amino acids of this region meet the requirements of a signal sequence (Heijne, 1987).

Antisera raised against two synthetic peptides, based on the deduced Bcp1 amino acid sequence, identified a ~12-kD protein in mature pollen. In germinated pollen, this protein is present at lower levels. This may be the result of the release of the Bcp1 protein from pollen into the germination medium. A number of proteins, including enzymatic proteins, have been reported to be released from the pollen wall during germination (Stanley and Linskens, 1965; Shayk and Kolattukudy, 1977). For example, the cutinases of nasturtium pollen show high activity in mature pollen, but after 3 hr of in vitro germination, almost all the activity was in the germination medium (Shayk and Kolattukudy, 1977). Previous cytochemical observations of the Brassica pollen wall have shown the presence of a number

```
                                                          ATCACAATCTC   11
TAACGCCAAACCCTAGAAATAAATAAAAAAGAGCGACGTAGAAAGAGAACAGAGAGAAGA   71
                                  10                        20
 M  G  R  Q  N  V  V  V  V  F  G  L  V  F  L  A  V  L  G  L
ATGGGTCGCCAAAACGTTGTTGTAGTTTTTGGCCTCCCGTGTTCTTGGCCGTCCTTGGCC  131
                                  30                        40
 A  A  A  A  S  S  P  S  P  S  A  S  P  S  K  A  P  S  T  S
TCGCCGCAGCTGCCTCCTCTCCGTCTCCTTCAGCGTCACCTTCCAAAGCTCCATCTACTT  191
                                  50                        60
 T  P  E  V  E  A  P  V  S  E  D  T  I  G  T  T  D  D  D  A
CCACACCTGAGGTCGAAGCTCCAGTGTCCGAGGACACCATTGGAACCACCGACGACGATG  251
                                  70                        80
 A  A  S  P  G  D  D  D  V  A  V  A  G  P  L  G  S  D  S  S
CAGCTGCTTCTCCTGGTGATGATGACGTAGCTGTGGCTGGTCCTCTAGGAAGTGACTCCT  311
                                  90                       100
 Y  G  S  N  G  P  S  G  S  A  D  S  A  D  S  G  A  A  A  L
CCTACCGGTAGCAATGGACCTTCAAGGCTCTGCCGATTCTGCTGACAGCGGCGCGGCGGC  371
                                 110                       120
 G  V  S  A  V  V  V  G  V  T  S  I  V  G  S  F  L  F  F  *
TCTTGGAGTCTCTGCGGTCGTTGTGGGTGTTACATCCATCGTCGGTTCTTTCTTGTTTTT  431
CTGAGGTGTTGTATTTTCATGAGAATATCATTTTTGAGTGAAGACTATTTATATATATGG  491
AATATTGTGATGATCATGTTGTAATTTATTTCTCCTTGGTTATGAGAAACGATATCCTTG  551
AAAATAAAGCTGAAAATAAATTAAACTTTCCTTAGCAATAAAAAAAAAAAAAAAAAAAAA  611
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  661
```

Figure 7. Nucleotide and Deduced Amino Acid Sequences of cDNA Clone Bcp1.

The possible consensus polyadenylation sequence is underlined.

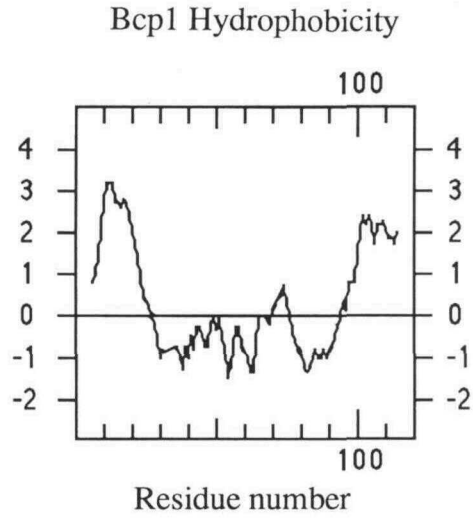

Figure 8. Hydropathy Plot of the Deduced Amino Acid Sequence of Bcp1.

Hydrophobicity was averaged over a window of 7 amino acids. Note the hydrophobic region at the N terminus.

of extracellular enzymatic proteins (Knox et al., 1975). These proteins are synthesized in the tapetum and transferred to the pollen wall late in development. Possibly, Bcp1 is in this category, or it may be a storage protein that is utilized as a nitrogen source during pollen germination. Immunobiological studies are in progress to clarify the function of Bcp1.

