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Gametophytic and Sporophytic Expression of Anther-Specific Genes in Developing Tomato Anthers

Virginia M. Ursin, Judy Yamaguchi, and Sheila McCormick[1]

United States Department of Agriculture/Agricultural Research Service, Plant Gene Expression Center, 800 Buchanan Street, Albany, California 94710

The tissue localization of transcripts corresponding to five anther-specific cDNA clones isolated from tomato was determined. Transcripts specified by three of the cDNA clones were first detectable in anthers containing mitotic-stage gametophytes and were localized to the gametophyte. Transcripts specified by the two other cDNA clones were not detectable until anthers had reached a later developmental stage; these transcripts were also localized to the (now bicellular) gametophytes. Transcript levels for all of the cDNAs increased during gametogenesis and reached maximal levels in mature pollen grains. These mRNAs persisted in in vitro-grown pollen tubes, concentrating toward the tips of the growing tubes. At flower maturity, transcripts specified by each of the cDNAs were also detected in the epidermal and endothecial cell layers of the anther wall. The spatial distribution of transcripts in the anther wall was confined to that region of the anther that surrounds the locule. Transcripts were not detected in the sterile tip of the anther or in the filament. mRNA levels for these cDNA clones were markedly reduced in the anthers of several independent male-sterile mutants of tomato. Our results provide evidence that these anther-specific cDNAs represent genes expressed in both the gametophytic and sporophytic phases of the plant life cycle. The patterns of mRNA accumulation observed support the hypothesis that the proteins encoded by these genes function during pollen development and pollen tube growth.

INTRODUCTION

In the anthers of higher plants, neither the developing pollen nor the surrounding tissues show autonomous growth and development. The developing anther is a system in which the gametophytic and sporophytic generations of the plant communicate, coordinate, and apparently co-participate in development. Our goal is to understand how development in these two generations is coordinated at the level of gene expression.

There is compelling genetic and biochemical evidence that pollen development is determined by genes expressed in both the sporophyte and the gametophyte. If tapetal degeneration occurs precociously, for example, pollen development is adversely affected (Vasil, 1967). Such sporophytic effects are not limited to nuclear genes. Cytoplasmic male sterility is a condition determined by an interaction between mitochondrial and nuclear genes in the sporophyte; the morphological outcome is the degeneration of developing pollen by the late microspore stage (Kaul, 1988).

Both the sporophytic tissues of developing anthers and the developing gametophyte are known to express tissue-specific sets of genes in a highly regulated manner. For example, cDNA clones corresponding to mRNAs expressed in all sporophytic cell layers of the anther, except the tapetum, have been isolated from tobacco (Goldberg, 1988), and tapetal-specific cDNA clones have been characterized in tomato (Smith, Hinchee, and Horsch, 1987) and tobacco (Goldberg, 1988). Additionally, pollen-specific cDNA clones have been isolated from corn and *Tradescantia* (Mascarenhas, 1988; Hanson et al., 1989).

Previously, we have described the cloning and preliminary characterization of anther-specific cDNA clones from tomato (McCormick et al., 1987). Five of these cDNA clones, pLAT51, pLAT52, pLAT56, pLAT58, and pLAT59, showed a progressive increase in steady-state levels of their corresponding mRNAs during anther and pollen maturation, and in isolated pollen these mRNAs were present at very high levels. This suggested that the RNA isolated from maturing anthers that hybridized to these cDNAs was from the developing pollen contained within the anthers (Twell et al., 1989; R.A. Wing, J. Yamaguchi, S. Larabell, V.M. Ursin, and S. McCormick, unpublished results).

Although no functions have been identified conclusively for any of the pLAT genes, two of the cDNAs, pLAT56 and pLAT59, share significant sequence homology to two bacterial pectate lyase genes (R.A. Wing, J. Yamaguchi, S. Larabell, V.M. Ursin, and S. McCormick, unpublished results). This suggests that pLAT56 and pLAT59 may

[1] To whom correspondence should be addressed.

function in pectin degradation. In addition, pLAT52 (Twell et al., 1989) shares significant amino acid identity (32%) with a recently described pollen-specific gene from corn (Hanson et al., 1989).

This paper reports on the results of in situ hybridizations using the five anther-specific cDNA clones, pLAT51, pLAT52, pLAT56, pLAT58, and pLAT59. We describe the localization of mRNAs complementary to these cDNA clones to specific cells of the sporophyte and developing gametophyte, including post-germinative pollen tubes. Finally, we report on the expression of these genes in mature anthers of a number of male-sterile mutants of tomato blocked at various stages of gametogenesis.

RESULTS

Developmental Expression of pLAT51 and pLAT58

The expression during anther development of the two uncharacterized anther-specific cDNAs, pLAT51 and pLAT58, was analyzed. RNA gel blots of polyadenylated RNA from developing anthers and pollen were hybridized with ^{32}P-pLAT51 and ^{32}P-pLAT58 cDNAs; the results are shown in Figure 1. The pLAT51 probe hybridized to an mRNA approximately 2.1 kb in size. mRNAs homologous to pLAT51 were present at low levels in immature anthers, increased progressively during anther development, and were most abundant in pollen (Figure 1A). When similar blots were probed with pLAT58 cDNA, hybridization to a 2.0-kb mRNA was observed (Figure 1B). The overall pattern of transcript accumulation observed for pLAT58 was similar to that observed for pLAT51. However, the ratio of mRNA levels in green petal (GP) anthers to that in mature anthers was less for pLAT58 than for pLAT51 (Figure 1B). These results indicate that the developmental expression of pLAT51 and pLAT58 is similar to that observed for the other anther-specific cDNAs, pLAT52, pLAT56, and pLAT59 (Twell et al., 1989; R.A. Wing, J. Yamaguchi, S. Larabell, V.M. Ursin, and S. McCormick, unpublished results).

