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Promoter analysis of genes that are coordinately expressed during pollen development reveals pollen-specific enhancer sequences and shared regulatory elements

David Twell,¹ Judy Yamaguchi, Rod A. Wing,² James Ushiba, and Sheila McCormick³

Plant Gene Expression Center, U.S. Department of Agriculture/Agricultural Research Service—University of California—Berkeley, Albany, California 94710 USA

We have investigated the functional organization and properties of *cis* regulatory elements in the promoter regions of two genes from tomato (LAT52 and LAT59) that are preferentially and coordinately expressed during pollen maturation. Promoter deletion analysis in transgenic plants demonstrated that only minimal (<200 bp) promoter proximal regions are required for developmentally regulated expression in pollen and in specific cell types of the sporophyte. *Cis*-acting regulatory regions of these two promoters and of a third pollen-expressed promoter (LAT56) were characterized in detail using a transient expression assay. We identified two upstream activator regions in the LAT52 promoter and further showed that a 19-bp segment from one of those regions enhanced expression of the heterologous CaMV35S promoter in pollen. Similarities in sequence between crucial *cis* elements provide evidence that shared regulatory elements are involved in the coordinate expression of the LAT genes during microsporogenesis.

[*Key Words*: Tomato; tobacco; male gametophyte; transcriptional control; enhancer; cell-specific gene regulation]

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Identifying the molecular components that control the spatial and temporal patterns of gene expression accompanying gametogenesis is an important step in understanding sexual reproduction in eukaryotes. We are interested in the development of the male gametophyte (pollen) of angiosperms because it provides a unique system with which to study cell-specific gene regulation and cellular differentiation during reproductive development in plants. Major cytological and biochemical events that occur during pollen development are well characterized, the mRNA populations in pollen have been analyzed, pollen-specific isoenzymes have been identified, and several genes that are preferentially expressed in pollen in a stage-specific manner recently have been cloned and sequenced (for reviews, see Mascarenhas 1975, 1989). Of particular interest is the asymmetric mitotic division of the haploid microspore (microspore mitosis), which results in the formation of two dimorphic cells with different developmental fates. The

vegetative cell supports pollen maturation and pollen tube growth, while the generative cell, completely contained within the vegetative cell, undergoes a further mitosis, resulting in the formation of two sperm cells. Microspore mitosis is therefore an important deterministic switch in pollen development, which is conserved among all known flowering plants. After mitosis a rapid synthesis of mRNA is initiated, resulting in the accumulation of mRNA in the mature desiccated pollen grain. Some of these mRNAs are presumably involved in pollen maturation, while others appear to be stored and translated upon pollen germination and/or pollen tube growth (for review, see Mascarenhas 1988).

We have characterized cDNA clones from tomato corresponding to mRNAs that accumulate following microspore mitosis and are either absent or present at highly reduced levels in sporophytic tissues (McCormick et al. 1987). Two of these cDNA clones (pLAT56 and pLAT59) encode proteins whose putative sequences are highly similar to one another (54% amino acid identity) and that show significant sequence similarity to the pectate lyase genes of the plant pathogen *Erwinia* (Wing et al. 1989). This suggests that pectin degradation may be involved in pollen germination and/or pollen tube

Present addresses: ¹Leicester Biocentre, University of Leicester, Leicester LE1 7RH UK; ²Plant Science Center, Cornell University, Ithaca, New York 14853 USA.

³Corresponding author.

growth through the pistil. Another clone, pLAT52 (Twell et al. 1989b), shows 32% amino acid identity to a pollen-specific cDNA clone isolated from maize (Hanson et al. 1989).

The isolation of several genes that are similarly regulated during microsporogenesis provides an opportunity to identify sequence elements that are required for pollen-specific expression and to test the hypothesis that common regulatory elements are involved in their coordinate patterns of expression. We showed that the 5'-flanking regions of the LAT52 and LAT59 genes, when fused to the *Escherichia coli* β -glucuronidase (GUS) reporter gene (Jefferson et al. 1987), are sufficient to direct expression in an essentially pollen-specific manner in transgenic tomato, tobacco, and *Arabidopsis* plants (Twell et al. 1990). The expression of the 52-GUS and 59-GUS gene fusions was developmentally and coordinately regulated during microsporogenesis, similar to the pattern of mRNA accumulation for the endogenous genes, and was closely correlated with the onset of microspore mitosis (Twell et al. 1990).

In this paper we describe the results of experiments designed to delimit sequence elements within the upstream regions of the LAT52, LAT56, and LAT59 genes that are required for regulated gene expression during pollen development. Analyses of promoter deletion mutants fused to reporter genes in stably transformed plants (for the LAT52 and LAT59 promoters), or with a transient expression assay in pollen (Twell et al. 1989a), have allowed the precise identification of several *cis*-regulatory elements that are important for gene expression during pollen development. We present evidence for the function of two conserved regulatory elements, each of which is shared among two of the three LAT gene promoters. An upstream region of the LAT52 promoter that contains one of these conserved regulatory elements has the properties of an enhancer and is able to activate a heterologous core promoter in pollen.

Results

Sequence comparison of the LAT gene promoters

As a first step in our analysis of sequence elements required for the regulated expression of the LAT52, LAT56, and LAT59 genes during pollen development, their 5'-flanking DNA sequences were compared to identify conserved motifs. The nucleotide sequence of 0.6 kb of 5'-flanking DNA of the LAT52 gene was described previously (Twell et al. 1989b). Sequences upstream of the coding regions of the LAT56 and LAT59 genes are shown in Figure 1. Pair-wise computer alignment of all three promoters showed no extensive regions of sequence similarity. However, the repeated sequence TGTGGTT, which we refer to as the PB core motif (Fig. 2a), was previously noted in the LAT52 promoter (Twell et al. 1989b) and is similar to the so-called SV40 enhancer core motif (Sassone-Corsi and Borrelli 1986) and to the binding site for the plant nuclear factor GT-1 (Green et al. 1988). The PB core motif is repeated three

times within the 492 bp 5' of the LAT52 transcription start site (Fig. 2a), and in the LAT56 promoter at position -166 bp (Fig. 1a) and the LAT59 promoter at position -508 bp (Fig. 1b). A 12-bp sequence that is shared between the LAT52 and LAT56 promoters (52/56 box; Fig. 2b) encompasses the PB core motif and is closely related to the box II sequence motif in the pea *rbcS-3A* gene promoter (Fig. 2a). Box II is representative of a class of *cis*-regulatory elements that are important for light-regulated and organ-specific gene expression (for review, see Gilmartin et al. 1990). A different sequence motif is shared between the LAT56 and LAT59 promoters. This motif, which we refer to as the 56/59 Box (Fig. 2b), is present at a similar position relative to the transcription start site of both genes.

Deletion analysis of the LAT promoters

We showed previously that 0.6 kb of the LAT52 and 1.4 kb of the LAT59 5'-flanking DNA regions are sufficient to direct expression exclusively in gametophytic cells (pollen) of developing anthers (Twell et al. 1989b). To identify and characterize *cis*-regulatory elements involved in promoter strength and specificity, 5' and internal deletion mutants of the LAT promoters were constructed in vitro and linked to the coding region of the GUS reporter gene (Jefferson et al. 1987). Transfer of the chimeric genes to tomato plants allowed us to localize promoter activity in transgenic plants. A transient assay system in tobacco pollen (Twell et al. 1989a) allowed us to examine the effect on expression of many more promoter mutations than is practical in transgenic plants.

