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Isolation of Tissue-Specific cDNAs from Tomato Pistils

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We have used a differential plaque hybridization screening procedure to isolate cDNA clones for genes that show elevated or exclusive expression in tomato pistils. Clones that showed maximal expression in immature pistils (premeiotic to early meiosis) and mature pistils (at anthesis) were isolated. Of nine clones that were characterized, four were found also to express at some stage of anther development. In situ hybridization experiments showed that expression of the genes we have identified is very tightly regulated both spatially and temporally within the pistil. One gene was identified that is expressed in the pistil only in the transmitting tissue of the style. A second gene was found to express exclusively in two to three cell layers of the ovules for a period of less than eight days.

INTRODUCTION

Although the development of a higher plant is responsive to environmental influences, the heritability of form and function in plants demonstrates that this process is primarily under genetic control. The organ-specific modulation of gene expression is necessarily responsible for the biochemical differentiation of the cells within an organ or tissue. Kamalay and Goldberg (1980) have shown that each plant organ contains numerous transcripts that are not present in other plant organs. Identification of the regulated genes and determination of the functions of their protein products are important steps in the formation of a complete model of plant development. Recent publications have reported the isolation of a number of organ-specific sequences from stems, petals, roots, and floral organs of tobacco (Goldberg, 1988), embryos of soybean (Goldberg, 1988), germinating embryos of *Brassica napus* (Harada et al., 1988), and various organs of *Arabidopsis thaliana* (Simoens et al., 1988).

We have chosen the process of floral differentiation as a model for studies on developmentally regulated plant genes. Flowering is a complex process that typifies essentially all of the major aspects of plant development: organogenesis, differential cell division, cellular differentiation, and alterations in gene expression. Flowering begins with a series of events that lead to the conversion of a vegetative meristem to a floral meristem. This conversion is followed by a shift in phyllotaxis and the development of the lateral floral organs: the sepals, petals, stamens, and carpels. As these organs differentiate, a number of specialized tissues and structures are produced. These include the carpel walls, the placenta, the transmitting tissue of the style, the stigma, the ovules, the anther walls, the tapetal cells within the anthers, and the micro- and megagametophytes. Each of these tissues contains novel substances that are not found in other plant organs. These substances are the direct or secondary products of floral specific genes.

We have successfully used a differential screening procedure to isolate cDNA clones for developmentally regulated genes from the pistils of tomato. RNA filter hybridizations demonstrate the organ-specific expression of these genes and show that the genes are temporally regulated within the pistils. In in situ hybridization experiments we show that expression of these genes is restricted to specific cells of the pistils.

RESULTS

Construction of cDNA Libraries

Pistils were dissected from mature flowers and from flower buds that had been separated into two classes according to size. Microscopic examination of the buds showed that the microspores and megaspores were in early meiosis to mid-meiosis in the 6- to 7-mm buds and in late meiosis to tetrad stage in the 8- to 9-mm buds (data not shown). Poly(A^+) RNA was extracted from the mature pistils and from the two stages of immature pistils, and a separate cDNA library was made from each class of RNA in the bacteriophage vector λ gt10 (Huynh et al., 1985). Prelimi-

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nary experiments to optimize the cDNA cloning procedure demonstrated that many of the intermediate purification steps included in previously published methods could be eliminated, resulting in higher cloning efficiency. The quantities of RNA and the numbers of cDNA clones in our libraries are given in Table 1. The numbers of clones obtained in the three libraries were large enough to represent rare messengers. These libraries therefore, were more than sufficient for our screening experiments.

Identification of Clones with Preferential Expression in Pistils

We used a direct screening procedure to identify and isolate clones for genes with enhanced expression in pistils relative to expression in vegetative organs. cDNA clones from each library were plated at low density, and duplicate nitrocellulose lifts were made from each plate (see "Methods"). One filter was hybridized with ^{32}P-labeled single-stranded cDNA synthesized from the RNA that had been used to construct the library. The second filter was hybridized with a similar probe made from RNA from 14-day-old tomato seedlings. Seedlings of this age have two well-developed leaves, a shoot apex with leaf primordia, a stem that has initiated secondary growth, and a well-developed root system. In tomato, floral development is not initiated until six to eight mature leaves have been formed (Chandra Sekhar and Sawhney, 1984). Seedlings with two leaves will include, therefore, all mRNA species normally found in the vegetative organs of tomato and should be deficient in mRNA species with enhanced expression in flowers. The plaques showing substantially stronger hybridization to the pistil cDNA probes than to the seedling cDNA probe were picked and plated at 100 to 200 pfu/plate. Nitrocellulose replicas were made from these secondary plates and were hybridized with the same probes. Single plaques that showed detectable hybridization with the pistil probes and weak or undetectable hybridization with the seedling probe were isolated for further characterization.

Final screening was performed using DNA gel blots of phage DNA isolated from the putative pistil-specific clones that had been digested with EcoRI to separate the cDNA inserts from the phage vector. Duplicate DNA gel blots were hybridized with the same probes used in the original screening steps. This tertiary screen proved to be a sensitive method for screening a large number of clones for pistil specificity. Although several of the clones that came through the first two rounds of screening showed approximately equal hybridization to the two probes, most showed preferential hybridization to the pistil probe (data not shown). In all, 23 clones with strong (greater than 10-fold) preferential expression in pistils relative to seedlings were identified from among the 136 primary isolates. The progressive elimination of clones through the screening process is summarized in Table 1.