The fact that Bcp1 gene expression is taxonomically restricted to a single family suggests that the gene products may be involved in pollen-stigma interactions characteristic of the family Brassicaceae. These include recognition events associated with the sporophytic self-incompatibility reaction, which is well developed in this family. The protein encoded by Bcp1 is unlikely to be a common enzyme. Brown and Crouch (1990) reported that a pollen-specific clone of Oenothera hybridizes with RNA from genera in both monocot and dicot families, and from sequence homology they were able to show similarities with polygalacturonase.

Several other pollen- and anther-specific genes have been reported to show sequences homologous to various hydrolytic enzymes. These sequence similarities include two tomato anther-specific cDNA clones, LAT56 and LAT59, to pectate lyases (Wing et al., 1989) and a pollen-specific gene, Bp19, from *B. napus* to pectin esterases (Albani et al., 1991). A search of existing data bases, using both the nucleotide and the deduced amino acid sequence of the cDNA clone Bcp1, revealed no significant homology with any known proteins. The biological function of the Bcp1-encoded protein is yet to be determined.

A homologous genomic clone has been isolated from Arabidopsis. The presence of this transcript in Arabidopsis will enable the use of this system as a genetic tool to understand further the anther-specific expression of this gene. It is of especial interest to investigate the nature of the promoter region of this gene, which may elucidate whether there are both tissue and family specificities. We are currently using the antisense RNA approach to block the synthesis of the Bcp1 polypeptide in anther tissues. We wish to down regulate the activity of this gene separately in tapetum and pollen grains. The potential exists to modify Bcp1 expression only in pollen or the tapetum and then to determine the distribution of Bcp1 products in the anther. Previously, the hypothesis has been advanced from structural studies that the pollen-coat proteins are secreted from the tapetum. Such experiments should enable a molecular hypothesis on protein transfer to the anther to be tested.

METHODS

Plant Materials

Plants of oilseed rape, *Brassica campestris* cv T15, were grown in a greenhouse, and mature pollen was collected and stored in

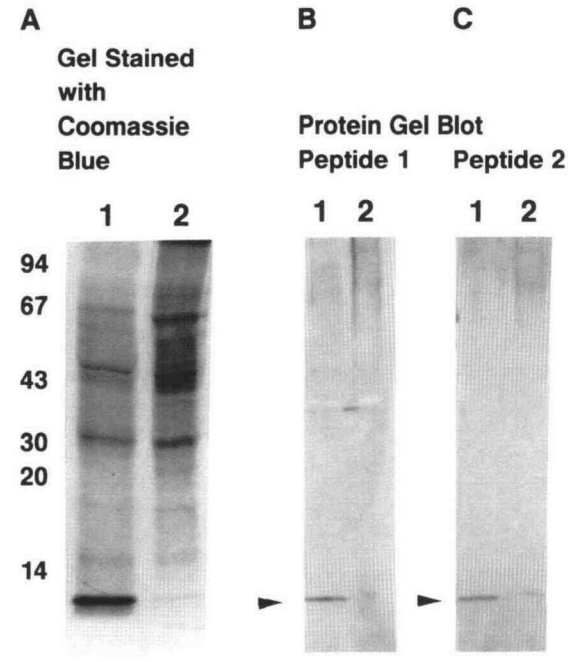

Figure 9. Identification of Bcp1-Encoded Polypeptides in Pollen.

(A) Coomassie Blue-stained 10% SDS-polyacrylamide gel of soluble proteins from *B. campestris* pollen. Lane 1, mature pollen proteins; lane 2, germinated pollen proteins.
(B) and **(C)** Immunoblots of the same proteins shown in **(A)** . The primary antibodies were 1:200 mouse antipeptide polyclonal

liquid nitrogen until needed for RNA isolation. Anthers at different developmental stages and various sporophytic organs were collected from plants as required for isolation of total RNA. Pollen of *B. napus*, *B. oleracea*, wallflower (*Cheiranthus cheiri*), tomato (*Lycopersicon esculentum*), and daffodil (*Narcissus sp*) was collected fresh from plants growing in the University garden. Pollen of birch, corn, rye-grass, and rye was obtained as stored dry pollen from Greer Laboratories (Lenoir, NC).