LAT52 promoter activity in transgenic plants LAT52 promoter activity in various tissues was initially examined by quantitative assay of GUS activity in extracts from pooled tissues of the primary transformants. No significant increase above background GUS activity (in extracts from untransformed plants) was detected in plants containing the 52-GUS deletion mutants for the following tissues: stem, leaf, sepal, petal, pistil, and pericarp of green or red fruit (data not shown), with the exception of the -3000- and -492-bp 52-GUS deletion mutants, which showed low levels of activity in those tissues relative to anthers. However, the sporophytic activity observed for the -3000- and -492-bp deletion mutants was accounted for by 1 of 4 independent -3000-bp transformants and 2 of 10 independent -492-bp transformants (data not shown), and are presumed to be due to position effects.

Whole anthers containing pollen can be assayed for GUS activity as a measure of the GUS activity in pollen (Twell et al. 1990). Deletion of the LAT52 promoter from -3000 to -492 bp did not significantly affect promoter activity in pollen (Fig. 3a). However, deletion to -145 bp reduced promoter activity ~10-fold. Deletion to -86 bp further reduced promoter activity 100-fold. The deletion mutants -86 bp 52-GUS and -71 bp 52-GUS showed similar levels of GUS activity, which was ~20-fold above background levels. These results demonstrate that

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a. LAT56

1-1153
 GTGGAAATCA ATTGCATGTG GACAAACAGAA ACAAAGAATT TATGTTGTCA AAAATATTGT AGAAAAGCAA TGGACATGAT GATTTTTTCA TCGCTTTTAT -1054
 GTTTTTTATC TTAATAAAAA GTAATAGAAA AAGCAAAGA CATAGTCCTC TAGTTTGTC ATTTTCTGCA TTTTAAAAAA AAATCCAAA AAGCGATGA -954
 ACTGCGACAT TTTTCCGTC GCTTCTACA TATTTAAAAA AAAGAAAAG AAAGGAATG TGTTCCCTG TCGCTCTCT TCTGTATTT TAAAAAAATA -854
 AAAGAGAAA AACCCACAC TCCATCGTTC TATAGGACA ACTGATAATT TTCTTCACAA AAAACACCCA TCAAAACTGT GGAGCTGTT GCTTTTTACA -754
 AAGTTTGAG GACAAAAGCA ACAGACTCAA CTATATCATT TTTTCAATA TTTTCTACA AAAAAAAAAC AACAAGTAT GGACTGTGT GCATTTTACA -654
 TCGCTTTTG CATATATCT TTTTAAAAA GTGACATACT GTACATTTT TGCATTTTAG AGTTTCTTT AAAAAGAAG ATAAAAAGT ATGCAATCCT -554
 TCGAAAAGT TCGAGGACCA AAGCGACAGA CTAGAGTGA TTCTTTCTG TCGATTTGA CCAAAAAGTA ATGCATTTCA TTGCTTTTGT CCATCTTTTT -454
 TTGATAGTCT TTTAGTCATA TGCAAAAT GAAAACGAC AATCTTTATA TACGGATTAA GAAGAAAGT AGGACAACA AATTAATAAG AAAGCAATAT -354
 TTATTTATT TATTTGTTT TTTAAACTT TTCTACCGT AGCAGTACT CATTTTTTAA CAAGTTGAT ATGATAAAT GAAAAAATA CGAAATAAGC -254
 TATTTGAT AATGACTTAT ATAATACAT TGATGACGTA AAAATTATTT AATGACTTAT ACACATACAT TGATAATGA TAATATGTT GGTATATAA -154
 ATAAAAGTG TTTGTTATTG GAAGGCTATA AGAAGAGAT ATATAAGCAA GAATTTGTA CACACATGT TTAATGTTCC TTAATTTGAG TAATGATCA -54
 AAATCATGTT CATTATGAG TATATAATA AGTGATGTT ATGAATGAAA ATAGCACAA AGAGGAACAA ATAAATGTTG TGTTTTAGA ATAATTAAT +47
 ACCCTATTT TCTCACACA CTTTTTAAAG GGTAGTAGT GTGTAAGGA TTGACCTTAC ATCAACTACC ATCAACACCC CTAGCAAAAT TTAACAATAA +147
 CCCCAACTTA CCTCAACCC TACCTACCT ATTAATTTAT AATAATG

b. LAT59

1-1305
 GAGCTCTCT TAAAGAAGTT GAAATACCTT CTCCAGTAA ATGGGACTGG AGGGGGAGAT TTGTGAATC CATCCCATTT TTAGTCAATT TTAGTATTTC -1206
 AATTGGTACA AGCAATTGTT AAAAAGGAAT AGTAATGGA GTTGAAAATA AAAGATTGG TGGTTGCCAA ATGGTAAGA TTTGCAACAA TCTTGACTTT -1106
 GCTATATTTA CATATGTCAT TAGTGACATA GGCATGGTTT TATTATCTTA TAAATAGAGC ATTTTGTGTC ATTTGTAGAA CACACAAGT TTAGAGAGAA -1006
 ATCATTGAG AGTAAAGTGA TGATGCATA GACTATACAA GAAATAGTCT CTCACAAAT AGAGTGTGAG CGATATTTTA GTAAGACGGA AACCAAAAGA -906
 GTGTGTGTC TTTTGAGGTG GTAGTAGTCA CTTTGGATAT TATATCCGTC ACTACACCGT GTAAAAATTC CTATATATG TAATATCAAT TGCCTCTCTT -806
 GGCCCGTGT TTTTCCCTT ATTCAAGG ATTCCACGT AAAATTATG GTGTGCTTAT TTTTCCATTT TATTCCGAT ACCTTTTACA TATATATTTT -706
 TGTGCTGTC CGGTGTTCC CAACAACCTC CTTCCCTTTA GAAAGAATAA TCTTAATTC TTTTGTAGT GTTTAAAAA AATAACTTTT TTCCTTTTTT -606
 GACAATACTT TAACTTTAA TTTAATCTC CACAAGGAT GTTAAAGAT ACAAGATTAA ATGACATTT GATACATTT ACATAACTTT ATTTTAGAAC -506
 CACAAGATA AAAAATTTCT TCTTTCTT AAACCTTGT TCAACTTTCA AGTCAAACTA GATCATTCT TTTTAAATG AGGGAGTACT AATTAAGAT -406
 TTTTTTTT TTTTGTATA GATCGATTT CCATGATTGA AGTTAAAAT ATAAATGTA GTATTTGTA TCTAATTTT TTATAATTGA AAATTAATAA -306
 AATCATCTTA AAAATTTGG CAATTTACT AGTGTCTTAT AACTAAAAA GATTATGTA ACTTCTGTC CACTTAATA ACCCTTGTGA GTTGTATAT -206
 TTTTCCICGA TTTCTGTAT TAITAAATG TTTGACTATT TCAATTTTGG CGAATATTC TCAATGCTT TCGACGAAAG AGCTATGGAA GAAATGTTG -106
 TGGACATTT AGTTTCAAAA CAAGTGACTG TCGCCAAAGT TAGAATTTAT GTGTGAGTG TTAATCACAA GTAATGAT TTAATAACA TTTACAAG -6
 TGTAAAAAA AAAAAAAA AAAAAAACC CTTTCAACT TGTGTCTCA TTGAGACGAG CCTTAAGTCT GAAATAAAC TTTTGTGTT GACCCAGGTT +95
 CAATAAAGG CCGAACCC TAGAGATTAA AAAGGTTGCA CCTTACCTT TGGTAACAAC AACGAGGAG TTTAAAGAAA ACGAAAAA ATAAAAATAT +195
 AATATAAAAA ATTTGTTTAA AAAAAAGGA AAAAAATAGA GATG