Table 1. Construction and Screening of cDNA Libraries

Developmental Stage	Starting RNA	Library Size	Clones Screened	Initial Isolates	Final Isolates
	μg				
Mature	2.2	4×10^6	25,000	55	9
8–9 mm	0.8	8×10^5	20,000	16	4
6–7 mm	1.0	1×10^6	20,000	65	10

Organ Specificity and Temporal Regulation of cDNA Clones

Nine clones that exhibited the strongest preferential hybridization to labeled pistil cDNAs were further characterized by RNA gel blot analyses. Since only a single stage of pistil RNA was used to make the probes in the screening procedures, it was possible that the clones we had isolated could be expressed at other stages of pistil development. Expression in other floral organs, as well as low-level expression in specific vegetative organs, was also a possibility because the screen we used would not have detected these patterns of expression. Figure 1A shows an RNA gel blot of poly(A$^+$) RNA that was hybridized with clone pMON9619 which was isolated from the library made from pistils of 6- to 7-mm buds. This clone hybridized most strongly to a 2200 base RNA present in immature pistils (Figure 1A, lane 8). The level of this RNA in pistils decreased to an undetectable level as the pistils matured (Figure 1A, lanes 8 to 10). The gene corresponding to pMON9619 was also expressed in anthers according to a similar temporal program, showing decreasing expression as the anthers mature (Figure 1A, lanes 5 to 7). Although no expression was detected in seedlings, weak hybridization (about 20-fold less than in pistils from 6- to 7-mm buds) was visible in the leaf lane (Figure 1A, lane 3).

A second clone, pMON9617, showed a very different pattern of hybridization (Figure 1B). In this experiment hybridization of pMON9617 to 5 μg of poly(A$^+$) RNA from the vegetative organs and petals was compared with the signal obtained with 1 μg of poly(A$^+$) RNA from reproductive organs. This clone hybridized very strongly to a 900-base RNA in mature pistils (Figure 1B, lane 2), but showed no hybridization to a mixture of RNA from pistils of 6- to 7- and 8- to 9-mm buds (Figure 1B, lane 3). The gene corresponding to this clone was expressed at a lower level in anthers (about 20-fold) but according to the same temporal program observed in pistils (Figure 1B, lanes 4 and 5). A signal was also seen with this clone in the nonreproductive organs (Figure 1B, lanes 6 to 9). The strongest of these signals was in the petal lane and represented a

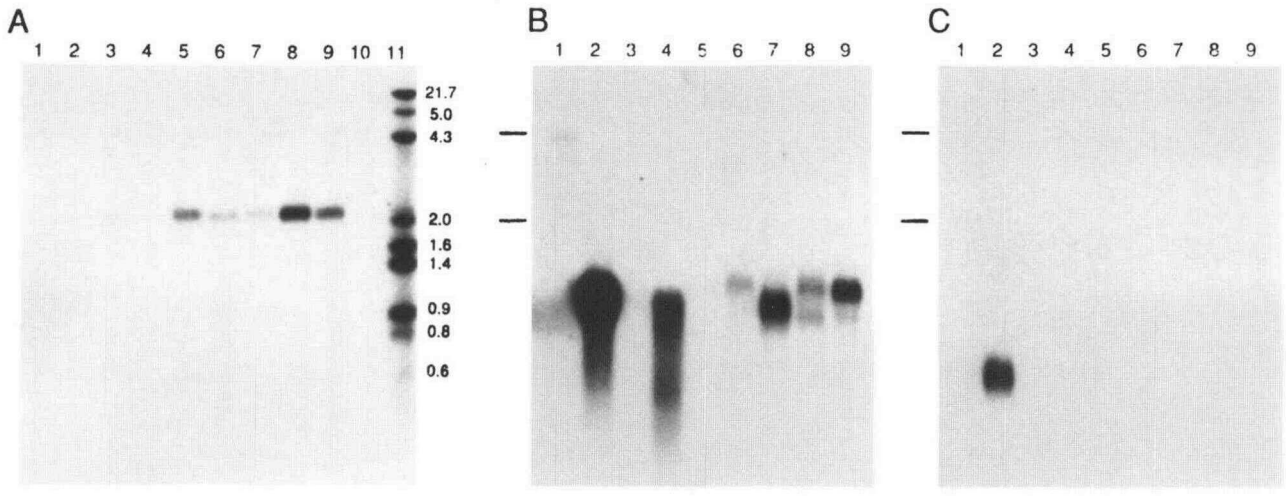

Figure 1. Representative RNA Gel Blots Probed with Pistil cDNA Clones.

(A) RNA gel blot of tomato poly(A^+) RNAs from the indicated organs hybridized with cDNA clone pMON9619 (from the cDNA library made from pistils of 6- to 7-mm buds). Lane 1, green fruit (2 μg); 2, breaker fruit (2 μg); 3, leaves (2 μg); 4, seedlings (1 μg); 5, anthers from 6- to 7-mm buds (2 μg); 6, anthers from 8- to 9-mm buds (2 μg); 7, mature anthers (2 μg); 8, pistils from 6- to 7-mm buds (1 μg), pistils from 8- to 9-mm buds (1 μg), mature pistils (1 μg). Lane 11 is a set of labeled DNA size markers. The sizes of the markers are given to the right of the autoradiogram in kilobase pairs.

