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Genes Expressed in *Pinus radiata* Male Cones Include Homologs to Anther-Specific and Pathogenesis Response Genes[1]

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We describe the isolation and characterization of 13 cDNA clones that are differentially expressed in male cones of *Pinus radiata* (D. Don). The transcripts of the 13 genes are expressed at different times between meiosis and microspore mitosis, timing that corresponds to a burst in tapetal activity in the developing anthers. In situ hybridization showed that four of the genes are expressed in the tapetum, while a fifth is expressed in tetrads during a brief developmental window. Six of the seven cDNAs identified in database searches have striking similarity to genes expressed in angiosperm anthers. Seven cDNAs are homologs of defense and pathogen response genes. The cDNAs identified are predicted to encode a chalcone-synthase-like protein, a thaumatin-like protein, a serine hydrolase thought to be a putative regulator of programmed cell death, two lipid-transfer proteins, and two homologs of the anther-specific A9 genes from *Brassica napus* and Arabidopsis. Overall, our results support the hypothesis that many of the reproductive processes in the angiosperms and gymnosperms were inherited from a common ancestor.

The gymnosperm *Pinus radiata* is monoecious, with male cones on the lower branches of the tree and female cones on the upper branches (see Fig. 1). Male cones consist of a spiral arrangement of tightly packed pollen-bearing structures or microsporophylls formed around a central axis. The lower surfaces of the microsporophylls have two chambers called microsporangia in which the pollen develops. Nutrients for the developing pollen are supplied from an inner layer of microsporangial cells called the tapetum.

Anther-expressed genes have been isolated from angiosperm flowers, nearly all of which are expressed in the microspores or tapetum during or after the burst in tapetal activity that occurs during meiosis (Williams and Heslop-Harrison, 1979; Pacini et al., 1985; Scott et al., 1991b). Most genes encode distinct proteins that share similar, tightly regulated temporal and spatial expression patterns (for reviews, see Scott et al., 1991b; Goldberg et al., 1993). Pollen development in gymnosperms shares several developmental and physiological processes with that of angiosperms (Stanley and Linskens, 1974; Pacini et al., 1985), and homologs to angiosperm floral transcription factors have been isolated from gymnosperm reproductive tissues (Tandre et al., 1995; Mellerowicz et al., 1998; Mouradov et al., 1998a, 1998b). However, there are no reports describing cDNAs that are differentially expressed in gymnosperm male reproductive structures.

Anther development can be divided into a series of defined developmental stages (Koltunow et al., 1990; Scott et al., 1991b; Goldberg et al., 1993) that correlate with aspects of male cone development. In *P. radiata,* male cone primordia first become visible in early summer (Wang, 1995); microsporophylls develop after 3 months, and the archesporial cells differentiate into the sporogenous cells and the parietal layer after 5 months. The parietal cells form a microsporangium wall four to five cells thick (the innermost layer is the tapetum) and the sporogenous tissue divides. After 6 months, the sporogenous cells are mature meiocytes and meiosis begins. Meiosis is complete after 7 months, the microspores are released from the tetrad, and the tapetum begins to degenerate (Wang, 1995). During degeneration, the cells of the tapetum release accumulated quantities of starch, crystallized protein, lipid, and polysaccharide, which are used by the microspores (Pacini et al., 1985). The two sacci of each pollen grain begin to inflate prior to the first division of the microspore as the three- to four-cell layer surrounding the sporangium collapses (Wang, 1995). Some time after 8 months, the pollen mother cells undergo three mitotic divisions. When dehiscence occurs, after about 9 months, each pollen grain contains two nonfunctional prothallial cells, a central vegetative cell, and a generative cell (Stanley and Linskens, 1974).

In New Zealand, Australia, and Chile, *P. radiata* is grown on large-scale plantations for timber and fiber. We were interested in engineering reproductive sterility as a means to manipulate biomass distribution and to control the spread and persistence of introduced genes (Kaul, 1988; Strauss et al., 1995). One strategy for engineering sterility involves directing the expression of cytotoxic genes to male cones using tissue-specific promoters. Such promoters are available from various angiosperm species, but their tissue specificity and long-term expression characteristics in conifers remain unknown.

We describe here the isolation and characterization of 13 cDNAs that are differentially expressed in *P. radiata* male cones. Six of the seven cDNAs identified by database searches are homologs of angiosperm genes expressed in

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Figure 1. *P. radiata* male (left) and female (right) cones photographed during male cone dehiscence.

anthers, indicating that a significant proportion of differentially expressed anther genes evolved prior to the divergence of gymnosperms and angiosperms. All of the seven cDNAs identified are also homologs of genes involved in the plant response to stress and pathogens.

MATERIALS AND METHODS

Tissue Collection

All tissues used in this study were collected from New Zealand Forest Research Institute nurseries in Rotorua (latitude 38° 24′, altitude 350 m). Vegetative shoot and cone tissue was harvested from mature trees. Root tissue was collected from 1-year-old cuttings, and needle tissue was collected from 4-year-old seedlings. Tissue was immediately stored in liquid nitrogen in transit to refrigeration at −80°C, or immersed in 10× volume of FAA fixative (ethanol 50% [v/v], glacial acetic acid 5% [v/v], and formalin 10% [v/v]).

Assessment of Microsporocyte Development

Male cones harvested during 1994 and 1995 were removed from FAA fixative, dissected, and the microsporangium contents were released into a solution of 3% (w/v) Suc. Samples were transferred onto microscope slides and viewed under a light microscope to assess microsporocyte development. The developmental stage of pollen in these cones (listed in Table I) was assessed according to the stages described by Scott et al. (1991b) and Koltunow et al. (1990). Images were captured with a video camera connected to a Power Macintosh 7500 computer using the Image program developed at the National Institutes of Health (available on the Internet at http://rsb.info.nih.gov/nih-image/).

RNA Extraction

RNA extraction of frozen tissue was by LiCl precipitation (Chang et al., 1993). Synthesis of cDNA for probing cDNA libraries was primed with oligo p(dTTP)15 primers (Boehringer Mannheim, Mannheim, Germany) and catalyzed by reverse transcriptase (Superscript II, Gibco-BRL, Gaithersburg, MD) according to the manufacturer's instructions.

cDNA Library Construction

Meiocyte (Me-i-stage) and tetrad (T1-stage) cDNA libraries were constructed using a plasmid cloning kit (Superscript, Gibco-BRL) according to the manufacturer's instructions. *Escherichia coli* DH10B cells competent for electro-poration were transformed with aliquots of the ligation reaction using an electroporation apparatus (Bio-Rad Laboratories, Hercules, CA) (2,500 V, 50 μFa, 200 Ω) according to the manufacturer's instructions with 2-mm gap cuvettes.

Differential Screening of the cDNA Library

E. coli transformants (500 per plate) from the meiocyte stage (16,000) and tetrad stage (8,000) libraries were grown on 132-mm colony/plaque screen nylon membranes (DuPont-NEN, Boston). Replica plating, colony lysis, and DNA fixation (using an alkali method) were carried out according to the manufacturer's instructions. Cellular debris were removed from the membranes according to the method of Vogeli and Kaytes (1987).

A differential screening strategy was used to select clones that hybridized strongly to a [^{32}P]dCTP-labeled cDNA mixture (Rediprime, Amersham, Buckinghamshire, UK) prepared from Me-i-stage- and T1-stage-male cone cDNA, but did not hybridize to a cDNA mixture prepared from needle, root, and shoot tissue. Hybridization, stringency washing, and autoradiography were carried out according to the manufacturer's instructions (DuPont-NEN). Duplicate filters were initially hybridized with the first probe, washed, and hybridized with the second probe. Selected clones that were highly expressed in male cones on both filters were picked and amplified for further analysis.

For secondary screening, plasmid DNA was purified from selected colonies, digested with *Not*I/*Sal*I, and fractionated on 1.2% (w/v) agarose gels. Nucleic acids were transferred to nylon membrane (Hybond N$^+$, Amersham) using the procedure outlined for Southern blotting. The filters were screened with the radiolabeled needle, root, and shoot cDNA mixture and then with the radiolabeled meiocyte and microspore cone cDNA as described above. Selected clones were further analyzed by northern blots of Me-i- and T1-stage male cone, needle, root, and shoot RNA (data not shown).

Dot-Blot Hybridization Analysis

Plasmid DNA samples were diluted 10-fold, denatured for 5 min (95°C), made up to 5× SSC, and then 2-μL

Table I. *Developmental stage of pollen extracted from male cones*

The harvest dates (mm/dd/yy) of *P. radiata* cones used in this study are shown. The approximate time that male cone primordia become visible is based on data collected in previous years (Wang, 1995). Morphological descriptions were determined by microscopic analysis, except the earliest stages, which are marked by asterisks and are based on data collected in previous years by Wang (1995). The abbreviations are based on the description of *B. napus* anther development (Scott et al., 1991). Numbers are used to distinguish samples at the same stage from different years. The last two samples were used for in situ analysis only. Note that there were clear differences in the rate of development over the 2 years sampled, since the Me-a stage was reached almost 1 month later in 1995 (7/3/95) than in 1994 (6/6/94).