Figure 1. DNA sequences of the 5'-flanking regions of the LAT56 (a) and LAT59 (b) genes. The transcription (arrows) and translation (underlined) start sites, putative TATA boxes (open boxes) and functionally conserved regulatory elements between the LAT52, LAT56, and LAT59 promoters (shaded boxes) are noted. The endpoints of the 5' deletion mutants are indicated above the sequence with a vertical line.

at least three distinct regions of the LAT52 promoter (Fig. 4a) regulate gene expression in pollen. Regions 52-A (-492 bp to -145 bp) and 52-B (-124 bp to -86 bp) contain sequences that strongly enhance expression in pollen. However, region 52-C (-71 bp to +110 bp) is sufficient to direct gene expression in pollen.

Previous analysis demonstrated that LAT52 promoter activity was localized in developing pollen grains after microspore mitosis (Twell et al. 1990). We found that all of the LAT52 deletion mutants directed GUS expression that was developmentally and spatially similar (Fig. 5B and data not shown) to that observed for the endogenous LAT52 gene (Twell et al. 1989b, 1990).

The 52-GUS deletion mutants all showed GUS activity in immature and mature seeds (Fig. 3b). GUS activity in the 52-GUS deletion mutants was restricted to the endosperm (Fig. 5F), whereas seeds from a transgenic tomato plant containing a cauliflower mosaic virus 35S (CaMV35S) promoter-GUS construct showed high levels of GUS activity in all seed tissues (not shown). Only 6 of the 26 independent transformants examined showed detectable staining in roots. We attribute this root expres-

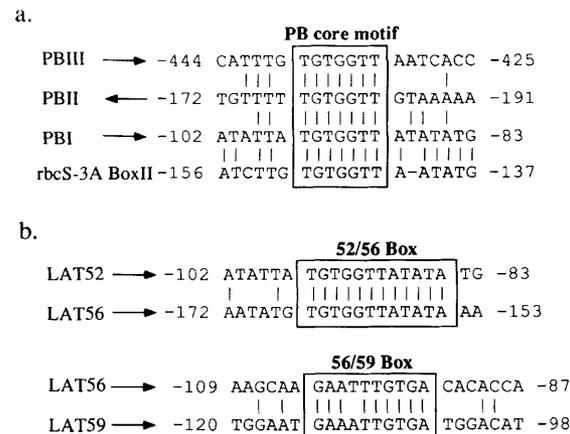


Figure 2. Conserved sequence motifs in the LAT gene promoters. Identities between pairs of sequences are shown as vertical lines, and orientation relative to the transcription start site is indicated by arrows. (a) Extended sequence similarity around the reiterated PB core motif (boxed) in the LAT52 promoter and in the rbcS-3A promoter. (b) Conserved 52/56 box and 56/59 box sequences.

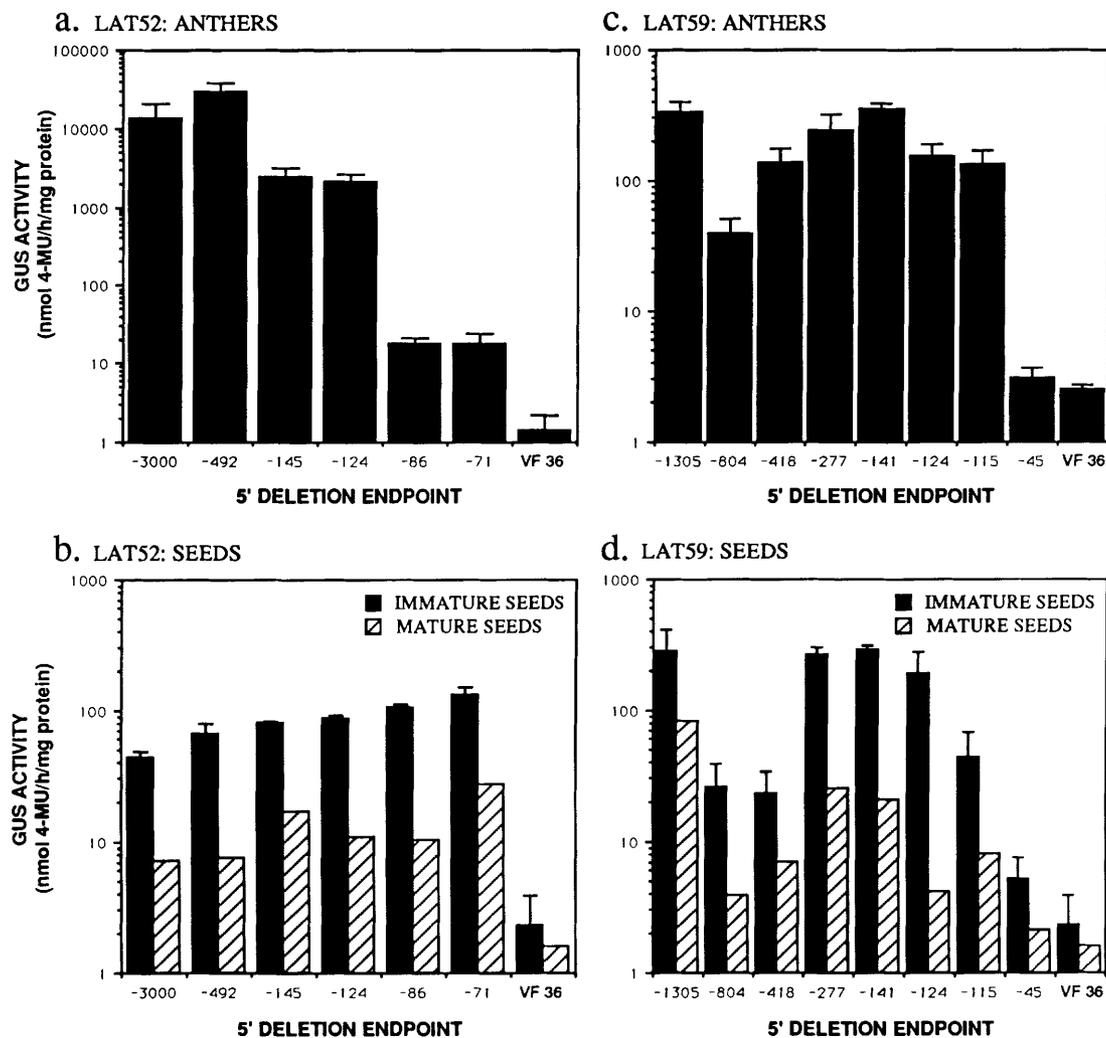


Figure 3. Promoter activity of 5' deletion mutants in transgenic tomato plants. GUS activity in anthers (*a* and *c*) and seeds (*b* and *d*) of LAT52 (*a* and *b*) and LAT59 (*c* and *d*) promoter deletion mutants. The data for anthers and immature seeds represent the mean activity of three separate determinations of GUS activity in extracts prepared from pooled tissues taken from 4–12 independent transformants; error bars indicate the s.d. The mature seed values are from one determination per deletion mutant. Control assays (VF36) were performed on untransformed tomato plants.

sion to position effects, because there was no correlation with the level of pollen expression, deletion end point, or cellular pattern of staining (data not shown).