To determine the copy number of pLAT51 and pLAT58 in tomato, genomic DNA was isolated from *Lycopersicon esculentum* and *L. pennellii* (parental species) and from several F_2 progeny and digested to completion with either HindIII (pLAT51) or BstNI (pLAT58). Following DNA gel blotting, the DNA was probed with either pLAT51 or pLAT58 cDNA. The results are shown in Figures 1C and 1D. pLAT51 cDNA hybridized to two differently sized pairs of bands in the *L. esculentum* and *L. pennelli* lanes (Figure 1C). These doublets segregated as single loci in the F_2 population. Single, differently sized bands in the *L. esculentum* and *L. pennelli* lanes hybridized to the pLAT58 cDNA (Figure 1D). These bands also segregated as single loci in the F_2 individuals. Similar results were obtained following digestion of the genomic DNA with several other restriction enzymes (data not shown). pLAT51 and pLAT58 map to single, unlinked loci on chromosome 1 of tomato (data not shown). pLAT52, pLAT56, and pLAT59 also segregate as single genes and have been mapped to specific loci in the tomato genome (Twell et al., 1989; R.A. Wing, J. Yamaguchi, S. Larabell, V.M. Ursin, and S. McCormick, unpublished results).

Localization of Expression of Anther-Specific cDNAs to Specific Cells in Anthers

To analyze expression of the pLAT cDNAs at the cellular level, ^{35}S-antisense transcripts of the pLAT cDNAs were hybridized in situ to sections of anthers taken at various stages of development. Because the distribution of silver grains on anther cross-sections was virtually identical for all of the pLAT clones, we present representative data. The results of the in situ hybridizations with all of the clones are summarized in Table 1.

When each of the five cDNA clones was hybridized to pistil, sepal, or petal sections, no hybridization was observed to sections from any flower stage. These results were consistent with results from RNA blot analyses (Twell et al., 1989; R.A. Wing, J. Yamaguchi, S. Larabell, V.M. Ursin, and S. McCormick, unpublished results). Similarly, no hybridization of the SP6 control transcript to any of these tissues was observed (not shown). Both of these observations confirm the specificity of the hybridization results. (See Methods for a description of the controls used.)

In cross-sections of the least mature flower stage examined (stage 1), no hybridization of the RNA probe was observed (not shown). In cross-sections of stage 2 flowers, the probe hybridized exclusively to the developing gametophytes in the anther locules (Figures 2A and 2B; Table 1). In cross-sections of stage 3 flowers, an increased level of hybridization to developing gametophytes was observed. At this flower stage, a low level of hybridization to the epidermal cells of the anther wall was also seen (Figures 2C and 2D). In sections of anthers from mature (stage 4) flowers, the probe hybridized to mature pollen grains (Figures 2E and 2F). The apparent tendency of mature pollen to burst during cryostat sectioning resulted in leaking of cytoplasm and a spreading out of the signal around the pollen grain (see Figure 4A). In stage 4 anthers, we also observed a strong hybridization signal in the epidermis and endothecium of mature anther walls (Figure 2D; Table 1).

The distribution of hybridization signal to transcripts in longitudinal sections of anthers from stage 4 flowers was also determined. A schematic representation of a longitu-