Harvest Date (Months after Cone Primordia Appear)	Morphological Description	Stage
3/23/94 (3.75)	Archesporial tissue not differentiated*	A2 (Archesporial)
4/12/94 (4.5)	Sporogenous and parietal cells differentiated*	SP (Sporogenous parietal)
4/25/94 (4.75)	Meiocytes at late interphase	Me-i (Meiocyte, interphase)
5/5/94 (5)	Meiocytes at late interphase, some early prophase	Me-iep (Meiocytes, interphase early prophase I)
5/26/94 (5.75)	Meiocytes at prophase I	Me-p (Meiocytes, prophase I)
6/6/94 (6.25)	Meiocytes at late anaphase I, early telophase I	Me-at (Meiocytes, anaphase I telophase I)
6/29/94 (7)	Tetrads	T1 (Tetrads)
7/15/94 (7.5)	Microspore sacci partially inflated	Mi (Microspores)
3/17/95 (3.5)	Archesporial tissue not differentiated*	A1 (Archesporial)
7/3/95 (7)	Meiocytes at anaphase I	Me-a (Meiocytes, anaphase I)
7/7/95 (7.25)	Meiocytes at anaphase I	Me-a′ (Meiocytes, anaphase I)
8/11/95 (8.25)	Microspore sacci inflated	Mi-si (Microspores, sacci inflated)
9/1/95 (9)	Mature pollen (two prothallial cells, a generative cell and a tube nucleus)	Mp (Mature pollen)
7/4/96 (7)	Meiocytes at telophase I and prophase II	Me-tIpII (Telophase I and prophase II)
7/16/96 (7.5)	Tetrads	T2 (Tetrads)

aliquots were placed on Hybond N^+ membrane prewetted in 10× SSC. Filters were probed with purified cDNA fragments released from pSPORT1 by *Not*I/*Sal*I digestion. Filters were hybridized, washed (final wash in 2× SSC, 75°C; 20× SSC is 0.3 M sodium citrate and 3 M NaCl), and exposed to film for 3 to 12 h.

Northern Hybridization Analysis

Aliquots (5 μg) of total RNA were glyoxylated, fractionated on agarose gels, and transferred to Hybond N^+ nylon membranes (Sambrook et al., 1989; Munch, 1994). Hybridization conditions were as described by Virca et al. (1990). Loading differences were assessed by probing blots with a probe to the 26S rRNA that was amplified using PCR with specific primers (kindly provided by M. Jacobs).

Southern Hybridization Analysis

Genomic DNA was isolated from cone or young needle tissue according to previously published methods (Doyle and Doyle, 1990; Fang et al., 1992). Southern hybridization was carried out using 10 μg of digested genomic DNA per lane according to standard methods (Sambrook et al., 1989).

Sequence Analysis

Plasmid DNA was prepared for automated sequencing or further analysis according to a previously published method (Feliciello and Chinali, 1993). Sequencing was carried out with automated sequencers (ABI Prism 373 or 377, Perkin-Elmer, Foster City, CA). Initial sequence data were obtained using M13/pUC forward and reverse sequencing primers; when required specific primers were designed to sites within each cDNA. Sequences were analyzed using the Genetics Computer Group package (versions 8.1 and 9.1, GCG, Madison, WI) and HOMED (Dr. Peter A. Stockwell, Department of Biochemistry, University of Otago, New Zealand) software packages. Unless stated otherwise, deduced amino acid sequences were translated from the first in-frame ATG in each cDNA and terminated at the first stop codon encountered. Homology inferences are based on the results of BLAST and FASTA database searches. Phylogenetic analysis was carried out using the GCG version of PAUP (version 4.0.0d55 for UNIX) with parsimony and heuristic search criteria and 100 boot strap replications to assess branching confidence.

In Situ Hybridization

In situ hybridization was based on existing protocols (Cox and Goldberg, 1988; Bochenek and Hirsch, 1990; Wilkinson, 1992) with the addition of proprietary reagents supplied in the RNA Color Kit (Amersham).

Tissue was fixed overnight in freshly prepared ice-cold fixative (gluteraldehyde 0.1% [w/v] and formaldehyde 4% [w/v], in phosphate-buffered saline [PBS], pH 7.2), washed in PBS (room temperature), dehydrated in an ethanol dilution series and infiltrated with xylene. Xylene was gradually replaced with molten paraffin wax (Paraplast, Sigma, St. Louis) and infiltration was continued for up to 2 d. The tissue was embedded, sectioned (8–10 μm), and baked onto Lys-coated slides (1–2 d, 42°C). Sections were rehydrated, incubated in 0.2 M HCl (20 min, room temperature), equilibrated in 10 mM Tris and 1 mM EDTA, pH 8.0

(TE), and then digested in 1 μg mL^{-1} proteinase K in TE buffer (37°C for 30 min). Digestion was stopped with 2 mg mL^{-1} Gly in PBS (5 min). Sections were equilibrated in an aqueous solution of triethanolamine (0.1 M, pH 8.0), acetylated with freshly prepared acetic anhydride (0.5% [v/v] in triethanolamine solution, 10 min), washed in PBS, and dehydrated prior to hybridization.

Probe preparation, hybridization, and signal detection were carried out using the Amersham RNA Color Kit (catalog no. RPN3300). Sections were probed with single-stranded fluorescein-labeled RNA probes corresponding to the sense and antisense strands of the cDNA. After hybridization, sections were rinsed in 2× SSC, treated with RNase A (10 μg mL^{-1}, 2× SSC, room temperature, 20 min), and washed under stringent conditions (1× SSC, 0.1% [w/v] SDS, 5 min, room temperature, then twice in 0.2× SSC, 0.1% [w/v] SDS, 55°C, 10 min). Sections were washed in TBS (100 mM Tris HCl, pH 7.5, and 400 mM NaCl, 5 min), incubated in block solution (0.5% [w/v] Amersham proprietary blocking agent in TBS for 1 h), rinsed in TBS then drained. Bound probe was detected using an anti-fluorescein antibody conjugated to alkaline phosphatase. Sections were incubated with the antibody for 1 h (1/1,000 in 0.5% [w/v] BSA in TBS), rinsed three times in TBS, washed in detection buffer (100 mM Tris HCl, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$) for 5 min, and drained. Detection buffer with 5-bromo-4-chloro-3-indolyl phosphate (0.18 mg mL^{-1}, Amersham) and nitroblue tetrazolium (0.34 mg mL^{-1}, Amersham) was added to each section and left to develop in the dark for up to 24 h. After the required development, the slides were rinsed in distilled water, dried, and mounted. Images were recorded on 35 mm Kodak Ektachrome 25t film.

RESULTS

Assessment of Male Cone Development

We use a system of abbreviations based on that of Scott et al. (1991b) to describe the stages of pollen development. A summary of cones collected is listed in Table I.

Thirteen Differentially Expressed cDNAs Were Isolated from the Microspore Library

Two cDNA libraries were prepared, one from Me-i-stage-male cones (the meiocyte library) and one from T1-stage-male cones (the microspore library). Sixteen thousand clones from the meiocyte library and 8,000 clones from the tetrad library were differentially screened by sequentially probing duplicate filters with each of two probes. One was prepared from mRNA isolated from Me-i- and T1-stage-male cones and one from a mix of mRNA isolated from roots, needles, and vegetative shoots. A total of 120 differential clones were selected from the microspore library and a strict secondary screen (see "Materials and Methods") reduced this number to 37 clones. No differential clones were obtained from screening the meiocyte library. The 37 differential clones were placed into 13 groups of cross-hybridizing clones (summarized in Table II).

Each of the 37 sequences was partially sequenced to confirm the placement into 13 hybridization groups. Of the

Table II. *Summary data for cDNAs isolated in this study*

The number of cross-hybridizing clones in each cDNA group is in brackets adjacent to the name of the largest clone. Transcript size was derived from northern analysis. Temporal and spatial expression data are summarized from northern-blot and in situ hybridization data; developmental stage abbreviations are from Table I. Copy number data are derived from Southern analysis. The accession numbers are for the sequence of the largest clone in each group. Asterisks indicate that the complete sequence of cDNA has been determined, the remaining sequences are lodged in the database as expressed sequence tags. Accession number of the largest clone in the second PrLTP1 subgroup is in parentheses. ND, Not determined.

Largest Clone (No. Cloned)	cDNA Size	mRNA Size	Temporal Expression	Spatial Expression	Homology	Genomic Copy No.	Accession No.
	bp						
PrMC6	2,020	2,300	T1 Mi	ND		Intermediate	AA220862
PrThL1 (3)	1,040	1,480	Me-a Me-at T1 Mi	ND	Thaumatin/permatin	Intermediate	AA220863
PrLTP1 (10*)	630	950	Me-a Me-at T1 Mi	Tapetum	Non-specific lipid transfer protein	High	U90342* (AI857146)
PrMC75	620	750	Me-a Me-at T1 Mi Mi-si	ND		Low	AA220866
PrMC1 (3)	611	650	Me-a Me-at T1 Mi	Tapetum	A9 tapetum-expressed gene	Low	U90350*
PrChS1 (4)	1,468	1,350	Me-a Me-at T1 Mi Mi-si	Tapetum	Chalcone synthase/ stilbene synthase	Low	U90341*
PrLTP2 (2)	582	800	T1	Tetrads	Non-specific lipid transfer protein	ND	AF110332*
PrMC103	1,375	2,100	Me-at T1 Mi	ND		Low	AA220871
PrMC104	703	620	Mi Mi-si	ND		Intermediate	AA220872
PrMC136	450	2,800	Me-a Me-at T1 Mi	ND		Low	AA220874
PrMC187 (5)	1,330	1,300	Me-at T1 Mi Mi-si	ND		Intermediate	AI857147
PrMC2	750	820	Me-a Me-at T1 Mi Mi-si	Tapetum	A9 tapetum-expressed gene	Low	U90343*
PrMC3 (4)	1,107	1,700	Me-a Me-at T1 Mi	ND	Ser hydrolase	Low	AF110333*

