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# Pollen Specificity Elements Reside in 30 bp of the Proximal Promoters of Two Pollen-Expressed Genes

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**Functional analyses previously identified minimal promoter regions required for maintaining high-level expression of the late anther tomato *LAT52* and *LAT59* genes in tomato pollen. Here, we now define elements that direct pollen specificity. We used a transient assay system consisting of two cell types that differentially express the *LAT* genes and both "loss-of-function" and "gain-of-function" approaches. Linker substitution mutants analyzed in the transient assay and in transgenic plants identified 30-bp proximal promoter regions of *LAT52* and *LAT59* that are essential for their expression in pollen and that confer pollen specificity when fused to the heterologous cauliflower mosaic virus 35S core promoter. In vivo competition experiments demonstrated that a common *trans*-acting factor interacts with the pollen specificity region of both *LAT* gene promoters and suggested that a common mechanism regulates their coordinate expression. Adjacent upstream elements, the 52/56 box in *LAT52* and the 56/59 box in *LAT59*, are involved in modulating the level of expression in pollen. The 52/56 box may be a target for the binding of a member of the GT-1 transcription factor family.**

## INTRODUCTION

Reproduction in angiosperms involves a double fertilization event in which two male gametes (sperm cells) combine with the egg and the central cell to yield a diploid embryo and triploid endosperm. Pollen, the male gametophyte, serves as a specialized vector for the delivery of the male gametes into the female gametophyte. This highly specific role undoubtedly involves proteins that are specifically expressed in pollen and that are necessary for pollen maturation, pollen–pistil communication, and growth of the pollen tube through the style for delivery of the sperm cells. Indeed, during the process of pollen maturation, several mRNAs have been found to be expressed at specific developmental stages (reviewed in Mascarenhas, 1990; McCormick, 1993). Some of these mRNAs exhibit overlapping expression in sporophytic tissue, and others have been found to be pollen specific. A number of so-called late genes, active at a late stage of pollen development, have been isolated in different species (reviewed in McCormick, 1991a, 1993). However, the mechanism by which their coordinate developmental expression is achieved is not yet understood. In this work, we have focused our studies on two such late pollen-specific genes, late anther tomato *LAT52* and *LAT59* (Twell et al., 1990), to help elucidate the mechanism(s) directing pollen specificity. We chose to study these two genes concomitantly to determine whether a common mechanism could be responsible for their coordinate expression.

Based on sequence homology with pectate lyases (Wing et al., 1989; McCormick, 1991a), the *LAT59* gene product is suggested to function during pollen germination, whereas recent results show that the *LAT52* gene product affects pollen hydration (Muschietti et al., 1994). RNA gel blot analyses (Twell et al., 1989a; Wing et al., 1989) as well as reporter gene analyses with the promoters of *LAT52* and *LAT59* (Twell et al., 1990) revealed that transcriptional activity in pollen was correlated with the onset of microspore mitosis and increased progressively until anthesis. A functional analysis of the promoters of both genes in transgenic plants revealed that high levels of expression and pollen specificity are maintained in the proximal regions: −115 bp for *LAT59* and −100 bp for *LAT52* (Twell et al., 1991). Although sequence comparisons of *LAT52*, *LAT59*, and other pollen-specific gene promoters yielded no common conserved regions (reviewed in McCormick, 1991a), conserved sequences at the 5′ end of the proximal promoter regions were noted between *LAT52* and an additional pollen-specific gene, *LAT56* (52/56 box), and between *LAT59* and *LAT56* (56/59 box) (Twell et al., 1991). The 52/56 box was found to be redundant in sequences farther upstream in *LAT52*, whereas the 56/59 box occurred only once in the *LAT56* and *LAT59* promoters. Mutagenesis of these boxes and analysis in transient assays confirmed that these sequences modulated expression levels in pollen. Promoter internal deletion experiments demonstrated that redundant upstream sequences can partly compensate for the loss of the *LAT52* proximal region 52/56 box and that constructs with upstream *LAT52* promoter sequences (−225 to −129) can somewhat activate a −89 cauliflower mosaic virus

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(CaMV) 35S core promoter (Twell et al., 1991). These results suggested the existence of positive elements in upstream sequences that could not alone confer high-level pollen-specific gene expression. Thus, although all of these experiments defined promoter sequences important for expression in pollen, they did not delimit pollen specificity elements in the promoter proximal regions. Functional analysis of other pollen-expressed genes (Guerrero et al., 1990; Albani et al., 1992; Kyozuka et al., 1994) similarly implicated promoter sequences necessary for expression in pollen without addressing tissue specificity.