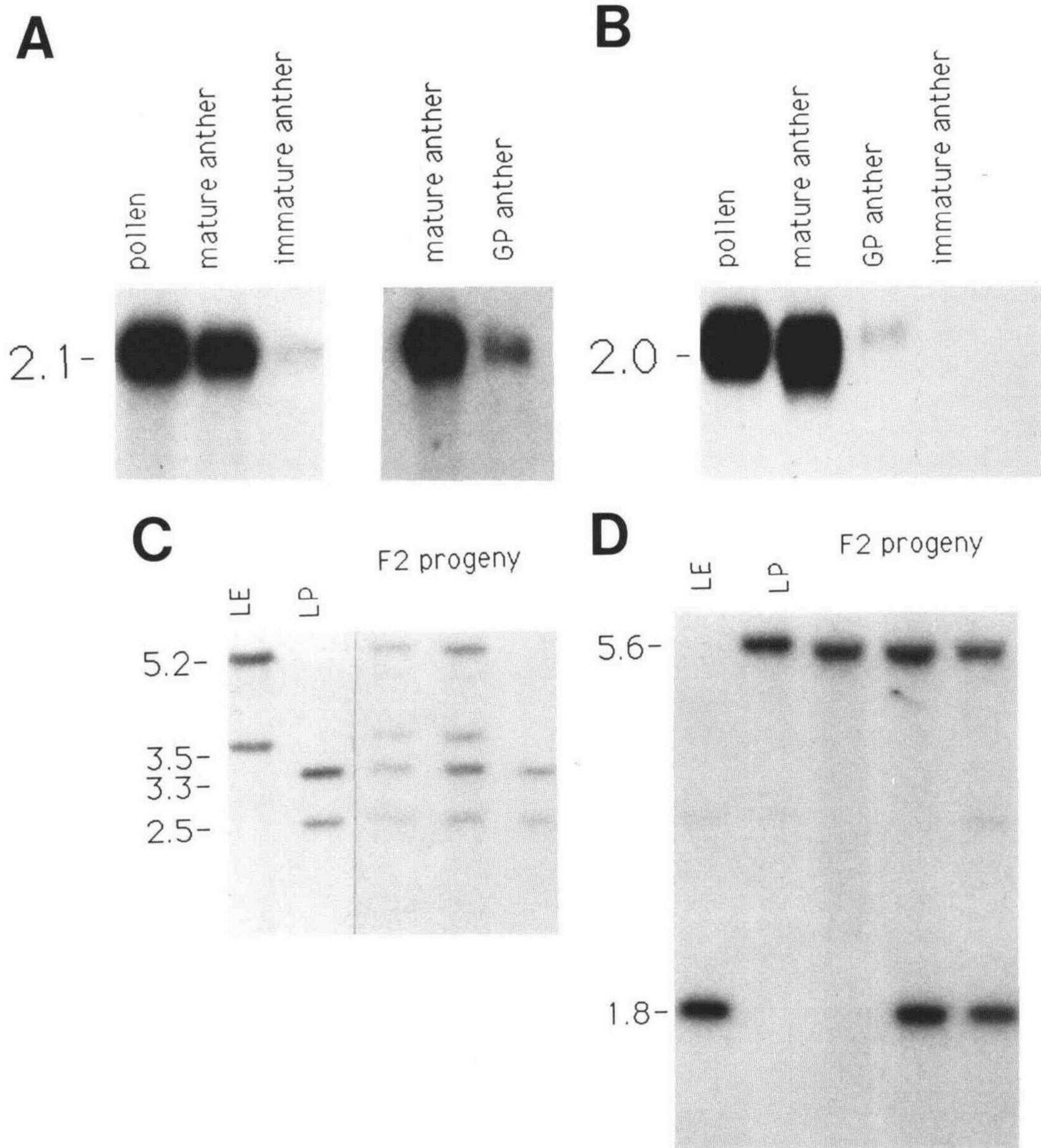

Figure 1. RNA Blots of mRNA from Developing Tomato Anthers and DNA Blots of Genomic DNA from F_2 Progeny of *L. esculentum* and *L. pennellii*.

(A) RNA blots of poly(A^+) RNA from pollen, mature anthers, and immature anthers (left) and mature anthers and GP anthers (right) hybridized with pLAT51. 2 μg of poly(A^+) RNA was loaded per lane. Numbers at left are size markers.

(B) RNA blots of poly(A^+) RNA from pollen, mature anthers, GP anthers, and immature anthers hybridized with pLAT58. 2 μg of poly(A^+) RNA was loaded per lane. Numbers at left are size markers.

(C) DNA blots of genomic DNA from *L. esculentum, L. pennellii* (parents), and F_2 progeny digested with HindIII and probed with pLAT51. Numbers at left are size markers.

(D) DNA blots of genomic DNA from *L. esculentum, L. pennellii* (parents), and F_2 progeny digested with BstNI and probed with pLAT58. Numbers at left are size markers.

Table 1. Contrast between the Temporal Regulation of Five Floral-Specific cDNAs In Gametophytic and Sporophytic Tissues of Developing Tomato Anthers.[a,b]

cDNA Clone		Developmental Stage of Flower			
		Stage 4	Stage 3	Stage 2	Stage 1
pLAT51	endothecium[c]	++++	+	–	–
	microspore	++++	+++	+	–
pLAT52	endothecium	++++	+	–	–
	microspore	++++	+++	++	–
pLAT56	endothecium	+++	–	–	–
	microspore	+++	+	–	–
pLAT58	endothecium	++++	+	–	–
	microspore	++++	+++	+	–
pLAT59	endothecium	+++	–	–	–
	microspore	+++	–	–	–

[a] Summary of in situ hybridization results assessing the abundance of transcript corresponding to the cDNA clones pLAT51, pLAT52, pLAT56, pLAT58, and pLAT59 in microspores and endothecium of developing anthers.
[b] ++++ indicates strongest hybridization signal observed; – indicates no detectable hybridization signal.
[c] Includes anther epidermal cell layer.

dinal section through a mature anther is shown in Figure 3A for reference. Hybridization was detected in the abaxial portion of the anther endothecium, as predicted by the hybridizations to anther cross-sections (Figures 2C and 2F). Reduced signal is observed in the adaxial portion of the anther, as the plane of dissection was near the stomium, where reduced hybridization is observed in cross-sections (Figures 2C and 2F). In all longitudinal sections, hybridization to the anther endothecium was closely associated with the locule, or the "fertile" region of the anther (Figure 3B). This apparent regional specificity within longitudinal sections was corroborated by the absence of hybridization of the pLAT probes to anther cross-sections from regions that were either below or above the locular region of the anther (not shown).

Expression of Anther-Specific cDNAs in Germinating Pollen

A representative result from in situ hybridizations of ^{35}S-antisense pLAT transcripts to mature pollen and germinated pollen grains is shown in Figure 4. Mature ungerminated pollen grains gave a strong hybridization signal (Figure 4A, pLAT58). After 18 hr of germination and pollen tube growth, in situ hybridization reveals a signal localized toward the tip of the growing pollen tube (Figure 4B, pLAT56). This is the region of the pollen tube where the cytoplasm is concentrated.