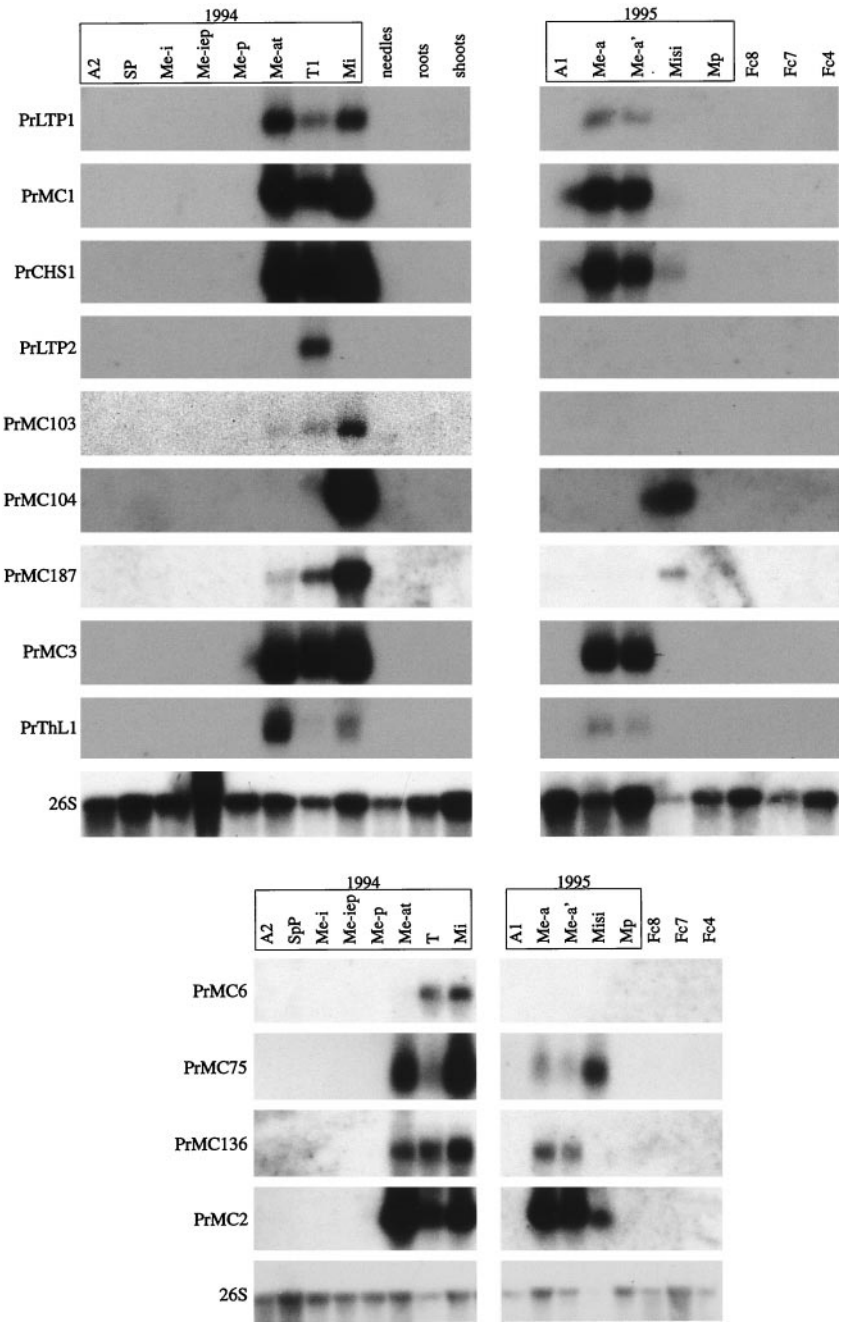

Figure 2. Temporal expression analysis. Northern blots of total RNA extracted from male cones during two flowering seasons. Abbreviations for male cones are given in Table I. Needle, root, and shoot data (where presented) are as indicated, and the abbreviations for female cones are as follows: Fc8, female cones 8 months before meiosis; Fc7, female cones 7 months before meiosis; Fc4, female cones 4 months before meiosis. Expression data and transcript size are summarized in Figure 3 and Table II. Loading differences were assessed by probing blots with a 26S rRNA probe as indicated. Note that there is wide variation in the loading of samples between lanes, with some samples underloaded (e.g. T1, Misi); this is the likely cause of the apparent transient down-regulation of genes such as PrLTP1 in T1-stage cones. Faint smears immediately adjacent to lanes containing strongly hybridizing bands (e.g. PrMC104, T1 sample) have been interpreted as noise in the summary in Figure 3.

seven groups with more than one member, six contain cDNAs that appeared identical in the overlapping regions that have been sequenced, and therefore are likely to represent independent clones from the same gene. One hybridization group (largest clone PrLTP1) had 10 cross-hybridizing clones that fell into two subgroups of five identical members. Identity between the two subgroups for the region corresponding to nucleotides 43 to 126 of PrLTP1 (predicted coding sequence is from 39–422) was 87%. Seven of the 13 cDNA clones shared homology to sequences in the databases; six of these genes were sequenced completely (Table II; see "Discussion").

Most cDNAs Are Expressed from Just Before to Just After Meiosis

Northern blots using male cones collected over two flowering seasons were used initially to characterize the expression of the 13 genes. The results are shown in Figure 2, and summarized in Table II and Figure 3. No transcripts of any of the cDNAs were detected in roots, vegetative shoots, needles or female cones. In male cones, the timing and duration of expression varied widely for the 13 genes, but in each case occurred between meiosis and the first mitotic division of the microspore. This timing is similar to that of

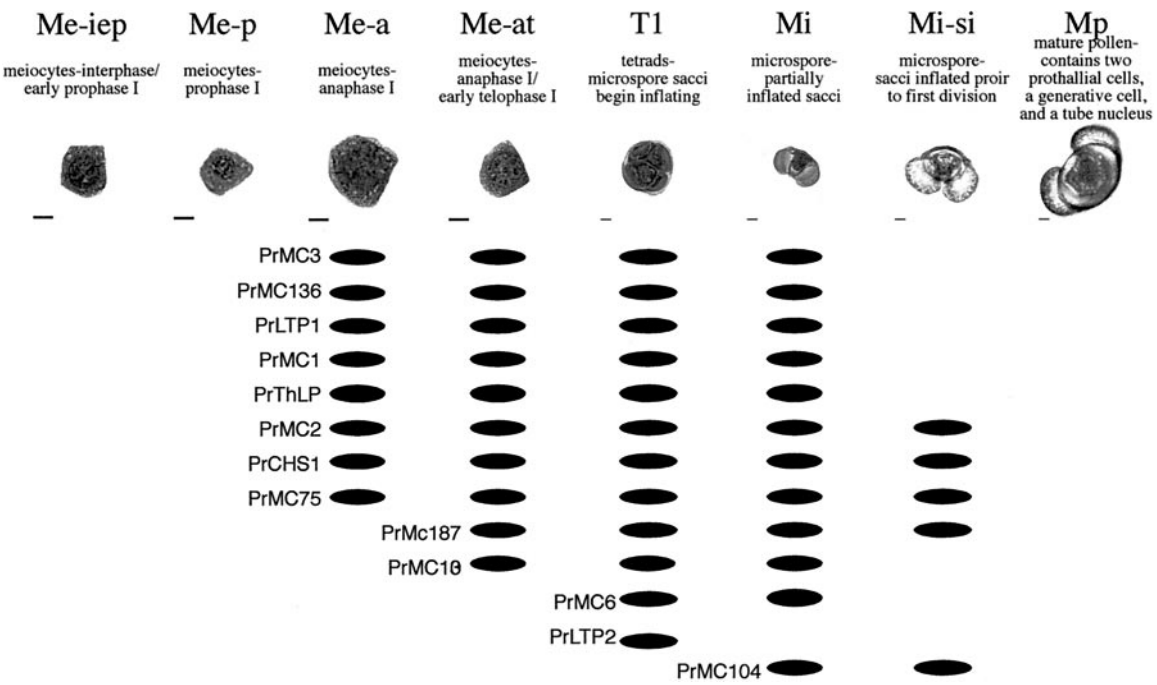

Figure 3. Most cDNAs are expressed from before to just after meiosis. This figure illustrates the expression of the 13 cDNAs at different stages of pollen development (top). Development progresses from left to right. The cDNAs are labeled on the left side of the figure. An oval spot represents expression. Abbreviations for male cones are given in Table I. Bars indicating the size of developing pollen are 9 μm. Me-iep- to Me-at-stage meiocytes are enlarged by a factor of two.

most angiosperm anther-specific genes (Scott et al., 1991b; Goldberg et al., 1993) and corresponds to the stage of a burst in tapetal activity in the developing anthers (Heslop-Harrison, 1968). The expression of five transcripts (PrThL1, PrLTP1, PrMC1, PrMC136, and PrMC3) was first detected in Me-a-stage cones and last detected in Mi-stage cones. Three other transcripts (PrMC75, PrCHS1, and PrMC2) were also first expressed in Me-a-stage cones, but their expression extended for a slightly longer period of development, lasting up to the formation of microspores with inflated sacci (Mi-si stage). Transcripts of PrMC103 and PrMC187 were first detected in Me-at-stage cones. The PrMC103 probe detected faint signals from Me-at- and T1-stage cones and was last detected in Mi-stage cones. PrMC187 was last detected in Mi-si–stage-cones. Transcripts of PrMC6 and PrLTP2 were expressed for short periods within the burst of tapetal activity, suggesting that their expression may be regulated differently. PrMC104 was notably later than all the other genes, with transcript undetected until the juvenile microspores were present and disappearing by the time mature pollen had formed.