Although many plant promoters have been studied at the functional level, only a few studies have directly addressed the mechanisms conferring tissue specificity by a combination of "loss-of-function" and "gain-of-function" approaches. Defined sequences have been shown to be involved in the aleurone-specific expression of a barley chitinase gene (Leah et al., 1994). The mechanism of regulation involves a combination of enhancing in aleurone tissue and silencing in other tissues. Sequences conferring tissue specificity by positive regulation have been localized in the tuber-specific patatin gene of potato (Grierson et al., 1994), in endosperm-specific zein genes of maize (Ueda et al., 1992), in the seed-specific lectin gene of pea (de Pater et al., 1993), and in the nodule-specific leghemoglobin *glb3* gene (Szczyglowski et al., 1994).

In this research, we used differential transient expression experiments and transgenic tomato plants to identify 30-bp regions in the proximal promoters of the *LAT52* and *LAT59* genes that are sufficient for directing high-level tissue-specific expression in pollen. We also provide evidence that these same sequences mediate their regulatory effect through the binding of a common *trans*-acting factor. Thus, we identified *cis*-acting elements determining pollen specificity and provide evidence for a common process regulating the coordinate expression of these *LAT* genes.

## RESULTS

### Distinct Positive Elements Reside in the *LAT52* and *LAT59* Proximal Promoters

To define sequences in the *LAT52* and *LAT59* promoters involved in expression in pollen, we dissected their proximal promoters by constructing a series of linker substitution mutants (designated LS) and testing their activities in transient expression assays using the particle bombardment system (Klein et al., 1987; Twell et al., 1989b). These experiments were performed by cobombarding the test plasmids (luciferase [LUC] fusions) with reference plasmids (β-glucuronidase [GUS] fusions). The latter served as a control for bombardment variability and allowed comparisons to be made between independent bombardments.

We chose the context of the −100 promoter in *LAT52* and the −115 promoter in *LAT59* because they appeared to be the minimal regions that still conferred high levels (25% relative to the available full-length promoter) of pollen-specific expression (Twell et al., 1991). These minimal promoters were then fused to the *luc* gene (Ow et al., 1986) coding region (see Methods), and the resulting plasmids served as a basis for creating the LS constructs. The *LAT52* linker substitutions were performed in p52LUC (see Methods), which contains the entire *LAT52* 5′ untranslated region (5′ UTR). A series of six 8- to 10-bp-long linker substitutions were made in p52LUC, spanning the region −84 to −29 (52LS1 to 52LS6; see Figure 1A). Because we have found that the *LAT59* 5′ UTR has an inhibitory effect on *luc* expression (C. Curie and S. McCormick, unpublished results), we opted to use p59ΔILUC (which retains only the upstream 10 bp of the 5′ UTR; see Methods) for construction of *LAT59* linker substitutions. Seven 10-bp-long linker substitutions were performed in p59ΔILUC, spanning the region −98 to −29 (59LS1 to 59LS7; see Figure 1B). The sequences of all linkers were designed to contain a restriction site (XhoI in *LAT52* and BglII in *LAT59*) in addition to as many base transitions as possible.

### *Analysis of LS Mutations by Transient Expression in Pollen*

Functional analysis of the LS constructs in transient expression assays in pollen yielded the results shown in Figure 1. In *LAT52*, the 52LS1, 52LS2, and 52LS3 substitutions resulted in a five- to ninefold decrease in LUC expression relative to p52LUC, whereas 52LS4, 52LS5, and 52LS6 did not affect the expression level (Figure 1A). In *LAT59*, the 59LS1, 59LS2, and 59LS3 substitutions all reduced LUC expression (Figure 1B), but the effect of 59LS2 and 59LS3 was four times greater (15-fold decrease) than the effect of 59LS1 (less than a fourfold decrease). In contrast, the 59LS4 substitution increased promoter activity by 2.5-fold. The most proximal mutations, 59LS5, 59LS6, and 59LS7, did not alter the level of expression. Thus, the linker substitutions in the proximal promoter regions of *LAT52* and *LAT59* showed essentially the same profile. In each, expression was decreased when substitutions were introduced in a region 30 to 40 bp upstream of the TATA boxes. These results define the locations of regulatory sequences essential for expression in pollen in the *LAT52* and *LAT59* proximal promoters.