LAT52 promoter activity in the transient assay system We showed previously that the activities of the LAT52 and CaMV35S promoters in transient expression assays, in which DNA is delivered into tobacco pollen by microprojectile bombardment, closely reflected the activities of the LAT52 and CaMV35S promoters in transgenic tomato plants (Twell et al. 1989a, 1990).

The 5' deletion analysis using this transient assay (Fig. 6a) confirmed the activity of the promoter regions 52-A, 52-B, and 52-C and further defined subregions of importance (52-A1, 52-A2, 52-B1, and 52-C1, Fig. 4a). The upstream activation properties of the 52-A region were analyzed (Fig. 7a) in the absence of 52-B. Two copies of 52-A in tandem led to an approximately twofold increase

in promoter activity (cf. pLAT52-20 and pLAT52-31; Fig. 7a), which demonstrates that this region can function in an additive manner when placed 0.5 kb upstream of the transcription start site. Removal of region 52-A1 (–492 bp to –225 bp) led to an approximately twofold decrease in activity (cf. pLAT52-20 and pLAT52-28; Fig. 7a). The activity of 52-A1 was not revealed by analysis of 5' deletion mutants when 52-B was intact (cf. –492 and –225 bp deletion mutants; Fig. 6a), so these data provide evidence for redundant enhancer sequences in the 52-A1 and 52-B regions. The region –225 bp to –129 bp (~52-A2), when placed upstream of the truncated –86-bp LAT52 promoter, enhanced expression additively and independent of orientation >10-fold (pLAT52-28, pLAT52-29, pLAT52-30, and pLAT52-17; Fig. 7a). A 50-bp fragment (–225 to –176 bp) containing the PB motif also enhanced the activity of the core promoter

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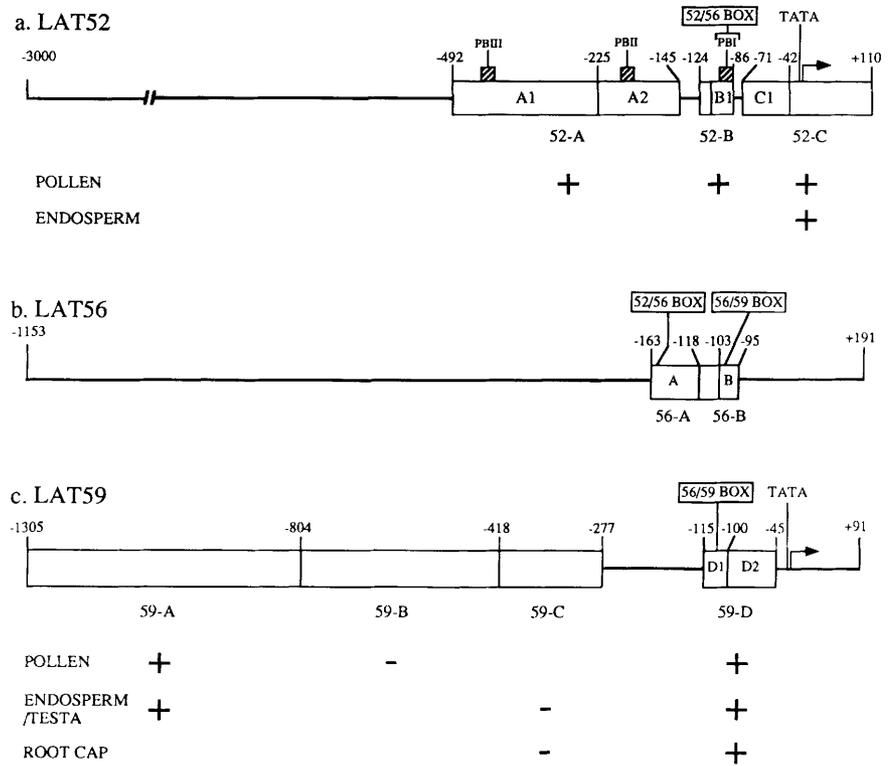


Figure 4. Functional maps of the LAT gene promoters. Diagrams of the LAT52, LAT56, and LAT59 promoter regions as defined by transient expression and/or transgenic plant assays are shown (see text).

approximately fivefold, independent of its orientation (pLAT52-49 and pLAT52-47; Fig. 7a).

To examine whether the 52-A2 region could enhance the activity of a heterologous promoter, the 52-A2 fragment and smaller portions of it were placed upstream of a truncated CaMV35S promoter (Ow et al. 1986) and tested using the transient expression assay. As shown in Figure 7b, a single copy of 52-A2 in reverse orientation enhanced the activity of the -89-bp CaMV35S promoter ~14-fold, and two tandem copies led to an ~25-fold enhancement. A 19-bp oligonucleotide containing the PBII sequence was placed upstream of the -89-bp CaMV35S-LUC fusion. A single copy of this sequence enhanced expression in the forward and reverse orientations ~4- and ~7-fold, respectively (Fig. 7b), while two copies in a tail-to-tail configuration enhanced expression ~15-fold. These results show that the PBII box acts as a transcriptional enhancer in pollen. Control experiments showed that increasing the length of the CaMV35S promoter to -148 bp or -1500 bp did not significantly activate gene expression in pollen (Fig. 7b).

The 5' deletion analysis within the 52-B region (-145 bp to -86 bp) identified *cis*-regulatory sequences in the region -100 to -86 bp (52-B1), which contains the 52/56 box. Partial deletion of the 52/56 box (cf. -100- and -93-bp deletions; Fig. 6a) led to a twofold decrease in promoter activity. Deletion of region 52-C from -71 to -42 bp (52-C1) led to a threefold decrease in activity (Fig. 6a). Since 52-C is sufficient for specific expression in pollen and endosperm of transgenic plants, these results indicate that the 52-C1 region is responsible, at

least in part, for the pollen component of this expression pattern. Further deletion from -42 to -17 bp (Fig. 6a), which removed the putative TATA box, abolished detectable expression.

LAT56 promoter activity in the transient assay system Because we had identified shared motifs between the LAT56 promoter and the LAT52 and LAT59 promoters, we used the pollen transient assay to perform a 5' deletion analysis of the LAT56 promoter to assess whether the shared motifs were important. Promoter activity of 5' deletion mutants of the LAT56 promoter in pollen identified two positive regulatory regions (Figs. 4b and 6b). Deletion of 56-A (-163 to -118 bp) led to an approximately fivefold decrease in promoter activity. The 56-A region contains the shared 52/56 box sequence (Fig. 2b). Deletion of 56-B (-103 to -95 bp) led to an approximately fourfold decrease in activity. This region contains the shared 56/59 box (Fig. 2b). Deletions in the -95- to -22-bp region showed no further decreases in promoter activity (Fig. 6b).