(B) A similar RNA gel blot hybridized with a single-stranded RNA probe made from cDNA clone pMON9617 (isolated from the cDNA library made from pistils of mature flowers at anthesis). Lane 1, total RNA from *Zea mays* (10 μg); 2, mature pistils (1 μg); 3, immature pistils (0.5 μg RNA each from pistils of 6- to 7- and 8- to 9-mm buds); 4, mature anthers (1 μg); 5, immature anthers (0.5 μg RNA each from 6- to 7-mm and 8- to 9-mm pistils); 6, leaves (5 μg); 7, petals (5 μg); 8, roots (5 μg); 9, seedlings (5 μg).

(C) The RNA gel blot used in **(B)** was eluted and rehybridized with a single-stranded RNA probe made from clone pMON9608 (isolated from the mature pistil cDNA library). The lines between the panels indicate the migration of the 26S and 18S ribosomal RNAs that were visualized in the lanes containing total RNA from *Z. mays*.

level of expression approximately 100-fold lower than that observed in mature pistils. The hybridization in the petal lane was to a lower molecular weight species of RNA than that observed in the pistil or anther lanes. This lower molecular weight RNA was also present in the RNA from roots and seedlings (Figure 1B, lanes 8 and 9). In DNA gel blotting experiments with tomato genomic DNA hybridized with pMON9617, only one set of sequences was detected, indicating that the gene for this cDNA is present in a single copy (data not shown). Differential splicing, or use of different transcription start or stop sites within a single gene, may account for the two size classes of message that hybridized to this clone.

Clone pMON9608 showed a very simple hybridization pattern. This clone hybridized exclusively to an RNA of approximately 600 bases in mature pistils (Figure 1C). Even in very long exposures, no RNA hybridizing to this probe was detectable in any of the other lanes (data not shown). Long exposures of the RNA gel blots would have detected a signal 1000-fold lower than the hybridization observed in the mature pistil RNA lane; thus, this gene was expressed at least 1000-fold less in anthers than in pistils. Since there was 5 times more RNA in the leaf, root, seedling, and petal lanes, this shows that this gene was expressed at least 5000-fold higher in mature pistils than in these organs.

Similar experiments were performed on six additional clones. The results of these and the above experiments are summarized in Table 2. Four of the nine clones tested showed some level of expression in anthers, and expression of six of the clones persisted as the pistil developed to form green fruits. The differences in the patterns of expression between the clones imply that each of the clones responds to a unique set of regulatory signals despite the fact that they all have enhanced expression in pistils. pMON9608 is the only clone that showed exclusive expression in pistils. DNA gel blots of tomato genomic DNA were also performed using each of the cDNA clones as probes. Based on copy number reconstructions, we found that the genes corresponding to all nine of these cDNA clones were present in one, or at most two, copies per haploid genome (data not shown).