Isolation of RNA and Construction of cDNA Library

Total RNA was isolated from plant tissue according to the method of Herrin and Michaels (1984). Polyadenylated RNA was isolated by chromatography over poly(U)-Sepharose (Pharmacia). Based on A260, the total RNA preparations were found to contain 1.0 to 1.6% poly(A)$^+$ RNA. A cDNA library was constructed in λgt10 according to the manufacturer's instructions (Amersham; cDNA cloning system λgt10). All platings were done on *Escherichia coli* NM514.

Differential Screens

For the initial screening, 10,000 plaque-forming units of an amplified library were plated onto 150-mm plates. Nitrocellulose replicas were prepared from each of the plates according to standard methods (Maniatis et al., 1982). For plaque purification, 100 to 300 plaque-forming units were plated on 90-mm plates. Hybridization probes were synthesized from poly(A)$^+$ RNA isolated from pollen and sporophytic tissues using similar reactions as described for first-strand cDNA synthesis (Amersham; cDNA synthesis system), with the exception that the unlabeled dCTP was replaced by 100 μCi of ^{32}P-dCTP. The 40-μL reaction mixture was incubated at 42°C for 60 min, and the reaction was terminated by adding 1/10 volume each of 0.25 M EDTA, 10% SDS, and 80 mg of herring sperm carrier DNA. The labeled cDNA was depleted of template RNA by hydrolysis with 0.25 M NaOH at 65°C for 5 min, followed by neutralization with 2 M Tris-HCl, pH 7.2. Plaque lifts were prehybridized for 4 hr at 65°C in 2 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 7% SDS, 0.5% Blotto, 1% PEG 20,000, 0.5 mg·mL^{-1} denatured herring sperm DNA. The solution was replaced with a fresh hybridization solution containing approximately 0.5 to 1.0 × 10^6 cpm·mL^{-1} labeled single-stranded cDNA and incubated at 65°C for 20 hr. Filters were washed at 65°C for 30 min each in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS, 2 × SSC, 0.1% SDS, and twice in 0.2 × SSC, 0.1% SDS. Filters were exposed to Kodak X-Omat film overnight at −70°C.

RNA Blot Hybridization

For RNA gel blot analysis, total RNA was denatured in 20 mM 3-(*N*-morpholino)-propanesulfonic acid, 50% deionized formamide, and 2.2 M formaldehyde at 65°C for 5 min, electrophoresed in 1.2% agarose gel containing 2.2 M formaldehyde, and electroblotted onto nitrocellulose. The RNA slot-blot analysis was carried out by denaturing the total RNA in 20 mM 3-(*N*-morpholino)-propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA at 65°C for 10 min and applying the samples onto nitrocellulose saturated with 20 × SSC fitted in the Minifold 11 Filtration Manifold (Schleicher & Schuell). Both filters were prehybridized for 2 to 6 hr at 42°C in a solution containing 50% deionized formamide, 2 × SSPE, 1% SDS, 0.5% Blotto, 10% dextran sulfate, and 0.5 mg·ml^{-1} carrier DNA. Hybridization was carried out for 24 hr at 42°C in an identical solution containing a 0.5 to 1.0 × 10^6 cpm·mL^{-1} ^{32}P-labeled cDNA probe prepared by random oligonucleotide priming using an oligolabeling kit (Bresatec, Adelaide, Australia). The filters were washed in four changes of 2 × SSC, 0.1% SDS at 42°C for 2 hr, and then exposed to x-ray film as described previously.

Figure 9. (continued).

antisera, and the secondary antibody was 1:500 goat anti-mouse horseradish peroxidase conjugate. The positive bands were visualized as described in Methods.

Subcloning and Sequencing of DNA

The λ DNA was isolated from phage lysate using LambdaSorb Phage Adsorbent (Promega). The cDNA inserts digested from the λgt10 DNA were subcloned into bacterial plasmids pGEM4-Z (Promega). The recombinant plasmid containing Bcp1 was used as template DNA prepared as described in the Qiagen manual (Qiagen Inc., Chatsworth, CA) and sequenced entirely along both strands using both the T7 and Sp6 primers. Dideoxy sequencing was performed using the T7 Sequencing Kit (Pharmacia) and according to the manufacturer's instructions.