### *Analysis of LS Mutations in Transgenic Tomato Pollen*

We next investigated whether the reduced expression resulting from the specific linker scan mutations could also be observed in pollen of stably transformed tomato plants. For these experiments, we replaced the *luc* gene with the *Escherichia coli uidA* gene encoding GUS because the latter allows histochemical localization in plant tissue sections (Jefferson et al., 1987). LAT52-GUS, LAT59ΔI-GUS, and the four most distal LS mutations of each of the promoters (LS1, LS2, LS3, and LS4) fused to *uidA* were subcloned into a pBin19 vector

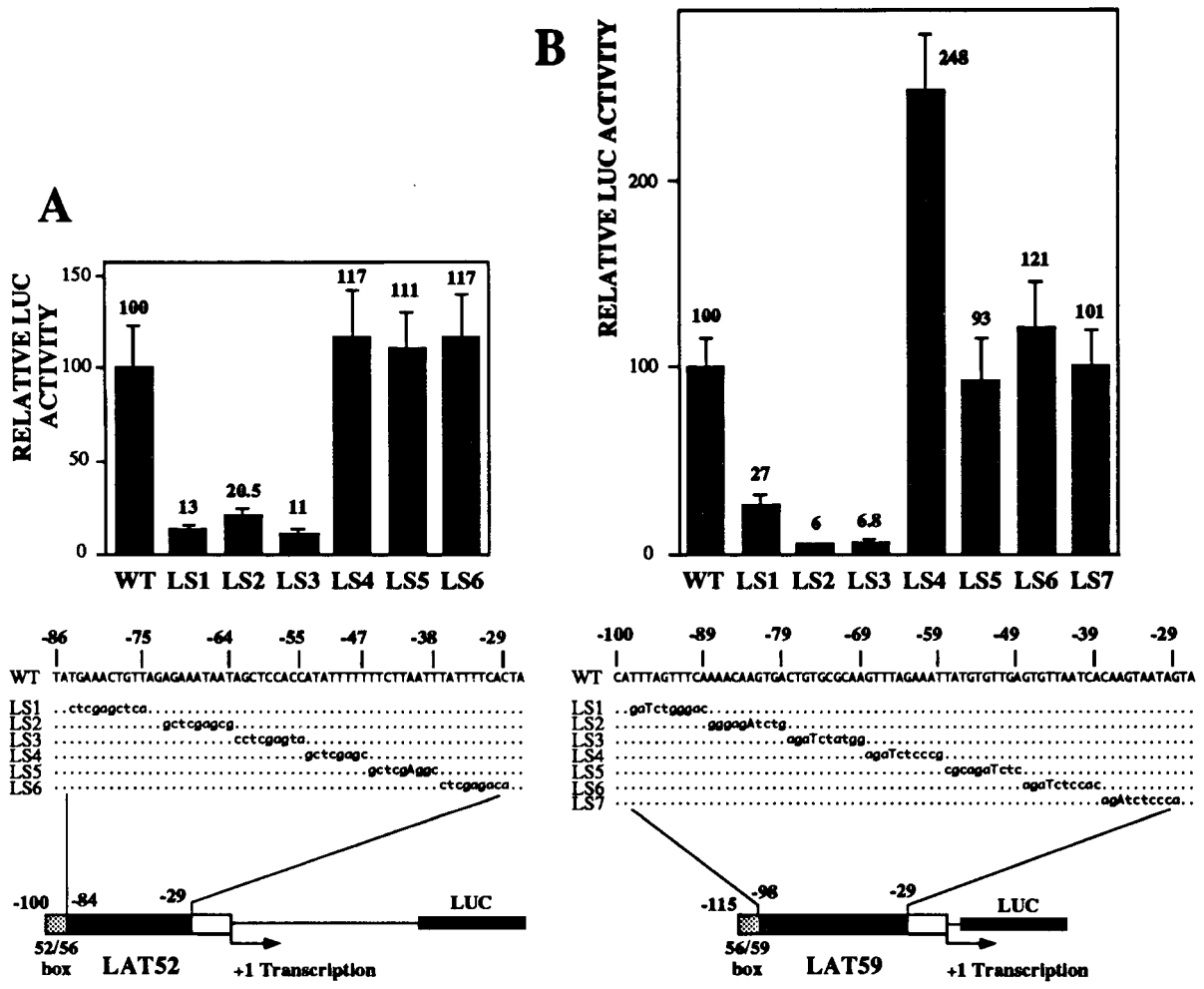


**Figure 1.** Analysis of LS Mutations by Transient Expression in Pollen: Loss-of-Function Approach.

**(A)** LUC expression of *LAT52* constructs. Linker substitutions (LS1 to LS6) represent mutations introduced in the proximal promoter region of *LAT52* in the plasmid p52LUC. WT represents the nonmutated p52LUC.

**(B)** LUC expression of *LAT59* constructs. Linker substitutions (LS1 to LS7) represent mutations introduced in the proximal promoter region of *LAT59* in the plasmid p59ΔILUC. WT represents the nonmutated p59ΔILUC.

The positions of the promoter mutations relative to the transcription start are indicated above the sequences; substituted bases appear in lowercase letters, and conserved bases are in uppercase letters. Each bar represents the mean of at least six independent transformation experiments. Relative LUC activity represents the ratio between the test (LUC) and the reference (GUS) plasmids.