LAT59 promoter activity in transgenic plants In contrast with the LAT52 promoter, deletion analysis identified no strong upstream activating sequences in the LAT59 promoter (Fig. 3c). Deletion of the LAT59 promoter to -115 bp gave only a slight decrease in promoter activity (two- to threefold) compared with the -1305-bp 59-GUS fusion. Further deletion to -45 bp, which retained the putative TATA box, abolished detectable expression. Regulatory elements located between -115 and -45 bp (59-D, Fig. 4c) thus appear to be largely responsible for the activity of the LAT59 pro-

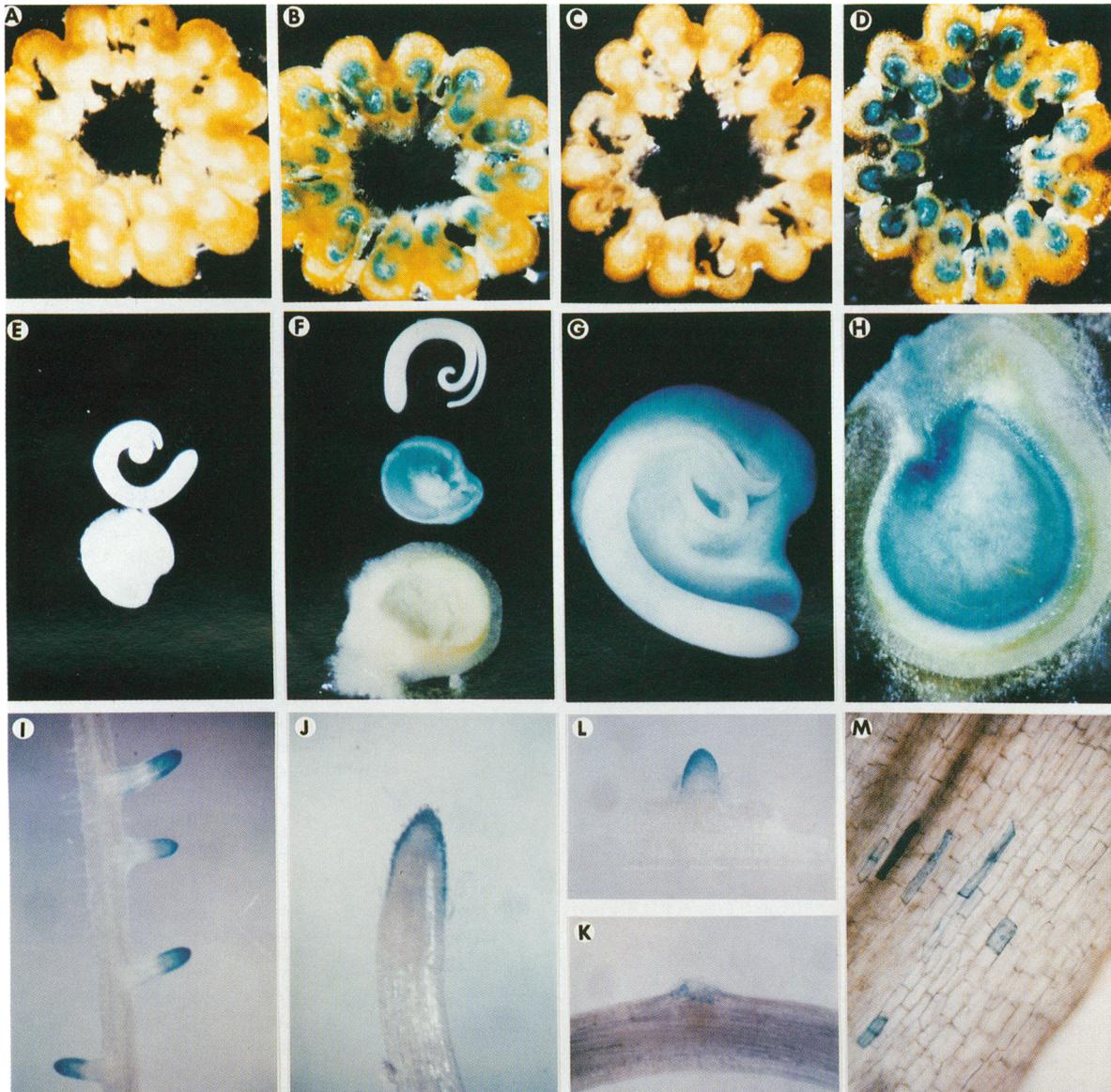


Figure 5. Localization of GUS activity in anthers, seeds, and roots of representative transgenic tomato plants. (A) Anther section from untransformed tomato cv. VF36; (B) anther section from -71 -bp 52-GUS plant; (C) anther section from -45 -bp 59-GUS plant; (D) anther section from -115 -bp 59-GUS plant; (E) embryo and endosperm section from VF36; (F) embryo, endosperm, and testa section from -71 -bp 52-GUS plant; (G) embryo and endosperm section from -124 -bp 59-GUS plant; (H) testa section from -140 -bp 59-GUS plant; (I) root segment from -277 -bp 59-GUS plant; (J) longitudinal section of tip of a primary root from -277 -bp 59-GUS plant; (K) root segment from -124 -bp 59-GUS plant before emergence of the lateral root meristem; (L) recently emerged lateral root from -124 -bp 59-GUS plant; (M) root segment from -1305 -bp 59-GUS plant.

motor in pollen. The data shown in Figure 3c also provide evidence for a negative regulatory element located between -804 and -418 bp (59-B, Fig. 4c), and a positive regulatory element located between -1305 and -804 bp (59-A, Fig. 4c), since the activity of the -804 -bp 59-GUS deletion mutant is reduced 5- to 10-fold relative to the flanking deletion mutants. As with the minimal LAT52 promoter, the minimal LAT59 promoter (-115 bp) directed GUS expression that was spatially and developmentally similar (Fig. 5D; data not shown) to that of the endogenous LAT59 gene (Wing et al. 1989).

Our previous analysis showed that the -1305 -bp 59-GUS fusion was also expressed at low levels in seeds and roots of transgenic tomato plants (Twell et al. 1990). Although we were unable to detect the endogenous LAT59 transcript in these tissues (Twell et al. 1990), this deletion analysis revealed the location of *cis*-acting regions that specify sporophytic expression of the 59-GUS fusion. GUS activity was detected in both immature and mature seeds of all but the -45 -bp 59-GUS deletion mutant (Fig. 3d). GUS activity in seeds was localized to both the endosperm and the testa (Fig. 5G,H). Deletion

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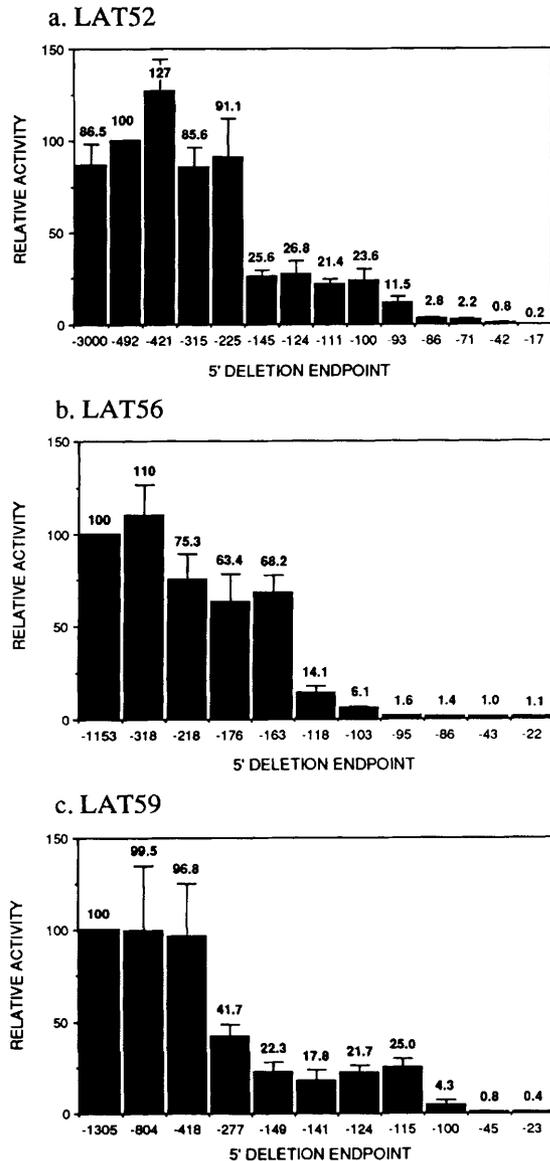