Sequence Analysis

Sequence analysis was carried out using the Melbourne data base system (MELBDBSYS), a collection of analysis programs developed at the Walter and Eliza Hall, Ludwig, and Howard Florey Institutes, University of Melbourne. This system incorporates data bases from the following sources: GenBank, EMBL, and NBRF nucleic acid libraries; NBRF PIR protein, PSD-Kyoto(Ooi), GBtrans, Swiss-Prot, and Doolittle protein libraries.

Analysis of Developmental Stages of Pollen

The developmental stages of Brassica microspores and pollen grains were determined by staining anther squashes with 20 μg·mL^{-1} DNA fluorochrome 4′,6-diamidino-2-phenylindole in 20% sucrose containing 0.1% Triton X-100 for 10 min in the dark (Vergne et al., 1987). Pollen grains were viewed by incident light fluorescence microscopy using the UV filter combination. The stage of pollen development corresponded with the length of the flower bud, as previously reported (Singh et al., 1985; Evans et al., 1990), and is as follows: 1-mm buds, meiosis complete and microspore tetrads present; 2-mm buds, microspore release from the tetrads followed by the vacuolate period, characterized by the formation of a single large vacuole in the cytoplasm of unicellular microspores; 3-mm buds, tapetum degenerates, microspore mitosis produces bicellular pollen; 4-mm buds, generative cell mitosis produces tricellular pollen; 5-mm buds, midtricellular pollen; 6-mm buds, near mature pollen grains, ovoid in shape, 28 × 35 μm.

Fixation, Embedding, and Sectioning of Anthers

The procedures for fixation and embedding of anthers were essentially as described by McFadden et al. (1988), with minor alterations. The aqueous fixative (4% paraformaldehyde and 0.25% glutaraldehyde in 50 mM Pipes, pH 7.3) was combined with an equal volume of *n*-heptane; the mixture was shaken for 1 min and left to stand until the phases separated. The heptane phase was carefully transferred to another container. Anthers dissected from fresh buds were immediately fixed in the heptane phase for 10 min, followed by fixation in the aqueous fixatives (4% paraformaldehyde and 0.25% glutaraldehyde in 50 mM Pipes, pH 7.3) for 2 hr at room temperature.

After fixation, anthers were washed in three changes of Pipes buffer for 30 min. The specimens were dehydrated in solutions of 10%, 30%, 50%, and 70% ethanol (30 min each), transferred to a mixture of ethanol:LR Gold (London Resin Company, England) (3:1), and rotated overnight at room temperature. Anthers were then infiltrated with a 1:1 followed by a 1:3 mixture of ethanol: LR Gold (3 hr each), transferred to 100% LR Gold, and again rotated overnight at room temperature. The infiltrated anthers were embedded in LR Gold in gelatin capsules and polymerized with benzoyl peroxide paste in the dark.

Semithin sections (1.5 to 2.0 μm) were cut on a Reichert Ultracut microtome with glass knives and transferred in drops of Millipore-filtered distilled water to glass slides coated with poly-L-lysine. The sections adhered to slides by placing on a warm plate (40°C) until dry.

cDNA Probes for in Situ Hybridization

The 500-bp EcoRI fragments from pollen-specific clone Bcp1 were labeled with either digoxygenin-dUTP (Boehringer Mannheim) or biotin-dUTP (Sigma) by random priming. The random-primed mixture was fractionated by gel filtration (Sephadex G-25 column equilibrated in 0.1 M NaCl, 10 mM Tris HCl, pH 8.0, 1 mM EDTA) in a 1-mL-graduated disposable pipette. The fractions were collected and ethanol precipitated. The cDNA pellet was resuspended in hybridization buffer to give a concentration of approximately 2 ng/μL.

In Situ Hybridization

Labeled probes were denatured at 95°C for 2 min and cooled on ice. Aliquots (3 to 4 μL) of the probes were dispensed onto 10-mm diameter coverslips, and the slides were inverted onto the drop of probe. Some sections were pretreated with 1 μg/mL proteinase K in 100 mM Tris, pH 7.5, 50 mM EDTA for 30 min, followed by treatment with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min before addition of the probes. Hybridization was for 18 to 20 hr at 43°C in an atmosphere of 50% formamide in hybridization buffer. For each developmental stage, sections pretreated with 0.05 to 0.5 mg/mL RNase for 2 hr were included as a negative control; the loss of RNA was checked by incubating sections in the fluorochrome acridine orange. After hybridization, the slides were washed in 2 × SSC for 30 min, 1 × SSC for 30 min at room temperature, 0.5 × SSC for 30 min at hybridization temperature, and in 0.5 × SSC for 30 min at room temperature.