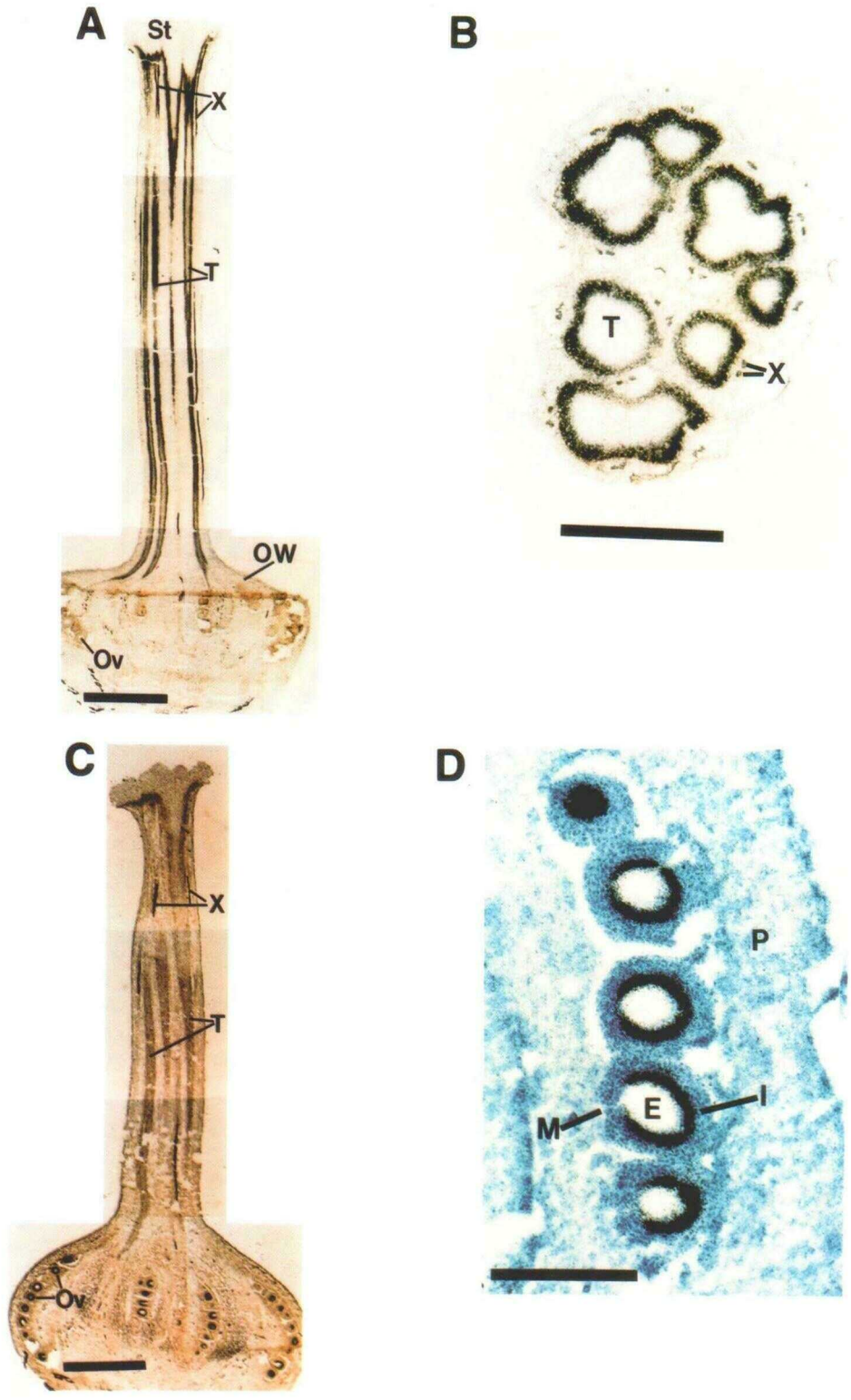

Figure 2. In Situ Hybridizations of Pistil Sections with ^{35}S-Labeled Single-Stranded RNA Probes from Pistil cDNA Clones.

(A) A longitudinal section of a mature tomato pistil was hybridized with a probe made from mature pistil cDNA clone pMON9617, coated with photographic emulsion, allowed to expose for 12 days, and developed. Strong hybridization is visible in the strands of transmitting tissue (T). The xylem vessel members (X) appear black in this bright field micrograph due to their refractile walls. Other indicated features of the pistil are the stigma (St), the ovules (Ov), and the ovary wall (OW). The scale bar is 1 mm.

(B) Transverse section through the central region of the style of a mature tomato pistil hybridized and processed as in **(A)**. Intense

Table 2. Hybridization Intensity in RNA Gel Blot Experiments. Relative Signal Is Indicated by the Number of + Symbols.

Clone No. (pMON)	cDNA	RNA	Mature Pistil	Immature Pistil	Mature Anther	Immature Anther	Green Fruit	Seedl.	Leaf	Petal
	bp	bases								
9601	1300	1300	+	++	–[a]	+++	+	–	±	nt[b]
9604	550	700	++	nt	–	nt	+	–	–	nt
9606	1820	2300	++	nt	–	nt	+++	–	+	++
9608	430	500	+++	–	–	–	–	–	–	–
9611	820	900	+++	nt	–	nt	++	–	+	++
9612	1300	1450	+++	–	–	++	+	–	–	+
9614	1350	2200	++	nt	–	nt	+++	+	++	nt
9617	850	900	+++	–	+++	–	–	+	–	++
9619	440	2200	+	+++	+	++	–	–	+	nt

[a] –, no detectable signal.
[b] nt, not tested.

Localization of Gene Expression to Specific Tissues

Pistils are complex organs consisting of numerous differentiated tissues. The style includes the stigma specialized for the capture and germination of pollen and the transmitting tissues through which the pollen tubes grow toward the ovary. The ovary contains tissues that are required for proper fertilization and to provide nutrition to the seeds as they develop, as well as those cells that will differentiate to form the layers of the fruit and the seed coat. The pistil even contains the next generation in the form of the megagametophytes within the ovules. More refined techniques than the RNA gel blots described above are required to determine which of the tissues within the pistil express specific mRNAs. To study tissue-specific expression of our pistil cDNA clones, we used in situ hybridization of ^{35}S-labeled RNA probes to tissue sections.

Figure 2 shows examples of the results we obtained from in situ hybridization of pistil sections with probes made from cDNA clones pMON9617 and pMON9608. In Figure 2A, a probe from pMON9617, which hybridized most strongly to mature pistils and anthers on RNA gel blots (Figure 1B), was hybridized to a longitudinal section of a mature tomato pistil. Strong hybridization was visible in tracks running the length of the style. In tomato, the transmitting tissue through which the pollen tubes grow is present as a number of strands within the style. The pMON9617 hybridization was localized to these strands of transmitting tissue. At the top of the style, the hybridization subtended but did not intrude into the stigma. Hybridization of the same probe to a cross-section of the style (Figure 2B) showed that the hybridization was confined to the cells in the peripheral layers of the transmitting tissue. Hybridization to serial sections from a large number of pistils showed that the hybridizing tissue comprised a series of tubes surrounding the inner transmitting tissue in the style and extending into the ovary. At the top of the style, the cylinders fuse to form a funnel-shaped structure below the stigmatic tissue.

When an anti-sense RNA probe from the cDNA clone, pMON9608, was hybridized to similar sections, there was no visible hybridization to the transmitting tissue (Figure 2C). This demonstrates that the hybridization seen with the previous probe was not due to nonspecific binding of RNA to cells of the transmitting tissue. The pMON9608 probe hybridized exclusively to the ovules (Figure 2C). Several ovules, and the adjacent placental tissue, are shown at higher magnification in Figure 2D. The section has been stained with toluidine blue to allow better visualization of the cellular structure of the ovules. It can be seen that the hybridization was confined to two to three cell layers of the integument. A layer of cells within the

Figure 2 (continued).

hybridization is visible at the periphery of the strands of the transmitting tissue (T). The refractile xylem (X) appears dark, but is not hybridizing. The scale bar is 0.5 mm.