Expression of Anther-Specific cDNAs in Male-Sterile Anthers

The two male-sterile (ms) mutants were chosen to span a range of severity of phenotype. The developmental block in *ms10* occurs at the premicrospore stage of pollen development; mature anthers have a collapsed locule and no microspore development. In contrast, gametophyte development proceeds to a late stage in *ms14*, and mature anthers have aborted pollen in otherwise well-formed locules (see Rick, 1948, and Rick and Butler, 1956, for morphological descriptions of male-sterile mutants). In Figure 5, RNA gel blot analysis of mRNA prepared from mature anthers of *ms10*, *ms14*, and VF36 shows that levels of mRNA corresponding to each of the five cDNAs was markedly decreased in the male-sterile mutants relative to VF36. After long exposures, signal was observed in the *ms10* lanes, and, to a lesser extent, in the *ms14* lanes on blots probed with each of the clones (Figure 5). Preliminary results with several other male-sterile mutants (*ms2*, *ms9*, *ms12*, *ms33*, and *ms47*) also show markedly reduced levels of mRNA corresponding to all five cDNAs (V.M. Ursin, J. Yamaguchi, and S. McCormick, unpublished results).

In an attempt to localize the source of the signal observed on the male-sterile anther RNA gel blots, in situ hybridizations were performed on mature anthers of *ms10* and *ms14*. No hybridization signal was observed in the *ms10* and *ms14* stage 4 anthers (not shown). These results indicate that cells of the male-sterile anthers do not express the pLAT transcripts at high levels.

DISCUSSION

We have previously described five cDNA clones that correspond to mRNAs expressed with a high degree of specificity in developing anthers of tomato (McCormick et al., 1987). In this paper we used in situ hybridization analysis to determine which tissues within the anther were contributing to the signal seen on RNA gel blots (Figure 1; Twell et al., 1989; R.A. Wing, J. Yamaguchi, S. Larabell, V.M. Ursin, and S. McCormick, unpublished results).

Transcripts Complementary to the pLAT cDNAs Are Expressed in the Developing Male Gametophyte

We first observed hybridization of the pLAT probes to the developing gametophyte in cross-sections from stage 2 flowers, providing evidence that the transcription of the pLAT genes occurs from the haploid genome. The pattern of transcript accumulation of the pLAT genes during gametogenesis is similar to that of a group of pollen-specific

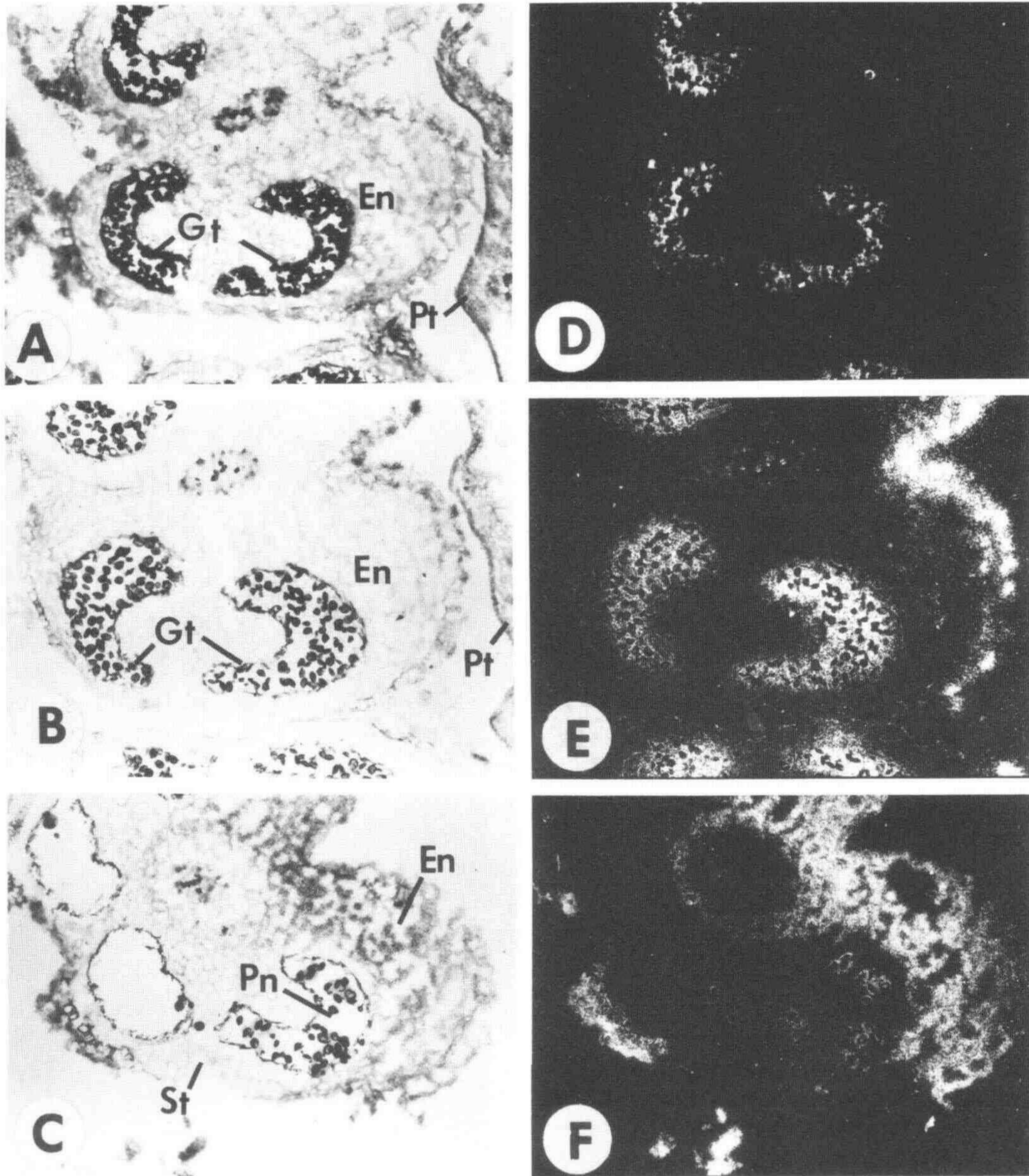

Figure 2. In Situ Localization of pLAT52 mRNA in Cryostat Sections of Developing Anthers.