Detection of Hybridization Signal

For the digoxygenin-labeled probe, hybridization was detected by treatment with polyclonal sheep anti-digoxygenin antibody, conjugated with alkaline phosphatase according to the manufacturer's instruction (Boehringer Mannheim). The sections were examined by bright-field microscopy. Sections hybridized with the biotinylated probe were incubated in 1% gelatin for 30 min. The excess gelatin was blotted off, and hybridization was detected by incubating the sections in streptavidin-colloidal gold (15-nm diameter, Amersham) diluted 1:50 with PBS for 30 min at room temperature. The streptavidin-gold complex was centrifuged at 12,000*g* for 10 min before use to remove aggregates. After 3 × 5-min washes in PBS and 3 × 5-min washes in distilled water, the colloidal gold marker particles were silver enhanced for 2 × 30 min using a silver lactate developer (Pettitt and Humphris, 1991) or for 15 to 20 min with Intense 11 (Amersham). The sections were examined by bright-field and dark-field microscopy or combined epipolarization and phase contrast microscopy.

Peptide Synthesis, Antibody Production, and Immunoblotting

Two 15-amino acid peptides (peptide 1, GTTDDDAAASPGDD; peptide 2, AVVGVTSIVGSFLFF) were synthesized by the *t*-butoxycarbonyl-polystyrene solid-phase procedure using an Applied Biosystems model 430 A peptide synthesizer. The assembled peptides were cleaved from the resin and the crude peptides purified by preparative reverse-phase HPLC. Peptide 1 was conjugated to Keyhole Limpet Hemocyanin (Muller, 1988). Peptide 2 was highly insoluble and was used unconjugated. Both peptides were mixed 1:1 with RIBI adjuvant system (Ribi ImmunoChem Research Inc., Hamilton, MT), and 100 μL of total volume was injected intraperitoneally into Balb/c mice at day 1 and day 15. Twenty-four days later, a booster injection was given without adjuvant; 10 days afterward, the mice were killed and the blood was collected for analysis. Pollen extract was prepared by grinding 500 mg of pollen in 2 mL of extraction buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride). The extract was microcentrifuged for 5 min and the supernatant was collected. In the case of germinated pollen, pollen was germinated for 2 to 3 hr in vitro in germination medium (Taylor et al., 1991), and extract was prepared as described for pollen. Protein was quantitated, and 20 μg of protein was electrophoresed on a reducing SDS-polyacryamide gel (10%). After electrophoresis, the gel was electroblotted onto nitrocellulose membranes that were then incubated in 1:250 dilutions of antisera. The membranes were washed in PBS containing 0.05% Tween 20, and bound antibody was visualized by incubation with goat anti-mouse IgG-peroxidase conjugate, followed by washing in PBS/0.05% Tween and incubation with horseradish peroxidase color development solution (Bio-Rad).

ACKNOWLEDGMENTS

We thank Dr. Stan Strother for help during the initial stages of this work, Dr. Sean Davies for supervising sequencing work, Jo Kenrick for supervising preparation of antibodies, Terryn Hough for valued technical assistance, and Dr. Richard Simpson, Head

of the Joint Protein Structure Laboratory of the Ludwig Institute for Cancer Research and Walter and Eliza Hall Institute, Melbourne, for peptide synthesis. We also thank the Department of Biology, Khon Kaen University, Thailand, for granting study leave and the University of Melbourne for award of a postgraduate scholarship to P.T. The financial support for this work was provided by the Australian Research Council.

Received July 10, 1991; accepted August 15, 1991.

REFERENCES

Albani, D., Robert, L.S., Donaldson, P.A., Altosaar, I., and Fabijanski, S.F. (1990). Characterization of a pollen-specific gene family from *Brassica napus* which is activated during early microspore development. Plant Mol. Biol. **15,** 605–622.

Albani, D., Altosaar, I., Arnison, P.G., and Fabijanski, S.F. (1991). A gene showing sequence similarity to pectin esterase is specifically expressed in developing pollen of *Brassica napus*. Sequences in its 5′ flanking region are conserved in other pollen-specific promoters. Plant Mol. Biol. **16,** 501–513.