(C) A longitudinal section of a mature tomato pistil was hybridized with a probe made from mature pistil cDNA clone pMON9608 and processed as in **(A)**. Hybridization is visible only in the ovules (Ov). The xylem (X) is black due to refraction in the cell walls, and the transmitting tissue (T) is dark due to the density of this tissue.

(D) Ovules of a longitudinal section of a mature pistil hybridized and treated as in **(C)** were stained with toluidine blue to reveal cellular detail and photographed at higher magnification. The nuclei stain most intensely with this dye. Hybridization is confined to approximately two cell layers of the integument (I). A gap in the hybridization is visible when the plane of section passes through the micropyle (M). No hybridization is visible in the placenta (P). The scale bar is 0.2 mm.

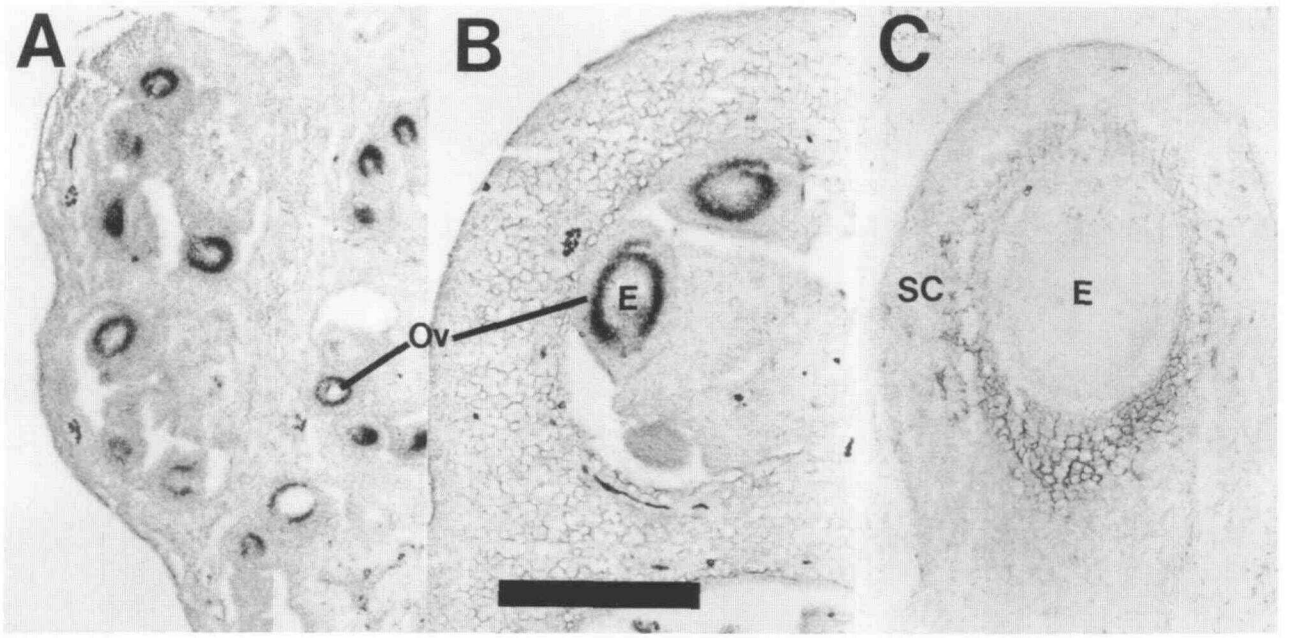

Figure 3. Time Course of Expression of the Gene Corresponding to pMON9608 in Tomato Ovules.

(A) A transverse section from a mature tomato pistil (at anthesis) was hybridized with an anti-sense RNA probe from pMON9608 and treated and developed as described in the legend to Figure 1. Hybridization is visible only in the integument of the ovules (Ov).
(B) A transverse section through a 10-mm tomato fruit hybridized and treated as in **(A)**. The hybridizing RNA is still present in the enlarging ovule (Ov). E is the enlarging egg/embryo sac.
(C) A transverse section through an ovule (young seed) of a 25-mm tomato fruit hybridized and treated as above. The integument has proliferated as it begins to form the seed coat (SC) that surrounds the expanding embryo sac (E). No hybridizing RNA is detected in any tissue of the young seed. All panels are at the same magnification, and the scale bar is 0.5 mm.

ovule, surrounding the egg sac, did not hybridize. When the plane of section passed through the micropyle, a gap in the hybridizing layer of cells was visible at the micropylar end of the ovule. Examination of numerous sections showed that the hybridizing cells form an oblong spheroid, approximately two cell layers thick, surrounding the inner layer of the integument and the egg sac. In each ovule there is a gap at one end of the hybridizing region through which the micropyle passes.

The sensitive in situ hybridization procedure allowed us to follow the developmental regulation of this gene without the necessity of isolating large amounts of material for RNA purification. Figure 3 shows sections of a series of stages in the development of the ovule as the ovary enlarges to form a young fruit. The sections were hybridized with ^{35}S-labeled anti-sense RNA from pMON9608. In the mature ovary (Figure 3A), the characteristic C-shaped pattern of hybridization due to the gap at the micropyle was visible in the ovules. After fertilization when the fruit was approximately 10 mm in diameter (Figure 3B), the embryo had begun to develop and the cells of the integuments had started proliferating. RNA hybridizing to the pMON9608 probe was still present at this time about 3 days postfertilization. Approximately 3 days later, the fruit had grown to about 25 mm and the fertilized ovules had increased in size 15-fold. The cells that contained the pMON9608 RNA had become enlarged and vacuolated and no longer hybridized to the probe (Figure 3C). We had previously shown that no RNA from the pMON9608 gene was present in the pistils of 8- to 9-mm buds (Figure 1C). Buds of this size are only 3 to 4 days younger than the mature pistils that show high-level expression of the pMON9608 gene. The RNA gel blot and in situ hybridization experiments show that this gene is very tightly regulated, expressing in only two to three cell layers for a period of 5 to 8 days.