(Bevan, 1984). The resulting plasmids were then used to transform tomato plants by Agrobacterium-mediated transformation (McCormick, 1991b). Pollen harvested from the flowers of the primary transformants was assayed quantitatively for GUS activity. The results of these analyses are shown in Figure 2.

Pollen from plants harboring the 52LS1, 52LS2, and 52LS3 substitutions contained 0.04, 0.4, and 0.04%, respectively, of the GUS activity of the wild-type promoter fusion plants (Figure 2A). In contrast, pollen from plants harboring the 52LS4 substitution contained 70% of the GUS activity of the wild-type promoter fusion plants (Figure 2A). These results are qualitatively the same as the results obtained by the transient assay. The differences are quantitatively more extreme, presumably due to the large difference in expression time of the transgenes (15 to 17 hr in the transient assay versus several days for transgenic pollen), resulting in more accumulation of the stable GUS enzyme in transgenic pollen. The region corresponding to 52LS123 was confirmed as essential for high-level expression in pollen.

Plants harboring the 59LS2 or 59LS3 substitution showed a reduction in GUS expression (Figure 2B), which is consistent with the results obtained in the transient assays. At the

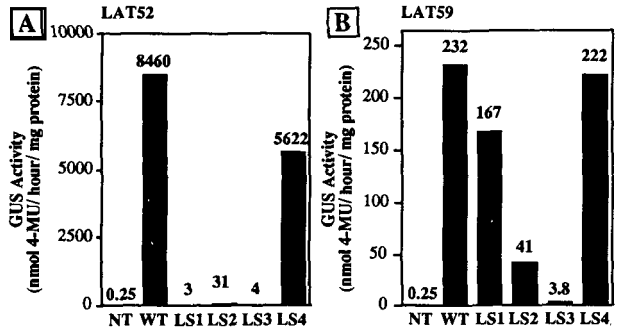


**Figure 2.** Analysis of LS Mutations in Pollen of Stably Transformed Tomato Plants.

**(A)** GUS expression levels in transgenic tomato pollen harboring *LAT52* (wild type [WT] and LS1 to LS4) constructs fused to the *uidA* reporter gene. NT represents nontransformed plants.

**(B)** GUS expression levels in transgenic tomato pollen harboring *LAT59* (wild type and LS1 to LS4) constructs fused to the *uidA* reporter gene. NT represents nontransformed plants.