Brown, S., and Crouch, M. (1988). Analysis of genes expressed during pollen development in *Oenothera organensis*. J. Cell. Biochem., suppl. 12C, UCLA Symposia on Molecular and Cellular Biology (New York: Alan R. Liss), p. 168.

Brown, S.M., and Crouch, M.L. (1990). Characterization of a gene family abundantly expressed in *Oenothera organensis* pollen that shows sequence similarity to polygalacturonase. Plant Cell **2,** 263–274.

Evans, D.E., Singh, M.B., and Knox, R.B. (1990). Pollen development: Applications in biotechnology. In Microspores: Evolution and Ontogeny, S. Blackmore and R.B. Knox, eds (London: Academic Press), pp. 309–338.

Goldberg, R.B. (1988). Plants: Novel developmental processes. Science **240,** 1460–1467.

Griffith, I.J., Smith, P.M., Pollock, J., Theerakulpisut, P., Avjioglou, A., Davies, S., Hough, T., Singh, M., Simpson, R.J., Ward, L.D., and Knox, R.B. (1991). Cloning and sequencing of *Lol pI*, the major allergenic protein of rye-grass pollen. FEBS Lett. **279,** 210–215.

Heijne, G. (1987). Sequence Analysis in Molecular Biology. (New York: Academic Press), pp. 81–121.

Herrin, D., and Michaels, A. (1984). Isolation and fractionation of intact, translatable RNA from walled algal cells. Plant Mol. Biol. Rep. **2,** 24–28.

Heslop-Harrison, J., Knox, R.B., Heslop-Harrison, Y., and Mattson, O. (1975). Pollen-wall proteins: Emission and role in incompatibility responses. In The Biology of the Male Gamete, J.G. Duckett and P.A. Racey, eds (London: Academic Press), pp. 189–202.

Knox, R.B. (1984). Pollen-pistil interactions. In Cellular Interactions, H.F. Linskens and J. Heslop-Harrison, eds, Encyclopedia of Plant Physiology, Vol. 17 (Berlin: Springer Verlag), pp. 508–608.

Knox, R.B., Heslop-Harrison, J., and Heslop-Harrison, Y. (1975). Pollen-wall proteins: Localization and characterization of gametophytic and sporophytic fractions. In The Biology of the Male Gamete, J.G. Duckett and P.A. Racey, eds (London: Academic Press), pp. 177–187.

Knox, R.B., Southworth, D., and Singh, M.B. (1988). Sperm cell determinants and control of fertilization in plants. In Eukaryote Cell Recognition, G.C. Chapman, ed (Cambridge: Cambridge University Press), pp. 175–193.

Koltunow, A.M., Truettner, J., Cox, K.H., Wallroth, M., and Goldberg, R.B. (1990). Different temporal and spatial gene expression patterns occur during anther development. Plant Cell **2,** 1201–1224.

Kyte, J., and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157,** 105–132.

Maniatis, J., Fritsch, E.G., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

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

Mascarenhas, J.P. (1990). Gene activity during pollen development. Annu. Rev. Plant Physiol. Plant Mol. Biol. **41,** 317–338.

McConchie, C.A., Jobson, S., and Knox, R.B. (1985). Computer-assisted reconstruction of the male germ unit in pollen of *Brassica campestris*. Protoplasma **127,** 57–63.

McConchie, C.A., Russell, S.D., Dumas, C., Tuohy, M., and Knox, R.B. (1987). Quantitative cytology of the sperm cells of *Brassica campestris* and *B. oleracea*. Planta **170,** 446–452.

McCormick, S., Smith, A., Gasser, C., Sachs, K., Hinchee, M., Horsch, R., and Fraley, R. (1987). Identification of genes specifically expressed in reproductive organs of tomato. In Tomato Biotechnology, D.J. Nevin and R.A. Jones, eds (New York: Alan R. Liss), pp. 255–265.

McFadden, G.I., Boning, I., Cornish, E.C., and Clarke, A.E. (1988). A simple fixation and embedding method for use in hybridization histochemistry of plant tissues. Histochem. J. **20,** 575–586.

Muller, S. (1988). Peptide-carrier conjugation. In Synthetic Polypeptides as Antigens, M.H.V. Van Regenmortel, J.P. Briand, S. Muller, and S. Plaue, eds (Amsterdam: Elsevier), pp. 101–102.