DISCUSSION

We have demonstrated that differential screening is an efficient method for the isolation of sequences that show enhanced expression in a particular organ. Although we

have characterized only those clones which showed very strong differential expression between pistils and seedlings, we were able to detect clones with differences in abundance between the two populations of RNA as small as fivefold (data not shown). On the basis of the frequency of cDNA clones detected in rescreening experiments on the pistil libraries, some of the clones we have isolated correspond to RNA species representing less than 0.05% of the poly(A^+) RNA. Thus, this method is useful for the isolation of clones for RNAs of intermediate abundance. We found that only 0.5% to 1.0% of the clones in our pistil cDNA libraries showed significantly (>10-fold) enhanced expression in pistils relative to seedlings. Thus far only a single clone that is expressed exclusively in the pistils has been identified (Table 2, Figure 1C).

Kamalay and Goldberg (1980) have shown that more than one-third of the complexity of the mRNA in ovaries is unique to that organ system. The majority of these mRNA species, however, are present at very low levels (0.001% of total mRNA) but should represent approximately 25% of the clones in our libraries (Goldberg et al., 1978). cDNAs for RNAs of this rare abundance class would be at such low concentration in our probe mixtures that they are unlikely to produce detectable signals in our screening procedure. Indeed, a significant fraction of the recombinant plaques in our libraries gave no signal above background with either of the probes used in screening. This may explain the relative paucity of identifiable pistil-specific clones in our libraries.

Although the clones that we have isolated correspond to genes with strong preferential expression in pistils, the majority appear to express at some level in other plant organs (Figure 1, Table 2). Four of the nine clones that were examined were expressed at some level in anthers. Such clones, showing expression in both reproductive organs, may have a general role in some aspect of gametogenesis or gametophyte development. The relatively low-level expression detected in the vegetative organs for several of the clones may be due to an important function of the gene products in these organs or to leakiness in the regulation of the genes. Alternatively, the signal detected in the vegetative organs may be the result of weak hybridization to RNA from diverged, related genes not detected in the stringently washed genomic DNA gel blots. Although stringent hybridization conditions were used for RNA gel blot analysis, the level of RNA from related genes could be high enough to produce a signal. We plan to perform a series of hybridizations at lower stringency to determine whether related genes are present in the tomato genome.

One of the primary goals of our studies is to determine the function of the pistil genes we have identified. The cellular specificity of the expression of the pMON9617 gene provides some indication of possible functions for this gene (Figure 2, A and B). The confinement of expression of this gene in the pistil to cells of the transmitting tissue and its appearance just prior to fertilization are consistent with a role for this gene in the differentiation of this tissue or in the process of pollen tube growth. This pattern of expression is superficially similar to the pattern observed for putative self-incompatibility genes (S-genes) of *Nicotiana alata* (Anderson et al., 1986; Cornish et al., 1987). However, the S-genes are expressed in the stigma and the entire transmitting tissue, whereas the pMON9617 gene is expressed only in tissues subtending the stigma and in the outer layers of the transmitting tissue. S-gene expression also is detected in the outer layers of the placenta (Cornish et al., 1987), whereas there is no detectable expression of the pMON9617 gene in these cells. The sequences of several self-incompatibility genes have been published (Nasrallah et al., 1985; Anderson et al., 1986; Cornish et al., 1987; Takayama et al., 1987). In sequence comparisons we find no detectable homology between pMON9617 and the S-genes (unpublished data). It is unlikely therefore that the pMON9617 gene is a remnant of a self-incompatibility system that may have existed in ancestors of the self-compatible cultivated tomato. A speculative hypothesis is that the product of this gene is involved in preventing the pollen tubes from leaving the transmitting tissue during elongation. The tubular arrangement of the expressing cells is consistent with this hypothesis.

Based on the frequency of hybridizing clones in our mature pistil cDNA library (prior to amplification), the transcript corresponding to pMON9608 represents 0.05% of the poly(A^+) RNA in a mature pistil. The pMON9608 gene is expressed exclusively in two to three cell layers of the integument of the ovules (Figure 2, C and D; Figure 3A). Morphometric calculations based on analysis of serial sections indicate that the cells expressing this gene represent approximately 0.5% of the volume of the pistil. If one assumes that distribution of RNA in the pistil is uniform, then this transcript would make up approximately 10% of the poly(A^+) RNA in the expressing cells. The dense cytoplasm and the lack of vacuolation in the cells of the integument make it likely that the total concentration of RNA in these cells is much higher than in the pistil in general, so that this number is an overestimate. However, it is clear that the level of RNA from the pMON9608 gene is very high in these cells. Since the nucellus does not persist in tomato (Cooper, 1931), one of the roles of the inner layers of the integument is thought to be the provision of nutritive material for the megagametophyte and development of the early embryo (Cooper, 1931). The product of this gene, therefore, may play a role in this process. In this role, the inner layers of the integument would be analogous to the tapetum in the anthers. It is interesting to note that cDNA clones for genes that express at a high level for only a short period of time, and then exclusively in the tapetum, have been identified (McCormick et al., 1987; Goldberg, 1988; Gasser et al., 1988b; A.G. Smith, unpublished data).