(A) Bright-field micrograph of stage 2 (10 mm to 14 mm) anther cross-section hybridized with ^{35}S-single-stranded RNA probe pLAT52 after 8 days' exposure.

(B) Bright-field micrograph of stage 3 (GP) anther cross-section hybridized with ^{35}S-single-stranded RNA probe pLAT52 after 8 days' exposure.

(C) Bright-field micrograph of stage 4 (mature) anther cross-section hybridized with ^{35}S-single-stranded RNA probe pLAT52 after 8 days' exposure.

(D) Dark-field micrograph of stage 2 (10 mm to 14 mm) anther cross-section hybridized with ^{35}S-single-stranded RNA probe pLAT52 after 8 days' exposure.

(E) Dark-field micrograph of stage 3 (GP) anther cross-section hybridized with ^{35}S-single-stranded RNA probe pLAT52 after 8 days' exposure.

(F) Dark-field micrograph of stage 4 (mature) anther cross-section hybridized with ^{35}S-single-stranded RNA probe pLAT52 after 8 days' exposure.

En, endothecium; Gt, gametophyte; Pn, pollen; Pt, petal; St, stomium.

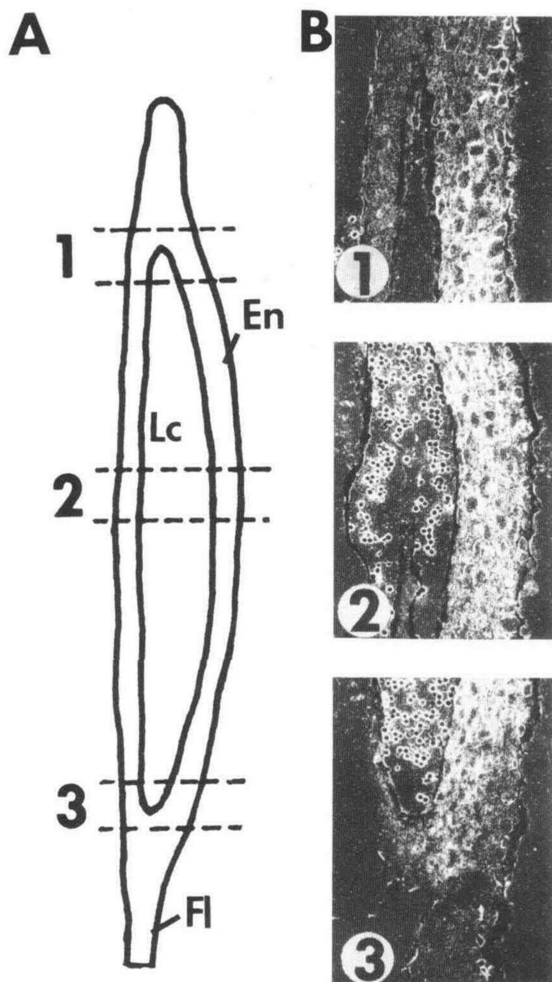

Figure 3. In Situ Localization of pLAT58 mRNA to Paraffin-Embedded Longitudinal Anther Sections.

(A) Line drawing of longitudinal section of an anther from a stage 4 flower. Regions identified are Lc, locule; En, endothecium; Fl, filament. Regions 1, 2, and 3 correspond to tip, middle, and base of anther.

(B) Dark-field micrograph of longitudinal anther sections from regions 1, 2, and 3 hybridized with ^{35}S-single-stranded RNA probe pLAT58 after 15 days' exposure.

genes in corn and *Tradescantia* (Stinson et al., 1987). Another group, typified by actin, is characterized by increasing transcript accumulation during microsporogenesis, followed by a decline at pollen maturity (Stinson et al., 1987).

It has been suggested, but not clearly demonstrated, that pollen grains utilize presynthesized mRNAs that can be rapidly translated upon pollen germination (Frankis and Mascarenhas, 1980), as has been observed in seeds of many flowering plants (see Payne, 1976, for review). The pLAT mRNAs are present in mature pollen grains and during pollen tube growth (Figure 4). Further experiments will be required to determine whether the pLAT clones correspond to stored messages in pollen and/or are synthesized de novo in pollen tubes.

Transcripts Complementary to the pLAT cDNAs Are Expressed in Sporophytic Cells of Mature Anthers

We observed expression of the pLAT transcripts in all cell layers of the mature anther wall (Figure 2). The sporophytic expression of pLAT52 late in anther development is apparently in contrast to the pollen-specific expression of the corn gene Zmc13 (Hanson et al., 1989), to which pLAT52 shares amino acid identity. It is interesting to note that the