Most Genes Are Expressed in the Tapetal Layer

Spatial expression patterns of five genes, PrChS1, PrLTP1, PrLTP2, PrMC1, and PrMC2 were analyzed in developing male cones by in situ hybridization analysis (Fig. 4). In Me-tIpII-stage cones, expression of PrChS1, PrLTP1, PrMC1, and PrMC2 was restricted to the tapetum. PrChS1, PrLTP1, and PrMC2 continued to be expressed in the tapetal tissues of T2-stage cones. PrLTP2, which was expressed only for a brief period in T1-stage cones, showed a different pattern, and appeared to be restricted to tetrads in a cluster of microsporangia in T2-stage cones. This pattern suggested that expression was restricted to a particular part of the cone or progressed acropetally as the tetrads reach a certain stage of development (Fig. 4).

Most cDNAs Represent Genes of Low or Intermediate Copy Number

Southern analysis was performed using the shortest isolate of each of the 12 hybridizing groups of cDNAs (no data were obtained for PrLTP2). Most cDNAs represented genes of low (1–2 bands per digest) or intermediate (3–6 bands) copy number (Fig. 5; Table II) except PrLTP1, which showed an average of nine hybridizing bands in each digest.

Nucleotide Sequence of the cDNAs

PrChS1 Belongs to a Tapetal-Specific Subgroup of Chalcone Synthase-Like Proteins

The longest clone of this group was sequenced completely in both directions and named PrChS1 because of its similarity to chalcone synthase (ChS) and stilbene synthase (StS) genes. The highest similarity was with five ChS sequences, which, together with PrChS1, form a distinct clade on phylogenetic trees derived from various ChS and StS sequences (Fig. 6). Transcripts of four of the five genes in the PrChS1 clade have been isolated from floral tissues

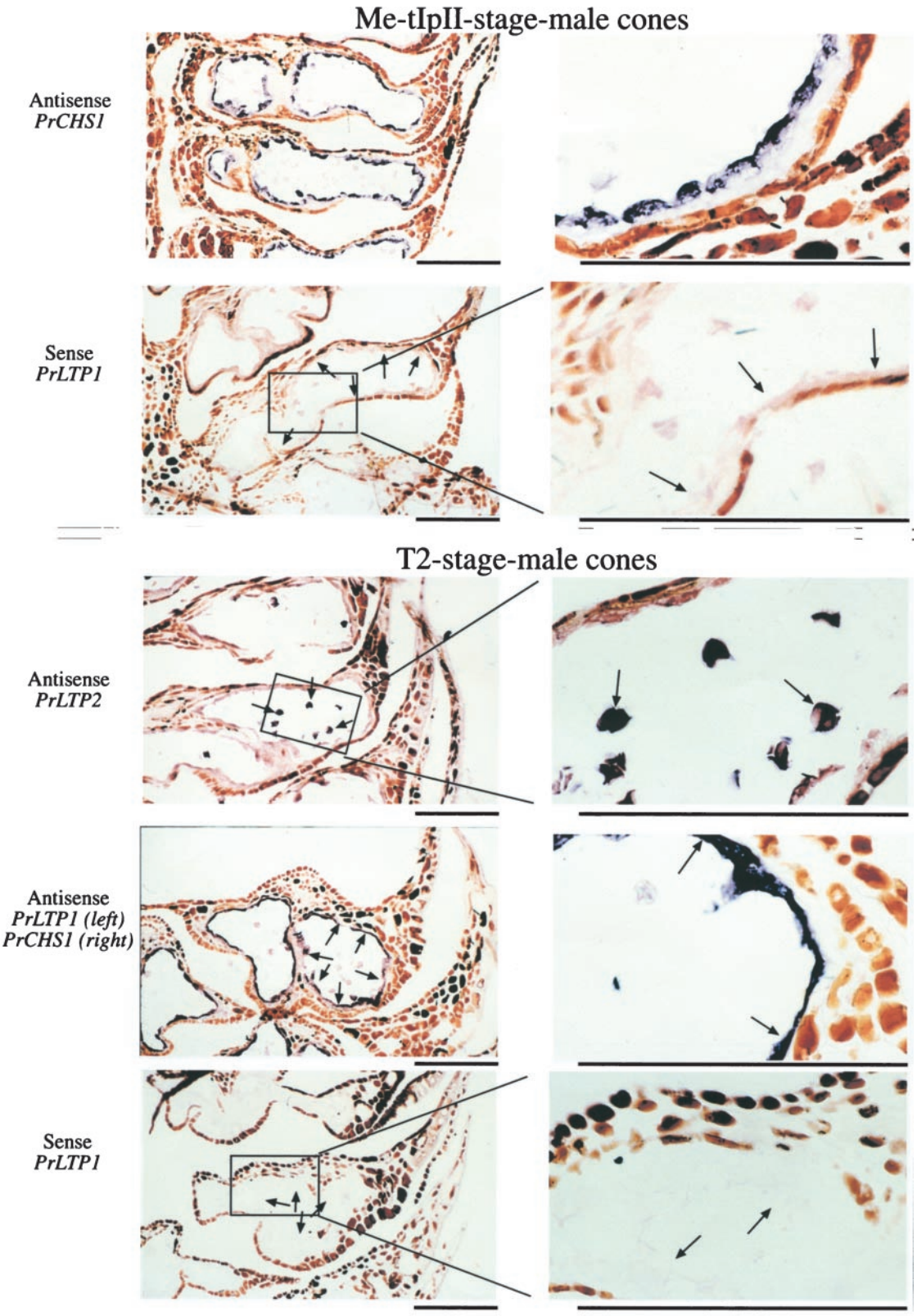

Figure 4. Spatial expression of five genes in *P. radiata* male cones. Me-tIpII-stage- and T2-stage male cones (top and bottom, respectively) were sectioned and probed with sense (control) and antisense transcripts of five cDNAs. The results for PrChS1, PrLTP1, PrMC1, and PrMC2 were similar and are represented above by the results of PrChS1 and PrLTP1. The result for PrLTP2 is illustrated separately above. The bar beneath each image represents 250 μm; rectangles indicate the origin of the enlarged images. Detection of transcript is indicated by the blue/black tetrazolium blue signal (arrow) and in the control sections arrows indicate corresponding tissue.

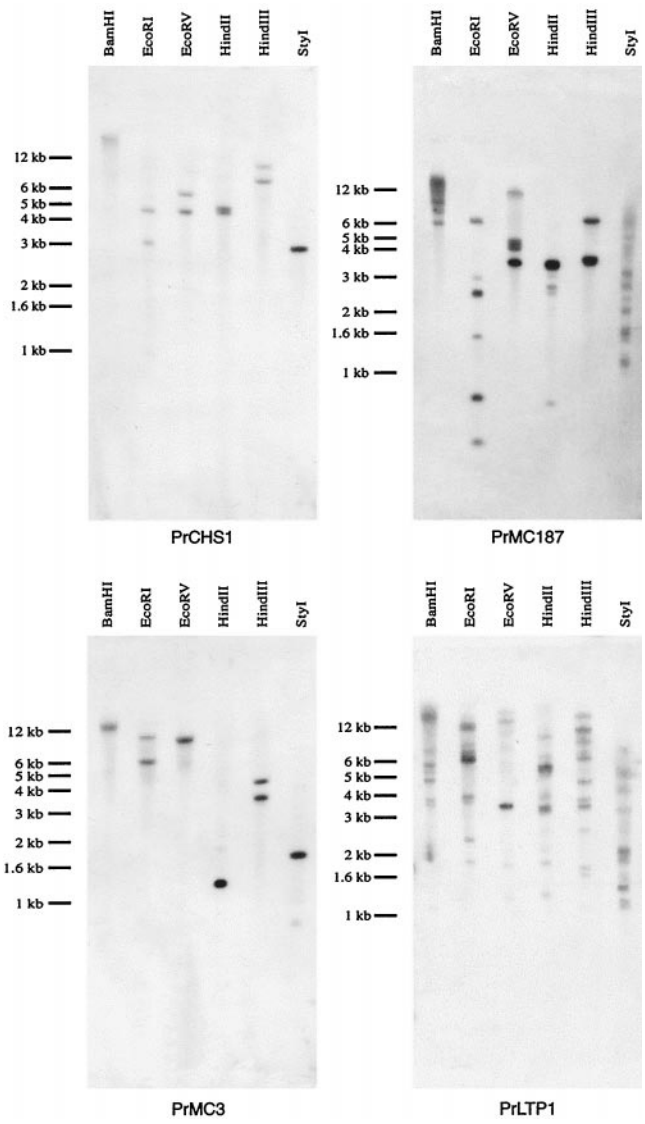

Figure 5. Southern-blot analysis. Blots of genomic DNA (10 μg per lane, digested as indicated) were probed with the cDNA clones indicated. Clones with an average of less than three signals per digest were scored as low (e.g. PrChS1 and PrMC3), three to seven bands were scored as intermediate (e.g. PrMC187), and eight or more bands were scored as high copy (e.g. PrLTP1, data summarized Table II).

(Shen and Hsu, 1992; Hihara et al., 1996; Turgut et al., 1996; Atanassov et al., 1998), and the fifth is from a genomic sequence (GenBank accession no. u89959). Expression of the rice gene was tapetal specific (Hihara et al., 1996), the *Brassica rapa* gene was expressed in the tapetum and vasculature of anthers as well as in young microspores (Shen and Hsu, 1992), the *Brassica napus* gene was isolated on the basis of its differential expression in anthers (Turgut et al., 1996), and expression of the tobacco gene was restricted to the tapetum and developing microspores (Atanassov et al., 1998). Both ChS and StS enzymes are induced in response to various pathogens and stresses (Koes et al., 1989a; Hain et al., 1993; Schubert et al., 1997) and ChS is developmentally regulated in various tissues including anthers (Koes et al., 1989b; van der Meer et al., 1990).