The genes that we have identified with enhanced

expression in pistils will be useful tools in future studies on gene expression and development in flowers. Currently, we are constructing plant transformation vectors for expression of anti-sense RNA to our cDNA clones. It has been shown that such vectors can lead to a significant reduction in the expression of the corresponding genes in transgenic plants (Rothstein et al., 1987; van der Krol et al., 1988). Reduction of expression of our pistil genes may produce a detectable phenotype that would aid us in assigning biological functions to the genes. In addition, we have isolated genomic clones for the pistil-specific genes. The promoter regions of the genomic clones have been identified and are being characterized in plant transformation experiments. The promoters will be used also in experiments to examine the effect of specific production of phytohormones in floral organs by fusion with hormone biosynthesis genes as described by Klee et al. (1987).

METHODS

Plant Material

Tomato plants (*Lycopersicon esculentum,* cv VF36) were grown under standard greenhouse conditions.

RNA isolation and cDNA cloning

RNA was isolated from plant tissues as previously described (Rochester et al., 1986). In a typical purification from isolated floral tissues, 500 mg of tissue was homogenized in 5 ml of lysis solution to yield 400 μg of total RNA. Poly(A^+) RNA was isolated from total RNA by chromatography over oligo-d(pT)-cellulose (Collaborative Research).

cDNA libraries were constructed by a modification of the procedures of Huynh et al. (1985) and Gubler and Hoffman (1983). The first cDNA strand was synthesized for 1 to 2 hr at 42°C in a 100-μl reaction containing: 40 μg/ml actinomycin D (Sigma; 50 mM Tris-HCl, pH 8.3; 30 mM KCl; 10 mM $MgCl_2$; 0.4 mM dithiothreitol; 10 μg/ml oligo-d(pT); 500 μM each dATP, dCTP, dGTP, TTP; 60 units of RNAsin (Promega); 0.5 to 1.5 μg of poly(A^+) RNA; 60 units of AMV reverse transcriptase (Life Sciences). A side reaction containing ^{32}P-dCTP (10 μCi) was carried out to estimate the size and quantity of cDNA synthesized. Typical reaction resulted in the synthesis of 500 ng of single-stranded cDNA. The first-strand reaction was extracted twice with an equal volume of phenol. The cDNA was desalted by chromatography over a 1.0-ml Bio-Gel P-60 (100 to 200 mesh, Bio-Rad) column in 1 mM Tris, pH 7.6/0.01 mM EDTA, and reduced to about 10 μl volume by lyophilization in a Savant Speed-Vac.

Second-strand synthesis was carried out in a 100-μl reaction containing 200 to 800 ng of single-stranded cDNA; 20 mM Tris-HCl, pH 7.4; 5 mM $MgCl_2$; 100 mM KCl; 10 mM NH_4SO_4; 150 μM β-NAD; 40 μM each dATP, dCTP, dGTP, TTP; 20 units of *Escherichia coli* DNA polymerase I (New England Biolabs); 2 units of *E. coli* DNA ligase (New England Biolabs); 50 μg/ml bovine serum albumin (Bethesda Research Laboratories); 30 μCi of ^{32}P-dCTP. The reaction was incubated at 14°C for 60 min and then at room temperature for 60 min. Of a mixture of 5 mM each dNTP and 1 μl of T4 DNA polymerase (New England Biolabs), 0.5 μl was added, and the reaction was incubated for an additional 30 min at room temperature to make the ends of the double-stranded cDNA flush. Approximately 50% of the single-stranded cDNA was converted to double-stranded cDNA in this reaction. To protect EcoRI sites within the cDNA molecules from digestion in subsequent steps, the following reagents were added: 1.2 μl of 1 mM *S*-adenosyl-L-methionine (Sigma), 1.0 μl of EcoRI methylase (New England Biolabs), 2.4 μl of 0.5 M EDTA, and the reaction was incubated for 45 min at 37°C. The reaction was heated to 68°C for 10 min to inactivate the enzymes, extracted twice with phenol, passed over a Bio-Gel P-60 column as above, and lyophilized to dryness.

The cDNA was dissolved in 3 μl of 1 mM Tris-HCl, pH 7.5/0.01 mM EDTA. EcoRI linkers were attached to the cDNA in a 10-μl reaction containing 500 ng of cDNA; 250 ng of EcoRI linkers (New England Biolabs); 1.0 mM ATP; 30 mM Tris-HCl, pH 7.6; 10 mM $MgCl_2$; 5 mM dithiothreitol; 400 units of T4 DNA ligase (New England Biolabs). The reaction was incubated at 14°C for 12 hr. To remove the linkers and produce cohesive ends, 2 μl of a solution consisting of 100 mM Tris-HCl, pH 7.6/100 mM $MgCl_2$/1.0 M NaCl, 6 μl of water, and 2 μl (40 units) of EcoRI was added, and the reaction was incubated at 37°C for 2.5 hr. Following digestion, the reaction was incubated at 68°C to inactivate the EcoRI.