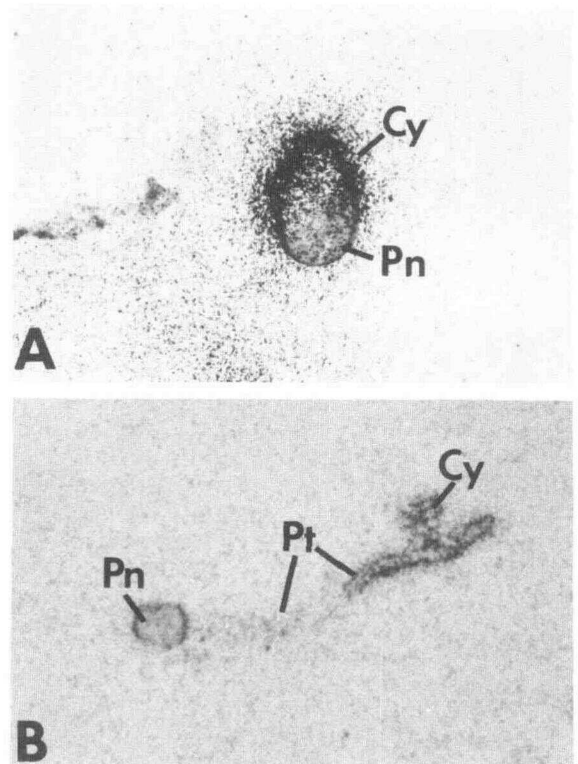

Figure 4. Localization of Anther-Specific Genes in Ungerminated and Germinated Pollen Grains.

(A) Bright-field micrograph of a cryostat sectioned pollen grain hybridized in situ to ^{35}S-single-stranded RNA probe pLAT58. Note that the pollen wall (Pn) is fractured and that hybridization has occurred primarily to the extruded cytoplasm (Cy).

(B) Bright-field micrograph of an 18-hr-old pollen tube squash hybridized in situ to ^{35}S-single-stranded RNA probe pLAT56. Note that most of the hybridization has occurred to the pollen tube (Pt) tip and the extruded cytoplasm (Cy), and not to the pollen grain (Pn).

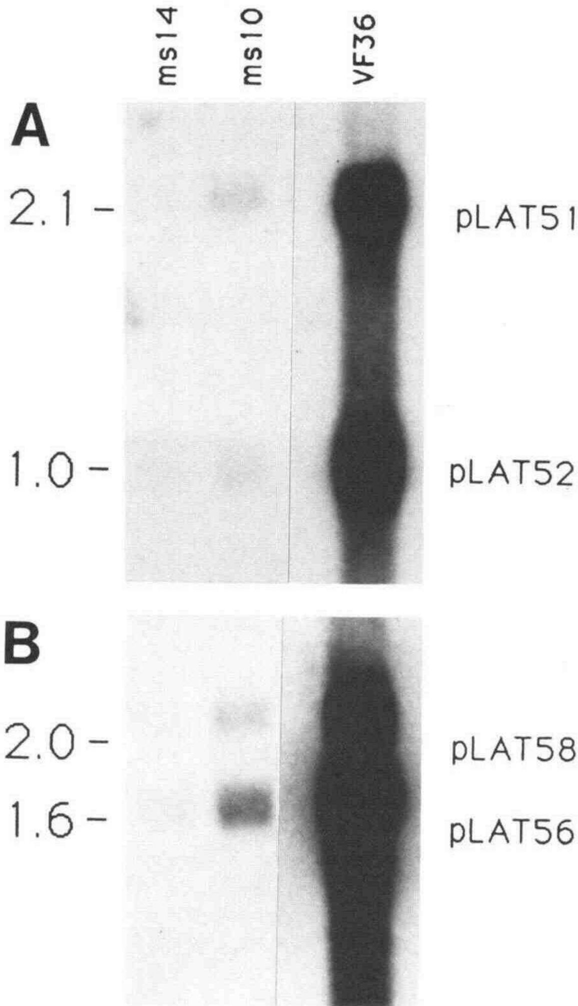

Figure 5. Expression of Anther-Specific Genes in Male-Sterile Anthers.

RNA blots of poly(A^+) RNA from wild-type and male-sterile anthers hybridized to floral-specific cDNAs. 1 μg of RNA was loaded per lane; lanes are labeled as to RNA source. Numbers at left are size markers; cDNA probes are identified at right. RNA gel blots were reprobed to confirm equivalent RNA loading.

sporophytic expression of the pLAT gene transcripts appears to cross cell lineages within the anther. The epidermal cell layer of the anther is derived from a different apical histogen (L1 of the apex) than the other cell layers of the anther (Esau, 1965). We also observed that transcript accumulation in the anther wall was confined to the region of the anther immediately adjacent to the locule, with no transcripts hybridizing in either the "sterile" tip of the anther or in the anther filament (Figure 3). These results suggest that the endothecial cells surrounding the locule, although anatomically similar, are biochemically distinct from those of the anther tip.

These LAT cDNAs correspond to single-copy genes (Figure 1; Twell et al., 1989; R.A. Wing, J. Yamaguchi, S. Larabell, V.M. Ursin, and S. McCormick, unpublished results), so the presence of message in both the anther wall and the gametophyte is direct evidence for sporophytic/gametophytic overlap in gene expression.

Genetic Male Sterility Alters the Abundance of pLAT Transcripts in Sporophytic Cells of Mature Anthers

Expression of the pLAT genes was analyzed in several recessive male-sterile tomato mutants. On RNA gel blots, a marked reduction, but not a complete absence, of transcripts in the anther wall was observed. The reduction in hybridization signal on the male-sterile RNA gel blots might be due solely to the lack of transcripts normally contributed by pollen. Alternatively, the reduction in signal observed might be due to reduced sporophytic expression of the pLAT transcripts in the male-sterile anthers. With in situ hybridization analysis, we confirmed that no cells in the anther walls of the male-sterile mutants expressed the pLAT messages at wild-type levels. The low levels of hybridization seen on the male-sterile RNA gel blots may be due to very low levels of expression of these genes in the male-sterile anthers.