Two Groups Encode Nonspecific Lipid-Transfer Proteins (nsLTPs)

PrLTP1 has homology with various plant nsLTPs, but is most similar to two *B. napus* anther sequences (34% amino acid identity). The first, bif38 (L31938), has not been characterized, and the second, BNE2 (Foster et al., 1992) (X60318), is expressed in the tapetum and microspores from meiosis to the first round of mitosis in the microspore. The deduced protein of PrLTP1 has a hydrophobic N-terminal secretory sequence with a predicted mature protein of 95 amino acids.

PrLTP2 also encodes a nsLTP showing 40% nucleotide and 25% amino acid identity with PrLTP1. Sequence alignments (Fig. 7) indicate that, although the PrLTP2 reading frame is missing eight to 16 amino acids at the N terminus, it encodes a preprotein with a hydrophobic N terminus and a secreted protein of 99 amino acids. The most similar homolog is the rice NLT4 gene (accession no. q42976), which has 33% amino acid identity with PrLTP2. Expression of NLT4 has not been described.

Two Groups Encode A9 Homologs

PrMC1 and PrMC2 are 42% identical and encode proteins with 29% amino acid identity. Their reading frames are both predicted to have secretory sequences that are cleaved to give secreted 6.7-kD proteins. Both deduced proteins have a nsLTP-like Cys motif (Fig. 7). However, compared with the nsLTPs, the length of some of the intervening stretches is reduced. PrMC1 and PrMC2 share best similarity with several anther-expressed cDNAs of which the most well characterized is A9 from *B. napus* (Scott et al., 1991a) and Arabidopsis (Paul et al., 1992). Within this anther-expressed group, the nearest homolog to PrMC2 is the *Silene latifolia* Men-8 mRNA that was isolated from anthers and is maximally expressed just prior to tapetum degradation; it was not detected in sepals, petals, filaments leaf, or root tissue (Scutt et al., 1997). PrMC1 is most similar to the tapetum-expressed M7 gene, which was isolated from a *Lilium henryi* meiocyte library and is expressed in the tapetum from early prophase until microspore mitosis. It was not expressed in gynoecia, leaf, or root tissue (Crossley et al., 1995).

PrThL1 Encodes a Thaumatin

Based on sequence alignments with various plant thaumatins, PrThL1 encodes a full-length protein with a hydrophobic N-terminal signal sequence. The secreted protein is a member of the 24- to 25-kD family of thaumatin proteins with 16 conserved Cys residues, rather than the deleted 17-kD version with 10 conserved Cys residues found in some monocotyledons (Hu and Reddy, 1997). The closest homolog to PrThL1 is Tomf216, a floral-specific transcript from tomato with 45% amino acid identity. Tomf216 is expressed in immature inflorescences, in flowers prior to meiosis, in stamens from tetrad dissolution through to anthesis, and in petals at anthesis. The closest non-floral homolog is an Arabidopsis sequence, ATLF (40% amino

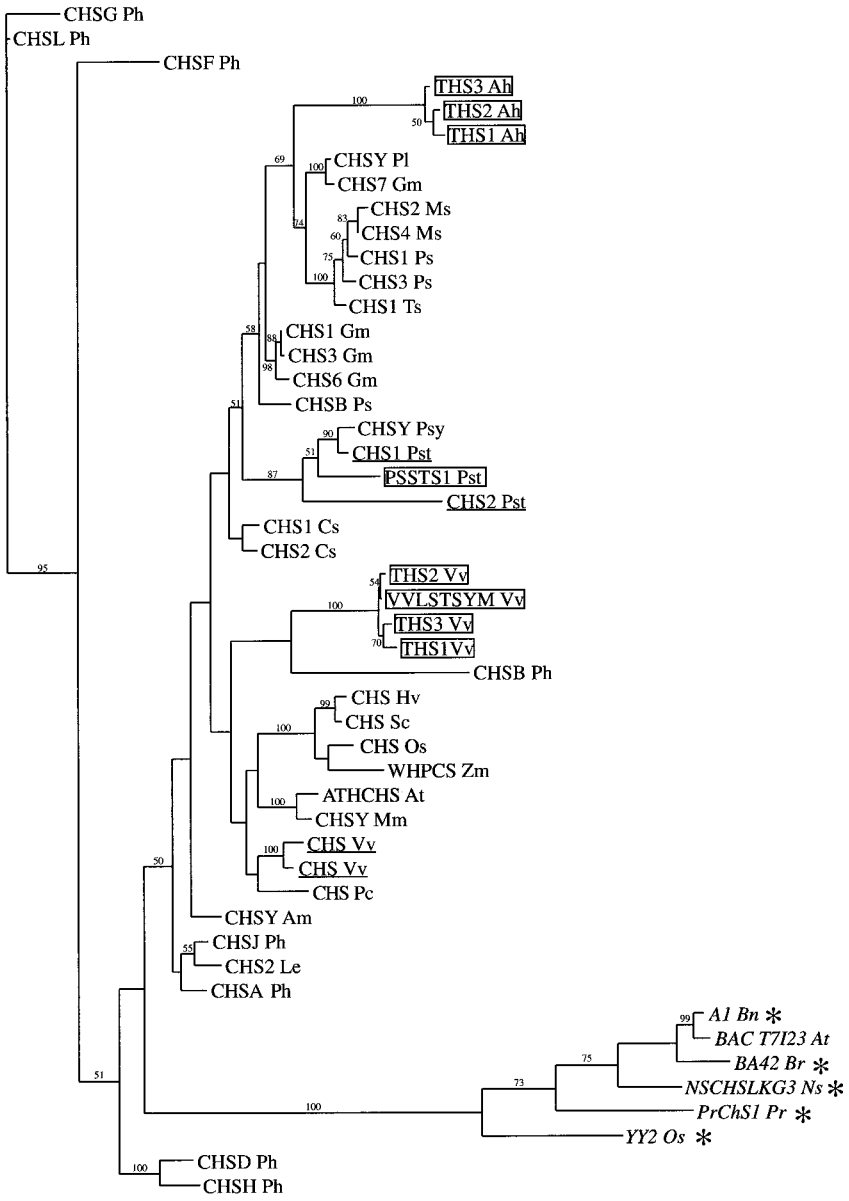

Figure 6. Phylogenetic analysis of ChS and StS-like sequences. The figure illustrates the tree generated when ChS and StS amino acid sequences corresponding to bases 8 to 386 of PrChS1 were aligned and subjected to a phylogenetic analysis. Five of the six sequences (indicated by asterisks) in the PrChS1 clade are specific to male reproductive tissues. ChS sequences from plant species corresponding to those represented in the PrChS1 clade are shaded gray. The distribution of StS sequences (boxed) throughout the phylogram suggests that StS evolved from ChS several times independently during evolution (also see Tropf et al., 1994). ChS sequences from plant species corresponding to the StS sequences are underlined. Abbreviations are as follows: Ph, *Petunia hybrida*; Ah, *Arachis hypogaea*; Pl, *Pueraria lobata;* Gm, *Glycine max*; Ms, *Medicago sativa;* Ps, *Pisum sativum*; Ts, *Trifolium subterraneum*; Psy, *Pinus sylvestris*; Pst, *Pinus strobus*; Cs, *Camellia sinensis*; Vv, *Vitis vinifera*; Bn, *Brassica napus*; At, Arabidopsis; Br, *Brassica rapa*; Ns, *Nicotiana sylvestris*; Os, *Oryza sativa*; Pr, *P. radiata*; Hv, *Hordum vulgare*; Sc, *S. cereale*; Zm, *Z. mays*; Mm, *Matthiola incana*; Pc, *Petroselinum crispum*; Am, *Antirrhinum majus*; Le, *Lycopersicon esculentum*.

acid identity; Arro et al., 1997), which is induced by parasites and by compounds inducing parasite resistance (Uknes et al., 1992).

PrMC3 Is Similar to a Hypersensitive Response Protein

PrMC3 is most similar to a tulip arylacylamidase (42% amino acid identity; GenBank accession no. e03271), a *P. radiata* expressed sequence tag from embryo tissue culture (accession no. AA220894) and the tobacco protein hsr203J (Pontier et al., 1994) (36% amino acid identity). The tulip and *P. radiata* homologs are not described in the literature. However, hsr203J is rapidly and specifically expressed in the hypersensitive response (HR) to various pathogens (Pontier et al., 1994). Other homologs in the database include a peptide encoded by an expressed sequence tag from elongating root hairs and root tips of *Medicago truncatula* (amino acid identity 40%; GenBank accession no. AA660803) and the peptide encoded by an unknown *Mycobacterium tuberculosis* gene (26% amino acid identity; GenBank accession no. Z80108). All of these sequences, including PrMC3, include the Ser hydrolase motif GXSXG (Fig. 8).