Figure 6. Transient expression analysis of 5' deletion mutants of LAT promoters in tobacco pollen. Test plasmids were cotransformed with the reference plasmid pLAT59-13 and assayed for GUS (test) and LUC (reference) activity after 6–12 hr. Relative activity represents the GUS/LUC ratio for each deletion mutant, normalized to the activity of the –492-bp 52-GUS deletion mutant (a), the –1153-bp 56-GUS deletion mutant (b), or the –1305-bp 59-GUS deletion mutant (c). Relative activity represents the mean of at least five independent bombardments. Error bars show the S.D.

from –1305 to –804 bp (59-A) led to a significant (~10-fold) decrease in promoter activity, and deletion from –115 to –45 bp led to a 26-fold decrease in GUS activity (Fig. 3d). These results indicate the presence of positive regulatory elements for seed expression in the 59-A and 59-D regions (Fig. 4c). Low levels of seed expression were observed for the –804-bp 59-GUS and –418-bp 59-GUS deletion mutants, which indicates

that negative regulatory elements are located in the –418- to –277-bp (59-C) region (Figs. 3d and 4c).

GUS activity was consistently detected in roots of tomato plants containing the –1305-, –277-, –141-, –124-, and –115- 59-GUS deletion mutants, but not in roots of the –804-, –418-, and –45-bp deletion mutants. These results suggest that the 59-D region (Fig. 4c) contains sequences required for expression of the 59-GUS fusion in roots, while region 59-C (Fig. 4c) appears to have a negative influence on root expression. With the exception of the –1305-bp 59-GUS deletion mutant, roots showed staining in the root cap of both primary and lateral roots (Fig. 5I,J) but not in the root meristem, cortex, or vascular tissue. In lateral roots, staining was first detected in epidermal cells that are dislodged during lateral root emergence (Fig. 5K) and then in the root cap immediately after emergence (Fig. 5L). In contrast, the –1305-bp 59-GUS deletion mutant roots showed a punctate pattern (i.e., staining in isolated cells) distal from the root tip (Fig. 5M).

Our results indicate that the expression of the 59-GUS fusion in roots is mainly associated with sites of lateral root emergence and the presence of the root cap. If the LAT59 gene product encodes a functional pectate lyase (Wing et al. 1989), we would propose a role for this protein in cell separation events that occur during the emergence of lateral roots, and in the sloughing off of root cap cells. Immunolocalization studies are required to establish whether the observed GUS staining patterns reflect the endogenous distribution of the LAT59 gene product in roots.

LAT59 promoter activity in the transient assay system Deletion of the 59-D region (Fig. 4c) led to a 31-fold decrease in promoter activity in the transient assay (Fig. 6c), similar to that observed upon deletion of 59-D in transgenic plants (Fig. 3c). Deletions from –115 to –100 bp, the region that contains the shared 56/59 Box sequence (Fig. 2b), and from –100 to –45 bp, each led to a five- to sixfold decrease in promoter activity (Fig. 6c). This indicates the presence of at least two regulatory regions within the 59-D region, which we refer to as 59-D1 and 59-D2 (Fig. 4c). Further deletion from –45 to –23 bp, removing the putative TATA box, led to a twofold decrease in activity (Fig. 6c). In the upstream region, deletion from –418 to –115 bp led to a fourfold decrease in promoter activity, due to two contiguous regulatory regions, –418 to –277 bp (59-C) and –277 to –149 bp (Figs. 6c and 4c, respectively).

In summary, the importance of the 59-D region was confirmed with both the transient and transgenic analyses. The positive influence of the –418- to –115-bp region was not observed in pollen of transgenic plants (Fig. 3c), although it influenced promoter activity in the transient assay (Fig. 6c). Furthermore, the negative influence of the 59-B region (–804 to –418 bp) observed in transgenic plants (Fig. 3c) was not detected using the transient assay (Fig. 6c). This lack of correlation between transient and stable analyses of the upstream regulatory elements of the LAT59 promoter could be due to insufficient levels of available *trans*-acting factors in mature or recently