Our results clearly indicate that the male-sterile mutations have altered normal gene expression in sporophytic tissues of the anther. This may be due to male-sterile gene action affecting development of both the sporophytic and gametophytic cell lineages in the anther. There are physiological lines of evidence for single-gene mutations affecting development of both the sporophyte and gametophyte; although the most obvious manifestation of *ms* gene action is pollen or microspore abortion, abnormal development of the sporophytically derived tapetum has been observed in several male-sterile mutants of tomato (Rick, 1948). Furthermore, a temperature-sensitive male-sterile mutation (*vms*) has pronounced effects on both tapetal and anther wall development (Rick and Boynton, 1967). Alternatively, the reduction in expression observed in the sterile anthers (Figure 5), combined with the apparent localization of the expression of the pLAT mRNAs within the "fertile" region of the anther (Figure 3), may be evidence for communication between the developing sporophyte and gametophyte. The relationship between development of the sporophyte and gametophyte will be tested by assaying the expression of these genes in anthers in which pollen has been physically or chemically ablated.

Each of the genes presented in this paper was selected only on the bases of abundance in mature anthers and tissue specificity (McCormick et al., 1987). The striking similarity in their developmental regulation suggests that sets of genes may be under coordinate control within the

developing floral organs of tomato. In addition, the similarity among corn, *Tradescantia*, and tomato in their patterns of transcript accumulation in developing pollen suggests that aspects of gene regulation in the developing male gametophyte may be conserved across plant species. The concept of coordinated control of gene expression in developing gametophytes is supported by the results of pulse-labeled RNA metabolism studies. A general purging of RNAs, including mRNAs, of *Lilium* meiocytes was observed during meiosis (Porter, Bird, and Dickinson, 1982; Porter, Parry, and Dickinson, 1983), followed by discrete periods of active RNA synthesis in the developing gametophyte (Stinson et al., 1987).

Our results on the abundance and tissue localization of anther-specific mRNAs represent a preliminary step toward analyzing the molecular basis of anther development. Immunolocalization of the proteins encoded by these genes and the elucidation of protein function during anther development will provide further insight into this developmental process.

METHODS

Plant Material

Wild-type tomato plants (*Lycopersicon esculentum* Mill., cv VF36 and *L. pennellii*, LA716) and male-sterile lines *ms10* (cv San Marzano) and *ms14* (cv Earlianna) were grown under normal greenhouse or field conditions. Seed was generously supplied by Dr. C.M. Rick (University of California, Davis). Male-sterile lines, maintained as heterozygotes, were scored for male sterility at anthesis. Flowers were staged according to developmental stage of the anthers and microspores as follows: Stage 1 flowers were unopened buds 6 mm to 10 mm (measured from tip to pedicle) in length containing immature anthers with uninucleate microspores; stage 2 flowers were unopened 10 mm to 14 mm buds with anthers containing microspores and binucleate gametophytes; in stage 3 flowers, termed green petal (GP), petals were emerging from the sepals but were still green, anthers were nearly full length with binucleate gametophytes; stage 4 flowers had mature anthers and dehiscent pollen. The filament of tomato stamens is greatly reduced, and the tip of the anther is sterile (no locule or pollen formed). For RNA gel blot analyses, the entire stamen, referred to as the anther, was used.

RNA Gel Blot and DNA Gel Blot Analyses

Anthers used for RNA gel blot analysis were dissected from flowers collected from either the field or greenhouse, immediately frozen on dry ice, and later stored at −80°C. Pollen was isolated from mature flowers by vibrating anthers. Total RNA was purified from anthers by phenol/chloroform extraction and LiCl precipitation, and poly(A+) RNA was selected by oligo(dT)-cellulose chromatography as described by Maniatis, Fritsch, and Sambrook (1982). RNA was quantitated spectrophotometrically and electrophoresed on 2% agarose gels containing formaldehyde. RNA was isolated from two separate collections of *ms10* and *ms14* anthers, and replicate RNA gel blots were prepared. DNA was isolated from tomato leaves as in Bernatzky and Tanksley (1986), digested with restriction endonucleases, and electrophoresed through 0.8% agarose gels. RNA and DNA gels were blotted to Nytran filters using standard procedures and probed with random-primer labeled cDNA inserts (Feinberg and Vogelstein, 1984). Hybridized blots were exposed to Kodak X-AR film at −80°C for autoradiography.