DISCUSSION

A differential screening strategy comparing gene expression in *P. radiata* male cones with expression in needles, roots, and vegetative shoots resulted in the isolation of 13 cDNAs, all of which were isolated from a tetrad-stage cone library. No differential cDNAs were identified among 16,000 clones from the early meiocyte library, indicating that there were few transcripts that were both abundant and differential in the Me-i-stage cones. All of the cDNAs

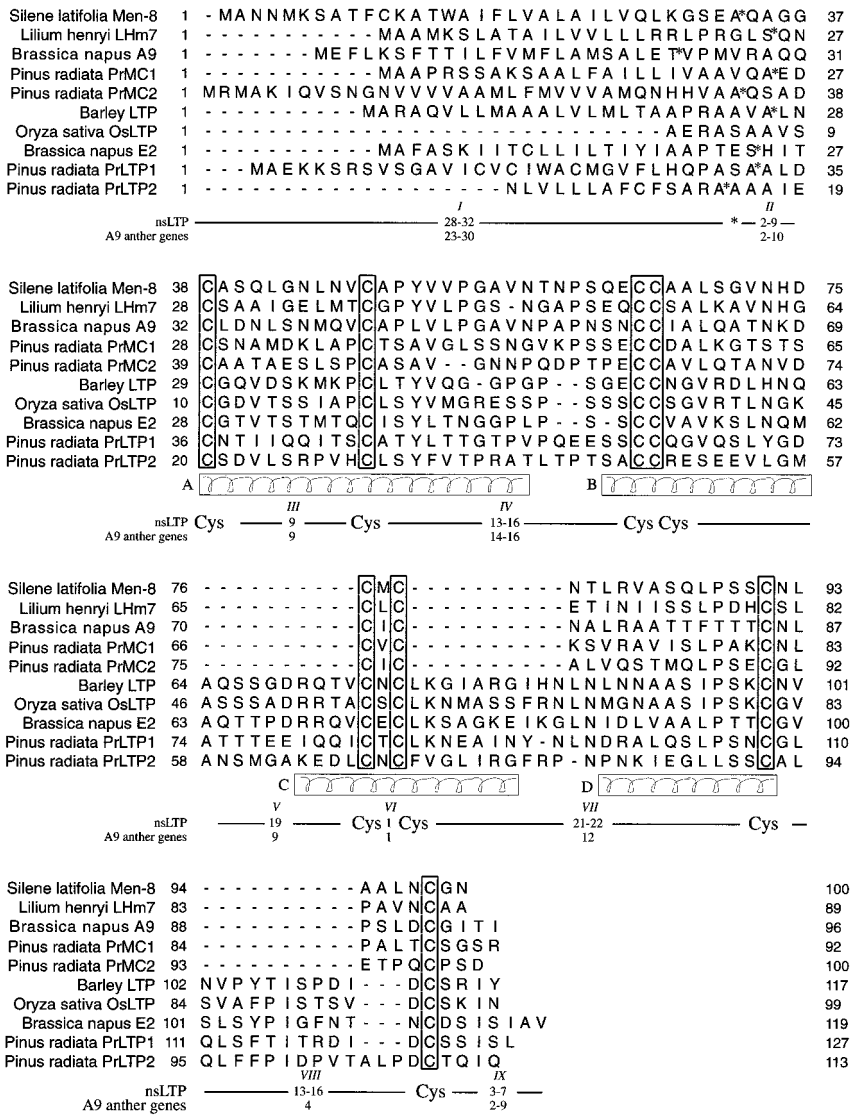

Figure 7. Comparison of deduced LTP and A9 anther-expressed proteins. Deduced sequences of nsLTPs (PrLTP1, PrLTP2, E2, OsLTP, and bLTP) and A9 homologs (PrMC1, PrMC2, Men-8, Lhm7, and A9) were aligned using the GCG program PileUp. The eight conserved Cys residues are shown beneath the alignment. The four helical domains of the barley LTP are labeled A, B, C, and D as indicated. The cleavage site for removal of the secretory sequence predicted by the GCG program SPSCAN is indicated by asterisk. The eight conserved Cys residues are shaded. Accession numbers are Men-8, y08780; LHm7, x80719; A9, q05772; barley LTP, p07597; OsLTP, u29176; and E2, x60318.

were expressed within the period in which the tapetum is known to undergo a burst in RNA synthetic activity, beginning with meiosis and ending before the first mitotic division in the microspore (Williams and Heslop-Harrison, 1979; Pacini et al., 1985). The temporal expression of 10 of the 13 genes (from meiosis until after the tetrads have dissolved) is consistent with their being expressed in the tapetum. In situ hybridization confirmed that transcripts of four genes (PrLTP1, PrMC1, PrChS1, and PrMC2) are expressed in the tapetum of Me-tIpII- and T2-stage cones. However, three cDNAs, PrMC6, PrLTP2, and PrMC104, were expressed only for short periods within this developmental window, suggesting that their expression is regulated differently and may not be part of the burst in tapetum expression. PrLTP2 transcript, with the shortest duration of expression (Figs. 2 and 3), was restricted to the tetrads (Fig. 4).

Of the 13 groups of cross-hybridizing cDNAs isolated in this study, six represented novel genes with no homologs in the databases. The remaining seven showed homology to angiosperm sequences, six of which (corresponding to PrChS1, PrLTP1, PrLTP2, PrMC1, PrMC2, and PrThL1) are specifically expressed in anthers or flowers (Table II). The pine sequences generally exhibit similar temporal and spatial expression patterns to their angiosperm homologs (for reviews, see Scott et al., 1991b; Goldberg et al., 1993). Considering the evolutionary distance between angiosperms and gymnosperms and the morphological differences between flowers and cones, the conservation of gene expression is striking and supports previous hypotheses that angiosperm and gymnosperm reproductive structures share common ancestry (discussed in Hickey and Taylor, 1996).

Microspores from both angiosperms and gymnosperms develop in a microsporangium surrounded by a tapetum, and the genes expressed in these organs have probably been retained through evolution because they play important roles in pollen development. Selective pressure appears to have maintained this intricate microspore-tapetum relationship, as disruptions to it are known to be a frequent

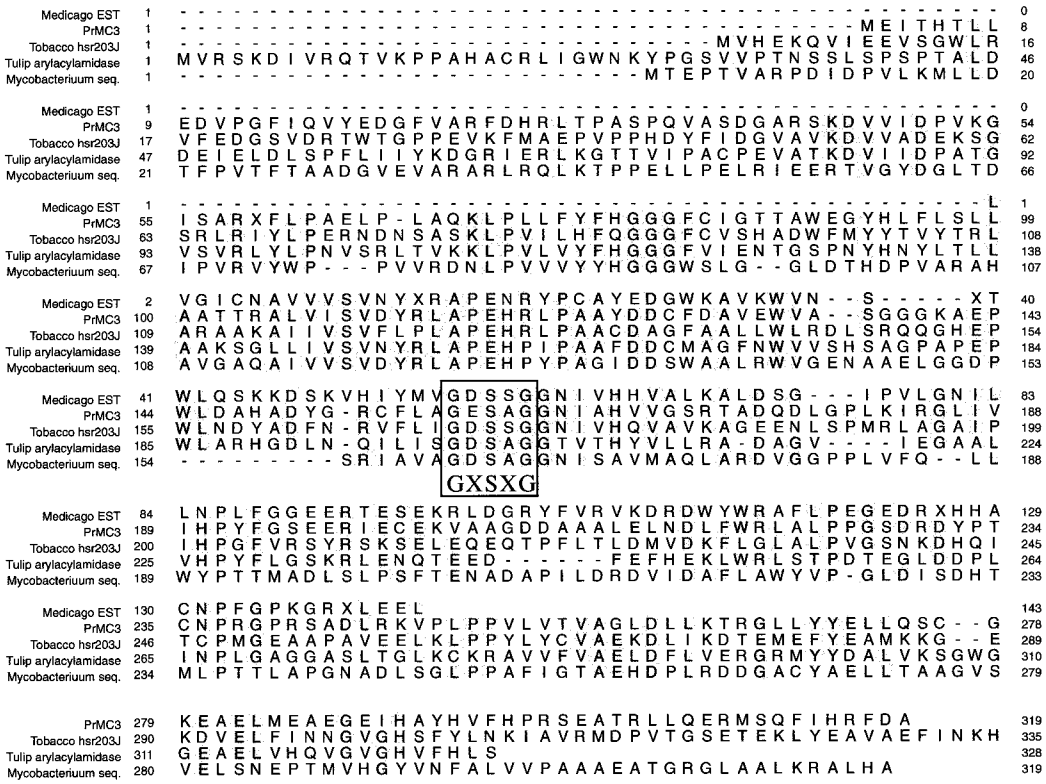

Figure 8. Alignment of PrMC3 with four homologs. The figure shows an alignment of deduced amino acid sequences for four homologs of PrMC3. The Ser hydrolase GXSXG motif is enclosed in a box, and bases identical in three or more sequences are shaded. Accession numbers for the sequences are as follows: *Medicago* expressed sequence tag, aa660803; tobacco hsr203J, s42807; tulip *arylacylamidase*, e03271; and *Mycobacterium* seq., z80108.

cause of male sterility (Kaul, 1988). The temporal and spatial expression patterns of the genes expressed in cones is also consistent with the idea that the conifer homologs (Tandre et al., 1995; Mellerowicz et al., 1998; Mouradov et al., 1998a, 1998b) of angiosperm floral meristem and floral organ genes play similar roles in regulating gymnosperm cone development.

A *P. radiata* homolog of ChS that grouped on a phylogenetic tree with a subset of five previously isolated ChS clones was isolated. The PrChS1 clade includes members from pine, rice, tobacco, and *Brassica* sp., so evolution of this clade must have occurred before the divergence of gymnosperms and angiosperms. In contrast, stilbene synthase genes evolved from ChS genes on several occasions in the course of evolution within both the angiosperm and gymnosperm divisions of the plant kingdom (Fig. 6; Tropf et al., 1994). Comparison of alignments of this clade with secondary structure predictions made by Tropf et al. (1994) supports the idea that genes in this clade may possess stilbene synthase activity. The role of the PrChS1 clade in male reproductive tissues remains to be determined. ChS enzymes have been demonstrated to be essential for pollen development. Disruptions to ChS activity in the anthers of petunia (Taylor and Jorgensen, 1992; van der Meer et al., 1992), maize (Coe et al., 1981), and tobacco (Fischer et al., 1997) resulted in the production of sterile pollen that could be rescued by the application of exogenous flavanoids (Taylor, 1995; Fischer et al., 1997).