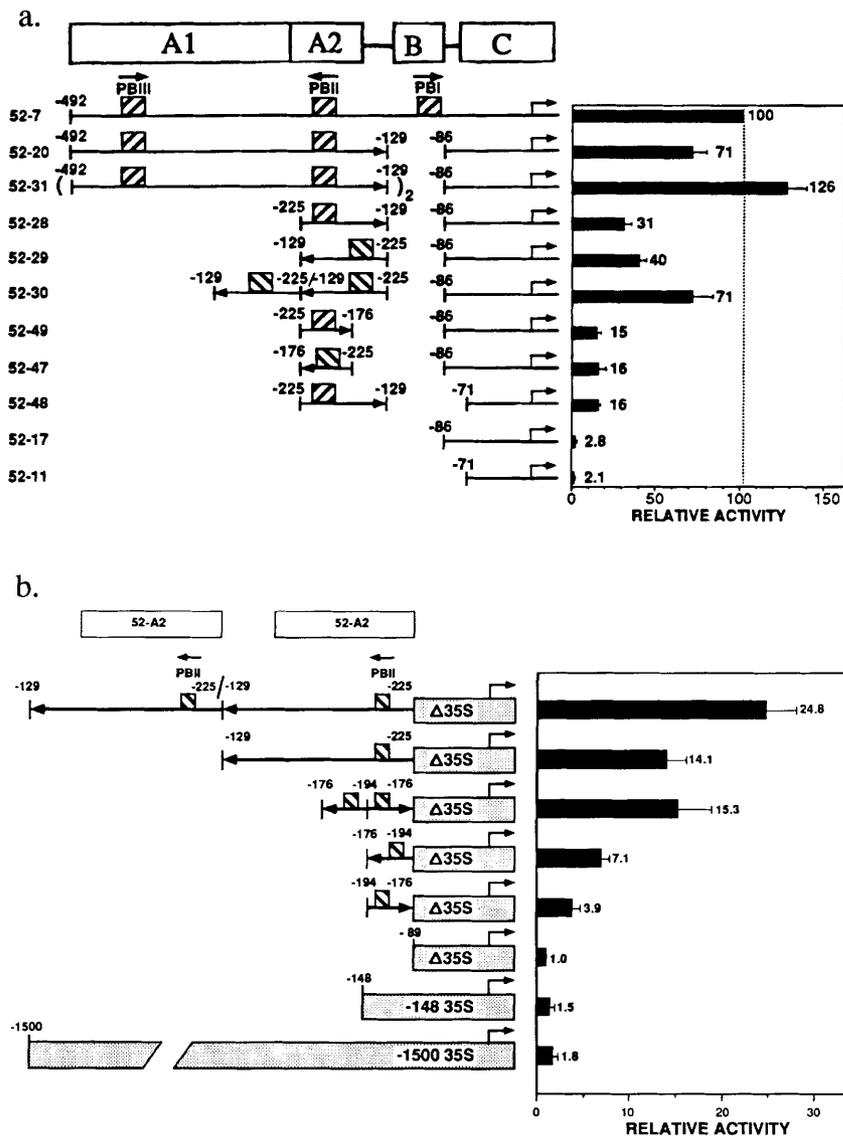


Figure 7. Analysis of LAT52 upstream elements. PBI, PBII, and PBIII (hatched boxes) represents the position and orientation of the reiterated TGTGGTT motif in the LAT52 promoter. Relative activity represents the mean of at least six independent bombardments. Error bars show the s.d. (a) Activation of a truncated LAT52 promoter by upstream sequences from the LAT52 promoter. Transient expression assays were performed as described in Fig. 6. Plasmid names are listed at left. (b) Activation of a truncated CaMV35S promoter by upstream sequences from the LAT52 promoter. The activities of hybrid promoter constructs linked to LUC were determined by transient expression assays as described in Fig. 6, using pLAT59-12 (–1305-bp 59-GUS fusion) as the reference plasmid. CaMV35S promoter sequences are indicated as stippled boxes. (Δ35S) –89-bp CaMV35S promoter.

hydrated pollen, or due to the use of pollen from tobacco, a heterologous plant. It is also possible that the lower GUS expression in the transgenic plants we analyzed for the –804 and –418-bp deletions was due to position effects.

Analysis of common regulatory elements in the LAT promoters

Mutation of the GG doublet in the 52/56 box sequence of the LAT52 promoter led to a 10-fold decrease in promoter activity (–100 5' del GG > CA; Fig. 8a); removing the GG doublet from the 52/56 Box in the LAT56 promoter resulted in a 2-fold decrease in expression (–161 5' del; Fig. 8b). Mutation of an additional downstream GG doublet in the LAT56 promoter did not lead to a further decrease in activity (–161 5' del GG > CA; Fig. 8b).

Substitutions of 2 or 3 bp were introduced in the 56/59 box of the LAT59 promoter (S1 to S8; Fig. 8c). Mutants of the core motif GTGA led to an approximately threefold decrease in promoter activity. The equivalent mutation in the 56/59 box in the LAT56 promoter also abolished the activity of the 56-B region (Fig. 8b). These experiments demonstrate the importance of the 56/59 box (Fig. 2b) in both the LAT56 and LAT59 promoters.

Conservation of LAT gene regulatory elements in other pollen-expressed genes

In addition to the LAT genes, the upstream regions of two other genes that are similarly regulated during pollen development have been sequenced: the maize Zmg13 gene (Hamilton et al. 1989), the coding region of which shares sequence similarity with that of LAT52, and the petunia chalcone flavone isomerase (*chi*) A gene (van

Tunen et al. 1989), the upstream promoter (*chiA* P_{A2}) of which is sufficient to direct pollen-specific expression in petunia (van Tunen et al. 1990). In the *Zmgl3* promoter, five sequences were found (at positions -495 bp, -439 bp, -170 bp, -141 bp, and -49 bp) that contained a GTGG motif and showed at least five of seven matches to the PB core motif. In the *chiA* P_{A2} promoter, there are three sequences (at positions -771 bp, -437 bp, and -353 bp) that contained a GTGG motif and showed six of seven matches to the PB core motif. One sequence was found in the *Zmgl3* promoter (at -81 bp) that showed 7- of 10-bp matches to the 56/59 box, while two such sequences (8- of 10-bp matches) were found (between -111 and -91 bp) in the *chiA* P_{A2} promoter.

Discussion

Pollen- and cell type-specific regulatory elements modulate LAT52 and LAT59 promoter activity

Our results (summarized in Fig. 4) demonstrate that only minimal promoter proximal regions of the LAT52 (-71 to +110 bp) and LAT59 (-115 to +91 bp) genes are required for their correct, regulated expression during pollen development. Several upstream regulatory elements (52-A, 52-B, 59-A) appear to modulate the activity of these minimal promoters in pollen. Although this is the first analysis of the organization of *cis*-acting sequences that regulate gene expression during gametophyte development in plants, the promoter proximal location of sequences that determines tissue-specific expression in the sporophyte has been described for other plant genes (for review, see Benfey and Chua 1989). For instance, the soybean β -conglycinin gene requires only 159 bp of upstream sequences to direct tissue- and stage-specific expression in seeds (Chen et al. 1986), while sequences farther upstream enhance transcription in a seed-specific manner. No evidence was obtained for negative regulatory elements that modulate LAT52 promoter activity in pollen or silence expression of the LAT52 promoter in sporophytic tissues. In contrast, deletion analysis of the LAT59 promoter in transgenic plants provides evidence for both positive (59-A) and negative (59-B) upstream regulatory elements that modulate gene expression in pollen (Fig. 4c). Because these putative regulatory elements did not display similar properties in the transgenic (in tomato) versus transient (in tobacco) analyses, further studies are required to confirm their properties and precise locations within the LAT59 promoter.

Our data also demonstrate that the LAT52 and LAT59 promoters are differentially activated in specific cell types of the sporophyte. Both promoters were expressed in the endosperm, but LAT59 was also expressed in the testa and root. The *cis*-acting sequences that mediate the observed activity of the LAT52 promoter in the endosperm (52-C) are clearly different from those that mediate high level expression in pollen (52-A and 52-B; Fig. 4a). However, within the 52-C region, either the same or different sequences could mediate the activity of the

LAT52 promoter in pollen and endosperm. Similar arguments can be made for the pollen and sporophytic activity of the "minimal" LAT59 promoter. Studies of other tissue-specific genes provide evidence for a modular arrangement of distinct regulatory elements that mediate expression in different tissues. For instance, the tissue-specific expression of the *Drosophila melanogaster white* gene is independently controlled by different regulatory elements that are active in either the testes, the Malpighian tubules, or the eye (Levis et al. 1985).

In summary, our data indicate that the LAT52 and LAT59 promoters share a functionally similar promoter core but are modulated by several upstream regulatory elements that show differences in their organization and ability to enhance or repress gene expression in pollen or in certain sporophytic cell types. The fact that cell specificity was not lost upon progressive deletion of either promoter suggests that cell-specific expression is not achieved through a release from transcriptional repression. In contrast, *in vitro* transcription studies indicate that such a mechanism is responsible for the testis-specific expression of the mouse protamine 2 gene (Bunick et al. 1990). While we cannot rule out the possibility that general silencer elements are located within the core LAT52 and LAT59 promoters, we favor a regulatory mechanism based on the promotion or repression of transcription by several cell-specific *trans*-acting factors, which exert additive or combinatorial effects to achieve cell-specific expression. A similar regulatory mechanism is proposed for the cell-specific and synergistic activities of enhancer subdomains within the CaMV35S promoter (Benfey and Chua 1990).