Nacken, W.K.F., Huijser, P., Saedler, H., and Sommer, H. (1991). Molecular analysis of tap2, an anther-specific gene from *Antirrhinum majus*. FEBS Lett. **280,** 155–158.

Nasrallah, J.B. (1989). Molecular genetics of self-incompatibility in *Brassica*. In Plant Reproduction: From Floral Initiation to Pollination, E. Lord and G. Bernier, eds (Rockville, MD: American Society of Plant Physiologists), pp. 156–164.

Pettitt, J.M., and Humphris, D.C. (1991). Double lectin and immunolabelling for transmission electron microscopy: Pre- and post-embedding application using the biotinstreptavidin system and colloidal gold-silver staining. Histochem. J. **23,** 29–37.

Rafnar, T., Griffith, I.J., Kuo, M-c., Bond, J.F., Rogers, B.L., and Klapper, D.G. (1991). Cloning of *Amb a I* (antigen E), the major allergen family of short ragweed pollen. J. Biol. Chem. **266,** 1229–1236.

Shayk, M., and Kolattukudy, P.E. (1977). Production of a novel extracellular cutinase by the pollen and the chemical

composition and ultrastructure of the stigma cuticle of Nasturtium (*Tropaeolum majus*). Plant Physiol. **60,** 907–915.

Singh, M.B., and Knox, R.B. (1984). Quantitative cytochemistry of β-galactosidase in normal (*Gal*) and enzyme-deficient (*gal*) pollen of *Brassica campestris* and application of the indigogenic method. Histochem. J. **16,** 1273–1296.

Singh, M.B., and Knox, R.B. (1985). A gene controlling β-galactosidase deficiency in pollen of oilseed rape *Brassica campestris*. J. Hered. **76,** 199–201.

Singh, M.B., O'Neill, P., and Knox, R.B. (1985). Initiation of post-meiotic β-galactosidase synthesis during microsporogenesis in oilseed rape. Plant Physiol. **77,** 225–228.

Singh, M.B., Hough, T., Theerakulpisut, P., Avjioglu, A., Davies, S., Smith, P., Taylor, P.E., Simpson, R.J., Ward, L., McCluskey, J., Puy, R., and Knox, R.B. (1991). Isolation of cDNA encoding a newly identified major allergenic protein of rye-grass pollen: Intracellular targetting to the amyloplast. Proc. Natl. Acad. Sci. USA **88,** 1384–1388.

Silvanovich, A., Astwood, J., Zhang, L., Olsen, E., Kisil, F., Sehon, A., Mohapatra, S., and Hill, R. (1991). Nucleotide sequence analysis of three cDNAs coding for *Poa p* IX isoallergens of Kentucky bluegrass pollen. J. Biol. Chem. 1204–1210.

Smith, A.G., Hinchee, M., and Horsch, R. (1987). Cell and tissue specific expression localized by in situ hybridization in floral tissues. Plant Mol. Biol. Rep. **5,** 237–241.

Stanley, R., and Linskens, H.F. (1965). Protein diffusion from germinating pollen. Physiol. Plant. **18,** 47–53.

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

Stieglitz G., and Stern, H. (1973). Regulation of β-1,3-glucanase activity in developing anthers of *Lilium*. Dev. Biol. **34,** 169–173.

Taylor, P.E., Kenrick, J., Blomstedt, C.K., and Knox, R.B. (1991). Sperm cells of the pollen tubes of Brassica: Ultrastructure and isolation. Sex. Plant Reprod. **4,** 226–234.

Ursin, V.M., Yamaguchi, J., and McCormick, S. (1989). Gametophytic and sporophytic expression of anther-specific genes in developing tomato anthers. Plant Cell **1,** 727–736.

Vergne, P.I., Delvalle, I., and Dumas, C. (1987). Rapid assessment of microspore and pollen development stage in wheat and maize using DAPI and membrane permeabilization. Stain Technol. **62,** 299–304.

Vithanage, H.I., and Knox, R.B. (1976). Pollen development and quantitative cytochemistry of the origins of intine and exine enzymes in *Brassica oleracea*. J. Cell Sci. **21,** 423–435.

Wing, R.A., Yamaguchi, J., Larabell, S.K., Ursin, V.M., and McCormick, S. (1989). Molecular and genetic characterization of two pollen-expressed genes that have sequence similarity to pectate lyases of the plant pathogen *Erwinia*. Plant Mol. Biol. **14,** 17–28.