We identified two homologs of nsLTPs and two A9 homologs that were expressed in male cones. nsLTPs have been isolated from various species based on their expression in anthers and in response to infection and stress (Nacken et al., 1991; Foster et al., 1992; Aguirre and Smith, 1993; Kader, 1996, 1997). Plant nsLTPs bind lipids with low affinity and are able to transfer them between membranes (for review, see Kader, 1996, 1997). Some inhibit proteases and limit the growth of microbial pathogens (Cammue et al., 1995; Kader, 1996, 1997). The A9 proteins might have a function related to the nsLTPs, based on their eight conserved Cys residues (see Fig. 7) and their overall structural similarity (Paul et al., 1992; Crossley et al., 1995). Alignments indicate that helix C of the nsLTPs is probably modified or missing in the A9 proteins; see Figure 7 and Paul et al. (1992). This helix is important in accommodating binding to large lipids (Shin et al., 1995; Lerche et al., 1997). We suggest that deletion of this helix might confer different substrate specificity on the smaller A9 proteins.

The high copy number of some of these nsLTP genes in the genome suggests that they may be in the process of acquiring new functions (Cammue et al., 1995; Kader, 1996). In vivo roles suggested for nsLTPs and A9 homologs in anthers include: shuttling lipids from the tapetum to the meiocytes and developing microspores (Foster et al., 1992; Kader, 1996, 1997), acting as a sulfur store (Aguirre and Smith, 1993), protecting the microspores from hydrolytic enzymes released during degradation of the tapetum

(Crossley et al., 1995), and protecting the pollen from pathogens either before or after dehiscence (Paul et al., 1992; Crossley et al., 1995). To date, no anther-expressed nsLTP or A9 protein has been tested for antimicrobial, proteinase inhibition, or lipid transfer activity. The expression of PrLTP2 in the tetrads during a brief developmental window suggests that it is unlikely to play a role in protection from pathogens, but rather plays a specialized role associated with early microspore development.

The screen for male-cone-specific genes also identified a *P. radiata* homolog of thaumatin. Many thaumatin proteins are induced by the plant in response to infection by pathogens and exposure to environmental stresses (Singh et al., 1989; Vigers et al., 1992; Stintzi et al., 1993; Abad et al., 1995; Griffith et al., 1997; Hu and Reddy, 1997). Some thaumatins have anti-microbial activity and are thought to permeabilize fungal hyphae by forming a pore or channel, allowing the release of the cytoplasmic contents (Abad et al., 1996; Cheong et al., 1997). Some thaumatin genes are expressed in floral tissues (Richard et al., 1992; Chen et al., 1996), but none of this group has been tested for antimicrobial activity. A possible role for PrThL1 in *P. radiata* may be to permeabilize the plasma membranes of the tapetum, facilitating transport of compounds to the developing microspores.

PrMC3 is a member of a family of proteins that all contain a Ser hydrolase motif (GxSxG) and have similarity to lipases and esterases of prokaryotic origin. PrMC3 is the first member of the family differentially expressed in male reproductive structures of a plant. The timing of PrMC3 expression, which occurs right through the burst of tapetal layer activity, would be consistent with a role hydrolyzing stored lipids for transfer from the tapetum to the microspores by nsLTPs and A9 homologs. However, the spatial expression of PrMC3 has not been determined, and the fact that the protein encoded by the tobacco homolog hsr203J is unable to hydrolyze lipids (Baudouin et al., 1997) suggests that this scenario is unlikely. Recombinant hsr203J protein degrades *p*-nitrophenylbutyrate, a general substrate for carboxylesterases, which suggests it is an esterase (Baudouin et al., 1997). The tobacco gene hsr203J is induced specifically and early in the hypersensitive response, after challenge by pathogens, and probably plays a role in regulating or limiting programmed cell death (Pontier et al., 1994, 1998). We suggest that PrMC3 might play a similar role in regulating the developmentally regulated programmed cell death of the tapetal layer during male cone development.

The association between genes expressed in *P. radiata* male cones and angiosperm genes involved in the pathogenesis response was striking. If the A9 proteins have activities related to the nsLTPs, then all seven of the genes that were identified in this study could be considered pathogenesis related. Vigers et al. (1992) proposed that tissues with vital reproductive capacities but limited resources for counterattack, such as seeds and tubers and, by inference, pollen, store thaumatin as protection from future microbial infections. Similar statements have been made in relation to the nsLTP and A9 proteins (Paul et al., 1992; Turgut et al., 1994; Crossley et al., 1995), and the same could be said of PrChS1. However, we consider it unlikely that the expression of these genes in male cones occurs solely in response to, or as protection against, pathogens or stress. First, the timing and pattern of expression of some of the genes (e.g. PrLTP2) is highly specific. Second, most of these genes are expressed in the tapetal tissue, which is protected from the external environment prior to anthesis and seems unlikely to be subject to large numbers of pathogen attacks. Third, the sheer number of genes involved in defense seems very high, given that pollen development involves an intense burst of metabolic activity over the period in which the genes are expressed (Pacini et al., 1985). Fourth, none of the nsLTP, A9, or thaumatin proteins expressed in male reproductive structures has been shown to have antimicrobial activity. Finally, most of the transcripts identified in this study have plausible functions in pollen development, such as shuttling lipids for the nsLTPs. In some cases, these functions are common to pathogen or stress responses. For example, both reproductive development and the plant response to stress or pathogens can involve the programmed reorganization and degradation of tissue (Greenberg, 1998). In male reproductive tissues the genes involved in tapetum degradation and mobilization are expected to be developmentally regulated, whereas in the pathogen response, the genes are induced as a result of an external stimuli.

In summary, we have shown that homologous genes are expressed differentially in male reproductive development in the angiosperm and gymnosperm divisions of the plant kingdom. The genes isolated here will prove useful for the isolation of tapetal-specific promoters and the genetic manipulation of male sterility in conifers.

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LITERATURE CITED

Abad AR, Mehrtens BJ, Mackenzie SA (1995) Specific expression in reproductive tissues and fate of a mitochondrial sterility-associated protein in cytoplasmic male-sterile bean. Plant Cell **7:** 271–285

Abad LR, D'Urzo MP, Liu D, Narasimhan ML, Reuveni M, Zhu JK, Niu X, Singh NK, Hasegawa PM, Bressan RA (1996) Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. Plant Sci **118:** 11–23

Aguirre PJ, Smith AG (1993) Molecular characterization of a gene encoding a cysteine-rich protein preferentially expressed in anthers of *Lycopersicon esculentum*. Plant Mol Biol **23:** 477–487

Arro M, Richard L, Van K, Ferrer A, Boronat A (1997) Cloning and characterization of a novel cDNA encoding thaumatin-like protein PR5 from *Arabidopsis thaliana* (accession no. L78079) (PGR 97–053). Plant Physiol **113:** 1463

Atanassov I, Russinova E, Antonov L, Atanassov A (1998) Expression of an anther-specific chalcone synthase-like gene is correlated with uninucleate microspore development in *Nicotiana sylvestris*. Plant Mol Biol **38:** 1169–1178

Baudouin E, Charpenteau M, Roby D, Marco Y, Ranjeva R, Ranty B (1997) Functional expression of a tobacco gene related to the serine hydrolase family-esterase activity towards short-chain dinitrophenyl acylesters. Eur J Biochem **248:** 700–706

Bochenek B, Hirsch AM (1990) In-situ hybridisation of nodulin mRNAs in root nodules using non-radioactive probes. Plant Mol Biol Rep **8:** 237–248

Cammue BP, Thevissen K, Hendriks M, Eggermont K, Goderis IJ, Proost P, Van Damme J, Osborn RW, Guerbette F, Kader JC, Borekaert WF (1995) A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer proteins. Plant Physiol **109:** 445–455

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep **11:** 113–116

Chen R, Wang F, Smith AG (1996) A flower-specific gene encoding an osmotin-like protein from *Lycopersicon esculentum*. Gene **179:** 301–302

Cheong NE, Choi YO, Kim WY, Kim SC, Cho MJ, Lee SY (1997) Purification of an antifungal PR-5 protein from flower buds of *Brassica campestris* and cloning of its gene. Physiol Plant **101:** 583–590

Coe EH, McCormick SM, Modena SA (1981) White pollen in maize. J Hered **72:** 318–320

Cox KH, Goldberg RB (1988) Analysis of plant gene expression. *In* CH Shaw, eds, Plant Molecular Biology: A Practical Approach. IRL Press, Oxford, pp 1–35

Crossley SJ, Greenland AJ, Dickinson HG (1995) The characterisation of tapetum-specific cDNAs isolated from a *Lilium henryi* L. meiocyte subtractive cDNA library. Planta **196:** 523–529

Doyle J, Doyle J (1990) Isolation of plant DNA from fresh tissue. Focus **12:** 13–15

Fang G, Hammer S, Grumet R (1992) A quick and inexpensive method for removing polysaccharides from plant genomic DNA. BioTechniques **13:** 52–55

Feliciello I, Chinali G (1993) A modified alkaline lysis method for the preparation of highly purified plasmid DNA from *Escherichia coli*. Anal Biochem **2312:** 394–401

Fischer R, Budde I, Hain R (1997) Stilbene synthase gene expression causes changes in flower colour and male sterility in tobacco. Plant J **11:** 489–498

Foster GD, Robinson SW, Blundell RP, Roberts MR, Hodge R, Draper J, Scott RJ (1992) A *Brassica-Napus* mRNA encoding a protein homologous to phospholipid transfer proteins is expressed specifically in the tapetum and developing microspores. Plant Sci **84:** 187–192

Goldberg RB, Beals TP, Sanders PM (1993) Anther development: basic principles and practical applications. Plant Cell **5:** 1217–1229