Function of the PB motif in the LAT52 upstream activation domain and its relationship with the GT-1 box

Analysis in transgenic plants indicates that the LAT52 promoter is composed of two distinct domains, a proximal domain (52-C), which determines tissue-specific expression (pollen and endosperm), and an upstream domain (52-A + 52-B) which is required for high-level expression specifically in pollen (Fig. 4a). Transient expression studies demonstrate further that sequences within the upstream domain 52-A2 (containing the PBII box) are able to activate either the minimal LAT52 promoter or the heterologous CaMV35S promoter in pollen, independent of orientation and, to some extent, independent of distance. It will be interesting to determine whether enhancer region 52-A2 or the PBII box alone is able to direct pollen-specific expression in transgenic plants. The reiterated PB motif in the LAT52 promoter domain is responsible, at least in part, for the functional redundancy of the LAT52 upstream region. Functional redundancy of upstream regions that mediate light-regulated and tissue-specific activity of the pea *rbcs-3A* gene appears to depend on the reiteration of the GT-1 box sequence (Kuhlemeier et al. 1988; Gilmartin et al. 1990). The similarity between the GT-1 box and the PB motif, together with the importance of the GG doublet

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in both sequences (this paper and Green et al. 1988) suggests that GT-1 may also bind to the PB motif.

Two shared regulatory elements modulate the activity of the LAT gene promoters in pollen

Our results provide evidence for the function of two regulatory elements, the 52/56 box and the 56/59 box, which are shared among two of the three LAT gene promoters. However, we also demonstrated that the 52/56 box is dispensable for the developmental regulation of the LAT52 gene in pollen (Fig. 5B). On this basis we propose a quantitative role for the 52/56 box, in which basal LAT52 and LAT56 promoter activity in pollen is coordinately enhanced by transcription factor(s) that recognize this sequence. The conserved 56/59 box may also serve to activate basal promoter activity, and/or it may have a more determinative role in turning on expression of the LAT56 and LAT59 genes in pollen.

The similarity between functionally important nucleotides within the 52/56 box (GTGG) and the 56/59 box (GTGA) raises the possibility that they are both recognized by the same transcription factor(s). If so, this would provide a mechanism for the coordinate regulation of the LAT genes and perhaps other genes that are expressed late during pollen development. Indeed, the conservation of both the PB core motif and the 56/59 box in the petunia *chiA* P_{A2} promoter would indicate a more "global" role for these sequences in coordinating gene expression late during pollen development. Isolation and characterization of *trans*-acting factors that regulate LAT gene expression will allow direct testing of regulatory mechanisms and their relationship to other factors, such as GT-1. Such studies should provide further insight into the molecular events that are required for the establishment of differential and coordinate patterns of gene expression during male gametophyte development.

Materials and methods

Plant transformation

Agrobacterium strains containing gene fusion constructs were used to infect cotyledons of *Lycopersicon esculentum* cv. VF36, and kanamycin-resistant plants regenerated as described (Twell et al. 1990), but using 375 μ M acetosyringone. Eight to 15 independently transformed plants were obtained for each gene fusion, except for the -3000-bp LAT52-GUS construct, for which only four transformants were obtained.

Construction of plasmids

To construct 5' deletion mutants of the LAT promoters, restriction sites were introduced by polymerase chain reaction (PCR), using oligonucleotide primers with restriction sites at their 5' ends and LAT promoter sequences with the desired mutation at their 3' ends. Along with each mutant primer, a second primer corresponding to the nonsense strand of the GUS gene at position -70 bp plus the appropriate template (-492 bp 52-GUS, -318 bp 56-GUS, or -1305 bp 59-GUS) was used to synthesize a PCR product that was cleaved, purified, and cloned into a promoterless GUS-NOS plasmid (pDAT-1 or pGP213; Twell et

al. 1989a, 1990). The LAT52 and LAT56 deletion mutants contained the entire 5'-most untranslated leader sequences fused to the translational start codon of the GUS gene. All of the LAT59 mutants contained only the 5' 91 bp of the 234-bp 5'-untranslated leader.

Selected 52-GUS-NOS3' and 59-GUS-NOS3' deletion mutants were cloned into binary vector pBIN19 (Bevan 1984) and transferred to the disarmed *Agrobacterium tumefaciens* strain PC2760 (pAL4404) as described (Twell et al. 1990). Internal deletion mutants of the LAT promoters were constructed by using a strategy similar to the PCR scheme described above. Hybrid LAT52-CaMV35S promoter fusions were constructed by cloning PCR fragments of the LAT52 gene or a double-stranded oligonucleotide into the *Xho*I site of plasmid pJO4X (-89 bp CaMV35S-LUC; Ow et al. 1986).

All PCR products were entirely sequenced. No errors were found in constructs containing LAT52 and LAT59 promoter sequences. However, two different base changes were detected in the untranslated leader sequence of the LAT56 constructs. These mutant constructs showed no significant differences in their activities in transient assays when compared with versions in which these changes were subsequently corrected to the wild-type LAT56 leader sequence.

The -300-bp CaMV35S-GUS fusion used as a control for seed expression was constructed (by Margaret Boylan) by cloning the GUS-coding region from pBI101.1 (Jefferson et al. 1987) into the polylinker of plasmid pMON316 (Rogers et al. 1987) between the CaMV35S promoter and *nos* gene polyadenylation/termination sequences.

Transient expression assays in pollen

Plasmids were introduced into mature tobacco pollen by microprojectile bombardment as described (Twell et al. 1989a), except that $\sim 10^7$ pollen grains (0.5 ml of a 20- to 40-mg dry weight/ml MS0 medium pollen suspension) were used per bombardment. Tobacco was used in these experiments because the LAT52 and LAT59 promoters were shown to function similarly in tomato and tobacco, both in transgenic plants and in transient assays (Twell et al. 1989a, 1990; McCormick et al. 1991) and because large quantities of tobacco pollen can be collected easily.

Test promoter constructs linked to the GUS gene were coprecipitated onto tungsten microprojectiles with a reference plasmid (pLAT59-13; Twell et al. 1990) containing the -1305-bp to +91-bp LAT59 promoter fused to the firefly luciferase (LUC) gene (Ow et al. 1986). Test promoter activity was expressed as a ratio of GUS to LUC activity and normalized to the activity of one of the LAT-GUS deletion mutant plasmids. Bombardment of pollen with microprojectiles coated with increasing amounts of test plasmid DNA led to a linear increase in activity in the range of 2-15 μ g for the 52-, 56-, and 59-GUS fusions (McCormick et al. 1991). The amount of DNA introduced (7 μ g of test plasmid and 2 μ g of reference plasmid) was chosen to be within the linear range. LAT52 and LAT59 bombardments were assayed after 6-7 hr. Longer incubations (12 hr) and higher amounts of DNA (14 μ g) were used for LAT56 constructs to increase the sensitivity of the assay, since the LAT56 promoter was approximately fourfold less active than the LAT59 promoter (McCormick et al. 1991). Pollen was extracted in 0.5 ml of buffer [0.1 M KPO₄ (pH 7.5), 1 mM dithiothreitol], and assayed for GUS and LUC activity as described (Twell et al. 1990).

Histochemical localization of GUS activity

Localization of GUS activity in sections of anthers, seeds, and root segments was carried out as described (Twell et al. 1990).

Seeds and anthers were incubated in 1 mM X-Gluc for 4 hr at room temperature; roots were incubated under the same conditions overnight.

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