The cDNA was size-selected, and the unattached linkers were removed from the cDNA by electrophoresis on a Sea Plaque agarose gel (FMC Corp.). The region of the gel containing cDNA greater than 500 bp in length (approximately 400 μl of agarose) was excised, diluted with 140 μl of 20 mM Tris-HCl, pH7.5/200 mM NaCl/1.0 mM EDTA, and 20 μl of 5 M NaCl, heated to 68°C for 10 min, and extracted twice with 500 μl of phenol. The DNA was purified from contaminants by chromatography on an EluTip D column (Schleicher & Schuell) according to the manufacturer's instructions. (Note: In more recent cDNA syntheses, we have had more consistent results eluting the cDNA from the gel onto DEAE membrane.) One μg of λgt10 arms (Stratagene) was added for each 35 ng of cDNA, and the nucleic acids were precipitated with ethanol. The cDNA/λgt10 mixture was dissolved in 7.2 μl of water, and combined with 1.0 μl of 300 mM Tris-HCl/100 mM $MgCl_2$/50 mM dithiothreitol, 1.0 μl of 10 mM ATP, and 0.8 μl of T4 DNA ligase (New England Biolabs). The reaction was incubated for 12 hr at 14°C. The DNA was packaged into phage using GigaPack packaging extracts (Stratagene) according to the manufacturer's instructions. All platings were done on *E. coli* BNN 102 (Young and Davis, 1983).

Differential Screens

For screening about 10,000 pfu were plated onto 22 × 22 cm plates. Duplicate nitrocellulose replicas were lifted from each of the plates and were treated according to standard methods (Davis et al., 1980).

Hybridization probes were synthesized from poly(A^+) RNA in reactions similar to the first-strand cDNA synthesis reactions described above. The reaction differed from standard first-strand conditions in that the unlabeled dCTP was replaced by 100 μCi of ^{32}P-dCTP for the 1st hr of the reaction. Unlabeled dCTP was

then added to a final concentration of 0.5 mM, and the reaction was continued for 30 min. In a typical reaction, 0.5 μg to 1.0 μg of RNA was used in a 25-μl reaction to synthesize 5×10^7 to 1×10^8 cpm labeled single-stranded cDNA. In some reactions, random hexanucleotides (Pharmacia LKB Biotechnology Inc.) at 100 μg/ml were substituted for the oligo-d(pT) primer. This substitution generally led to the synthesis of a larger amount of labeled cDNA. The labeled cDNA/RNA hybrids were purified by chromatography over Bio-Gel P-60 or Sephadex G-50 columns, extracted twice with phenol, precipitated with ethanol, and dissolved in 200 μl of water. In preparation for hybridization, the probes were denatured and the RNA was hydrolyzed by the addition of 60 μl of 1.0 N NaOH, followed by a 10 min incubation at room temperature. The base was neutralized by the addition of 60 μl of 1.0 N HCl and 60 μl of 20 × SSPE (1 × SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, pH 7.5, 1 mM EDTA).

Filters were prehybridized overnight in 2 × SSPE, 4 × Denhardt's solution (1 × Denhardt's solution is 0.02% each Ficoll, bovine serum albumin, and polyvinylpyrrolidone), 0.1% SDS, 10 μg/ml poly(A), 100 μg/ml denatured salmon DNA at 68°C. The solution was replaced with a fresh mix containing 10^5 to 10^6 cpm/ml labeled cDNA and incubated at 68°C for 36 to 48 hr. Filters were washed twice for 20 min at room temperature in 0.3 × SSPE, 0.1% SDS, and once for 20 min in the same solution at 68°C. Exposure times ranged from 12 to 72 hr.

Inserts of selected cDNA clones were isolated and subcloned into plasmid vectors by standard methods (Crouse et al., 1983).

DNA and RNA Gel Blots

DNA gel blots (Southern, 1975) to ZetaBind membrane (AMF-Cuno) were performed as previously described (Gasser et al., 1988a). The membranes were hybridized with single-stranded cDNA as described above. Samples of poly(A^+) RNA were quantitated by measuring acid-insoluble incorporation of ^{32}P-dCTP into first-strand cDNA [primed with oligo-d(pT)]. Tests of this method with poly(A^+) RNA available in sufficient quantity to measure by optical density demonstrated that, in our cDNA reactions, 40% to 60% of the RNA was converted to cDNA. Our quantitation of poly(A^+) RNA is therefore ±20%. RNA gel blots were performed as previously described (Gasser et al., 1988a). Cloned DNAs were labeled by the random oligonucleotide priming method of Feinberg and Vogelstein (1983) and were hybridized to the filters as described (Gasser et al., 1988a). Alternatively, labeled RNA probes were made by first recloning the cDNA inserts into Bluescript plasmids (Stratagene) and then transcribing with T3 or T7 RNA polymerase according to methods included with the plasmid vectors.

In Situ Hybridizations

For production of labeled RNA probes, cDNA inserts were isolated and cloned into Bluescript KS^- (Stratagene Cloning Systems). This plasmid includes promoters for the T7 and T3 RNA polymerases, allowing production of RNA probes from either strand of an insert. In preliminary experiments individual probes were made for each strand and were hybridized to blots of pistil RNA. In each case, only one of the two probes hybridized to the RNA gel blot, identifying that probe as being the anti-sense strand. The anti-sense probes were used in all of the illustrated in situ hybridization experiments.

Isolated pistils were quick-frozen in O.C.T. compound (Tissue Tek), cryogenically sectioned, treated, hybridized with ^{35}S-labeled RNA probes, and autoradiographed as previously described (Smith et al., 1987).

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