Greenberg JT (1998) Programmed cell death: a way of life for plants. Proc Natl Acad Sci **93:** 12094–12097

Griffith M, Antikainen M, Hon WC, Pihakaski Maunsbach K, Yu XM, Chun JU, Yang DSC (1997) Antifreeze proteins in winter rye. Physiol Plant **100:** 327–332

Hain R, Reif HJ, Krause E, Langebartels R, Kindl H, Vornam B, Wiese W, Schmelzer E, Schreier PH, Stocker RH, Stenzel K (1993) Disease resistance results from foreign phytoalexin expression in a novel plant. Nature **361:** 153–156

Heslop-Harrison J (1968) Pollen wall development: the succession of events in the growth of intricately patterned pollen walls is described and discussed. Science **161:** 230–237

Hickey LJ, Taylor DW (1996) Origin of the angiosperm flower. *In* LJ Hickey, DW Taylor, eds, Flowering Plant Origin, Evolution and Phylogeny. Chapman and Hall, New York

Hihara Y, Hara C, Uchimiya H (1996) Isolation and characterization of two cDNA clones for mRNAs that are abundantly expressed in immature anthers of rice (*Oryza sativa* L.). Plant Mol Biol **30:** 1181–1193

Hu X, Reddy ASN (1997) Cloning and expression of a PR5-like protein from *Arabidopsis*: inhibition of fungal growth by bacterially expressed protein. Plant Mol Biol **34:** 949–959

Kader JC (1996) Lipid transfer proteins in plants. Annu Rev Plant Physiol Plant Mol Biol **47:** 627–654

Kader JC (1997) Lipid-transfer proteins: a puzzling family of plant proteins. Trends Plant Sci **2:** 66–70

Kaul M (1988) Male sterility in higher plants. Springer Verlag, Berlin

Koes RE, Spelt CE, Mol JNM (1989a) The chalcone synthase multigene family of *Petunia-Hybrida* V30 differential light-regulated expression during flower development and UV light induction. Plant Mol Biol **12:** 213–226

Koes R, Spelt C, Van Den Elzen P, Mol J (1989b) Cloning and molecular characterization of the chalcone synthase multigene family of *Petunia-Hybrida*. Gene **81:** 245–258

Koltunow AM, Truettner J, Cox KH, Wallroth M, Goldberg RB (1990) Different temporal and spatial gene expression patterns occur during anther development. Plant Cell **2:** 1201–1224

Lerche MH, Kragelund BB, Bech LM, Poulsen FM (1997) Barley lipid-transfer protein complexed with palmitoyl CoA: the structure reveals a hydrophobic binding site that can expand to fit both large and small lipid-like ligands. Structure **5:** 291–306

Mellerowicz EJ, Horgan K, Walden A, Coker A, Walter C (1998) PRFLL-a *Pinus radiata* homologue of FLORICAULA and LEAFY is expressed in buds containing vegetative shoot and undifferentiated male cone primordia. Planta **206:** 619–629

Mouradov A, Glassick T, Hamdorf B, Murphy L, Fowler B, Marla S, Teasdale RD (1998a) NEEDLY, a *Pinus radiata* ortholog of FLORICAULA/LEAFY genes, expressed in both reproductive and vegetative meristems. Proc Natl Acad Sci USA **95:** 6537–6542

Mouradov A, Glassick TV, Hamdorf BA, Murphy LC, Marla SS, Yang Y, Teasdale RD (1998b) Family of MADS-Box genes expressed early in male and female reproductive structures of monterey pine. Plant Physiol **117:** 55–62

Munch S (1994) Nonradioactive northern blots in 1.5 days with substantially increased sensitivity through "alkaline blottting. Biochemica **3:** 30–32

Nacken W, Huijser P, Beltran J, Saedler H, Sommer H (1991) Molecular characterization of two stamen-specific genes Tapl and Fil1 that are expressed in the wild type but not in the deficiens mutant of *Antirrhinum majus.* Mol Gen Genet **229:** 129–136

Pacini E, Franchi GG, Hesse M (1985) The tapetum: its form, function and possible phylogeny in Embryophyta. Plant Syst Evol **149:** 155–185

Paul W, Hodge R, Smartt S, Draper J, Scott R (1992) The isolation and characterisation of the tapetum-specific *Arabidopsis thaliana* A9 gene. Plant Mol Biol **19:** 611–622

Pontier D, Godiard L, Marco Y, Roby D (1994) hsr203J, a tobacco gene whose activation is rapid, highly localized and specific for incompatible plant/pathogen interactions. Plant J **5:** 507–521

Pontier D, Tronchet M, Rogowsky P, Lam E, Roby D (1998) Activation of hsr203, a plant gene expressed during incompatible plant- pathogen interactions, is correlated with programmed cell death. Mol Plant-Microbe Interact **11:** 544–554

Richard L, Arro M, Hoebeke J, Meeks Wagner DR, Van KTT (1992) Immunological evidence of thaumatin-like proteins during tobacco floral differentiation. Plant Physiol **98:** 337–342

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schubert R, Fischer R, Hain R, Schreier PH, Bahnweg G, Ernst D, Sandermann H Jr (1997) An ozone-responsive region of the grapevine resveratrol synthase promoter differs from the basal pathogen-responsive sequence. Plant Mol Biol **34:** 417–426

Scott R, Dagless E, Hodge R, Paul W, Soufleri I, Draper J (1991a) Patterns of gene expression in developing anthers of *Brassica napus*. Plant Mol Biol **17:** 195–208

Scott R, Hodge R, Paul W, Draper J (1991b) The molecular biology of anther differentiation. Plant Sci **80:** 167–192

Scutt CP, Li Y, Robertson SE, Willis ME, Gilmartin PM (1997) Sex determination in dioecious *Silene latifolia*: effects of the Y chromosome and the parasitic smut fungus (*Ustilago violacea*) on gene expression during flower development. Plant Physiol **114:** 969–979

Shen JB, Hsu FC (1992) *Brassica* anther-specific genes: characterization and in situ localization of expression. Mol Gen Genet **234:** 379–389

Shin DH, Lee JY, Hwang KY, Kim KK, Suh SW (1995) High-resolution crystal structure of the non-specific lipid-transfer protein from maize seedlings. Structure **3:** 189–199

Singh NK, Nelson DE, Kuhn D, Hasegawa PM, Bressan RA (1989) Molecular cloning of osmotin and regulation of its expression by Aba and adaptation to low water potential. Plant Physiol **90:** 1096–1101

Stanley RG, Linskens HF (1974) Pollen. Springer-Verlag, Berlin

Stintzi A, Heitz T, Prasad V, Wiedemann-Merdinoglu S, Kauffmann S, Geoffroy P, Legrand M, Fritig B (1993) Plant 'pathogenesis-related' proteins and their role in defense against pathogens. Biochimie **75:** 687–706

Strauss SH, Rottmann WH, Brunner AM, Sheppard LA (1995) Genetic engineering of reproductive sterility in forest trees. Mol Breed **1:** 5–26

Tandre K, Albert VA, Sundas A, Engstrom P (1995) Conifer homologues to genes that control floral development in angiosperms. Plant Mol Biol **27:** 69–78

Taylor LP (1995) Flavonols: effects on fertility and fecundity. Crop Sci **35:** 1521–1526

Taylor LP, Jorgensen R (1992) Conditional male fertility in chalcone synthase-deficient petunia. J Hered **83:** 11–17

Tropf S, Lanz T, Rensing SA, Schroeder J, Schroeder G (1994) Evidence that stilbene synthases have developed from chalcone synthases several times in the course of evolution. J Mol Evol **38:** 610–618

Turgut K, Barsby T, Craze M, Freeman J, Hodge R, Paul W, Scott R (1994) The highly expressed tapetum-specific A9 gene is not required for male fertility in *Brassica napus*. Plant Mol Biol **24:** 97–104

Turgut K, Hodge R, Paul W, Scott R (1996) An anther-specific transcript from *Brassica napus* L. shows similarity to chalcone and stilbene synthase sequences. Turk J Bot **20:** 305–309

Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S, Chandler D, Slusarenko A, Ward E, Ryals J (1992) Acquired resistance in *Arabidopsis*. Plant Cell **4:** 645–656

van der Meer IM, Spelt CE, Mol JN, Stuitje AR (1990) Promoter analysis of the chalcone synthase (chsA) gene of *Petunia hybrida*: a 67-bp promoter region directs flower-specific expression. Plant Mol Biol **15:** 95–109

van der Meer IM, Stam ME, van Tunen AJ, Mol JN, Stuitje AR (1992) Antisense inhibition of flavonoid biosynthesis in petunia anthers results in male sterility. Plant Cell **4:** 253–262

Vigers AJ, Wiedemann S, Roberts WK, Legrand M, Selitrennikoff CP, Fritig B (1992) Thaumatin-like pathogenesis-related proteins are antifungal. Plant Sci **83:** 155–161

Virca DG, Northemann W, Shiels BR, Widera G, Broome S (1990) Simplified northern blot hybridisation using 5% sodium dodecyl sulphate. Biotechniques **8:** 370–371

Vogeli G, Kaytes PS (1987) Amplification, Storage and Replication of Libraries. *In* SL Berger, AR Kimmel, eds, Methods In Enzymology Guide To Molecular Cloning Techniques. Academic Press, New York, pp 407–415

Wang Y (1995) Male cone development in *Pinus radiata*. PhD thesis. Massey University, Palmerston North, New Zealand

Wilkinson DG (1992) In Situ Hybridisation a Practical Approach. IRL Press, Oxford

Williams EG, Heslop-Harrison J (1979) A comparison of RNA synthetic activity in the plasmodial and secretory types of tapetum during the meiotic interval. Phytomorphology **29:** 370–381