In Situ Hybridization

Probe Preparation

Insert DNAs from cDNA clones pLAT51, pLAT52, pLAT56, pLAT58, and pLAT59 were subcloned into Promega transcription vector pGEM7Zf(+). Antisense transcripts, determined by hybridizing both SP6 and T7 transcripts to mature anther RNA gel blots, were synthesized *in vitro* at a high specific activity using a modification of the protocol described in Melton et al. (1984). Each 10-μL transcription reaction contained 40 mM Tris (pH 8.0), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 7 mM DTT, 500 μM ATP, CTP, GTP, 2000 units/milliliter RNasin, 0.1 mg/mL linearized plasmid DNA, 31 μM ^{35}S-UTP (800 Ci/mmol), and 1000 units/milliliter RNA polymerase. DNA template was removed by DNase treatment (1 unit/microgram of DNA for 15 min at 37°C). Probes were then base hydrolyzed to approximately 150 bases by the method of Cox et al. (1984). Following base hydrolysis, probes were put through Sephadex G-50 spun columns as described by Maniatis, Fritsch, and Sambrook (1982). Although this probably resulted in the loss of smaller transcript fragments, it was found to be superior to ethanol precipitation in removing unincorporated nucleotides, and significantly reduced nonspecific hybridization. Originally, sense-strand transcripts for each cDNA were hybridized to tissue sections as controls for nonspecific hybridization. With some of the sense-strand probes (pLAT51 and pLAT58), high nonspecific background, i.e., signal over petal, sepal, pistil, and anther, was observed. On RNA gel blots, these sense-strand transcripts were found to hybridize to ribosomal RNAs. The corresponding antisense strands did not exhibit any homology with ribosomal RNA on identical RNA gel blots. To circumvent the problem of sense-strand hybridization to ribosomal RNA, and for consistency in the in situ experiments presented, an SP6 control template from New England BioLabs, which exhibited no nonspecific hybridization, was substituted as a control for all of the probes.

Tissue Preparation

Flowers were prepared differently for cryostat and paraffin embedding. For the former, fresh flowers were immersed in OCT compound (Tissue Tek) in either gelatin capsules or plastic scintillation vials and placed under vacuum to remove trapped air bubbles. Vials or capsules were immersed in liquid N_2 until nearly frozen through (if frozen too long, fracturing of the OCT compound occurred), and placed into the prechilled cryostat until equilibrated to −18°C to −20°C. Tissue sections (12 μM) were attached to poly-D-lysine-coated slides (Langdale, Rothermel, and Nelson, 1988) and dried at room temperature for 15 min to 30 min. Slides

were then placed on a slide warmer at 40°C for 2 min, immediately fixed in ethanol:acetic acid (3:1) (Smith, Hinchee, and Horsch, 1987), and dehydrated through an ethanol series. Flowers prepared for paraffin sectioning were fixed in 1% glutaraldehyde in PBS for 1 hr, rinsed in two changes of PBS, and dehydrated in an ethanol series, as described by Angerer and Angerer (1981). Tissue was then transferred through an ethanol:tertiary butyl alcohol (TBA) series to 100% TBA and through a TBA:paraffin (Paraplast) series to 100% Paraplast and embedded in Paraplast, as described by Langdale, Rothermel, and Nelson (1988). 8 μM sections were transferred onto poly-D-lysine-coated slides and heated on a slide warmer at 40°C overnight. Paraffin was removed with Histoclear (National Diagnostics) prior to prehybridization. For each experiment, a single flower from each stage was sectioned and hybridized to all five antisense probes and the control probe. An average of three serial sections were placed on each slide and two to four replicate slides were used for each probe. The results presented are consistent with results obtained over several months of optimizing the protocol for this tissue system.

Pollen was germinated on poly-D-lysine-treated microscope slides in pollen germination media (300 mg/L $CaNO_3$, 100 mg/L boric acid, 15% sucrose) for 18 hr. A coverslip was placed over the pollen tubes and pressed in place, and the slide was frozen on dry ice. After freezing, the coverslip was removed and slides were dried at 40°C for 10 min. Slides were fixed in ethanol:acetic acid (3:1) for 10 min and 70% ethanol for 5 min.

Hybridization of ^{35}S-Probe to Tissue Sections

Cryostat and paraffin sections and pollen squashes were treated in an identical fashion from this point. Slides were prehybridized essentially as described by Smith, Hinchee, and Horsch (1987), except that the 70°C wash was omitted for cryostat sections to reduce the loss of sections from the slides. Labeled transcript was added at 5×10^3 cpm/μL to the hybridization buffer described by Langdale, Rothermel, and Nelson (1988). Generally, the addition of more labeled transcript in the hybridization buffer resulted in increased background signal. Approximately 20 μL of hybridization mix was added to sections per 22 mm^2 coverslip, and slides were incubated 50°C overnight. Sections were also incubated with hybridization mixture without probe and treated exactly as described below to control for exposure of emulsion due to autofluorescence of tissues. Post-hybridization treatments were a modification of methods described by Langdale, Rothermel, and Nelson (1988). Washing temperatures were optimized to maximize the signal to noise ratio while minimizing tissue loss from the slides. Coverslips were removed in 2 × SSPE at room temperature, and slides were washed in prewarmed wash buffer [50% formamide, 0.3 M NaCl, 100 mM Tris (pH 6.8), 100 mM NaH_2PO_4 (pH 6.8), 50 mM EDTA, and 10 mM DTT] for 4 hr to 6 hr. Slides were incubated with 20 μg/mL RNase A in NTE [0.5 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA] for 30 min at 37°C and washed for an additional 30 min in NTE at room temperature. Slides were then transferred into fresh, prewarmed wash buffer and incubated overnight (but not more than 16 hr). After washing, slides were dehydrated through an ethanol series (50%, 70%, 95% ethanol), air-dried and dipped in warmed Kodak Nuclear Track Emulsion (NTB2), dried at room temperature in the dark for 2 hr to 4 hr, and placed at 4°C in foil-wrapped slide boxes to expose. After 8 (cryostat) days or 15 (paraffin) days, slides were developed in Kodak D19 developer at 16°C for 2.5 min, washed briefly in water, fixed in Kodak fixer for 5 min, and rinsed in water for 10 min. Sections were very lightly stained with 0.05% toluidine blue (stain obscured bright-field detection of silver grains over pollen) and photographed through a Zeiss Axiophot microscope using Kodak Panatomic-X film with a green filter in place.

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