GUS levels are presented in nanomoles of 4-methylumbelliferone (4-MU) generated per hour per milligram of protein in the pollen extract. The numbers above the bars show the average GUS activity in pollen of 20 to 37 independent transgenic plants for each *LAT52* construct and of 22 to 39 independent transgenic plants for each *LAT59* construct.

quantitative level, we noted some discrepancies between the data obtained from transgenic plants and those obtained by transient assay. Whereas both 59LS2 and 59LS3 resulted in a 15-fold decrease in LUC activity in the transient assay, the drop in GUS activity measured in the transgenic plants was only fivefold for 59LS2 but more than 60-fold for 59LS3. The results from 59LS1 and 59LS4 transgenic plants did not confirm the transient assay data: 59LS4 plants displayed a wild-type level of activity, but we measured a 2.5-fold increase in transient expression; 59LS1 plants showed an insignificant decrease, but we observed an almost fourfold decrease in transient expression. Thus, the alteration of the expression by the 59LS1 and 59LS4 substitutions obtained in transient expression experiments was not reproduced in the stable transformation system. Because the results using the *LAT52* promoter constructs were consistent in both the transient assays and transgenic plants, we can only speculate that the weaker promoter (*LAT59*) was more prone to a position effect. Indeed, the variation in expression we observed between plants carrying any given *LAT59* construct was by far greater (10-fold) than that between plants harboring *LAT52* constructs. Although we cannot draw any definitive conclusion regarding the 59LS1 and 59LS4 regions of the promoter, we did confirm that the region corresponding to 59LS2 and 59LS3 is essential for high-level expression in pollen by using both the transient and transgenic plant systems.

## Tissue-Specific Expression Is Not Achieved through Transcriptional Repression

### *Analysis of Tissue Specificity by Transient Expression in Two Cell Types*

The results obtained by transient expression in pollen and in transgenic plants provided us with information on the effect of the various constructs on expression in pollen but not on their effect on tissue specificity. We therefore added a tobacco cell culture, TXD (maintained as described by Howard et al. [1992]), as an additional component of the transient assay system. The TXD cell culture was initiated from tobacco mesophyll cells and therefore represents somatic tissue, as opposed to the gametophytic tissue represented by pollen. We chose cells in culture, rather than intact tissue, for our somatic tissue source because they superficially resemble pollen in that they can be spread out as a monolayer on a plate before bombardment. Table 1 illustrates the differential expression observed between the two types of cells. In this experiment, translational fusions between the *luc* coding region and either the CaMV 35S "somatic" promoter or the *LAT52* promoter were introduced into both types of cells by bombardment. The CaMV 35S promoter drove strong expression in cell culture but negligible expression in pollen, whereas the *LAT52* promoter showed the opposite pattern of strong activity in pollen and negligible activity in cell culture. Thus, the transient assay system mimics the expression pattern observed for these promoters in transgenic plants (Twell et al., 1991). This differential expression provided us with a tool with which to address tissue specificity.

### *The Linker Substitutions Do Not Affect the Pattern of Expression Directed by the* LAT *Gene Promoters*

*LAT52* and *LAT59* genes are expressed primarily in the pollen grain, with the exception of weak expression in the endosperm of immature seed for both genes and in the root tips for *LAT59* (Twell et al., 1991). Tissue-specific expression of these genes

**Table 1.** Differential Expression of Promoters in Pollen and TXD Cell Culture

| | Relative LUC Activity | |
|---|---|---|
| Plasmid | Pollen | Cell Culture |
| p35SLUC | 15 ± 3.4 | 9460 ± 984 |
| p52LUC | 62,233 ± 7234 | 24 ± 3.1 |

Plasmids p35SLUC and p52LUC were introduced into pollen and TXD cell culture by particle bombardment. Transient expression was assayed as described in Methods. Relative LUC activity represents the ratio between the test plasmid (LUC) and the reference plasmid (GUS) and is the mean of at least nine independent experiments.

could be explained by either a negative or positive mode of regulation or a combination of both. A negative mode of regulation would mean that their expression elsewhere in the plant is repressed. We then hypothesized that a substitution destroying a putative repressor binding site in a pollen-specific promoter would result in derepression, or increased activity, in somatic tissue. Therefore, we tested each of the LS substitutions of *LAT52* and *LAT59* for expression in TXD cell culture by transient assay. Because no increase in LUC expression was observed (data not shown), we concluded that a negative mode of regulation involving a repressor was not likely.

To extend tissue specificity analysis to all tissues of the plant, we tested the effect of the LS substitutions introduced into transgenic plants on the previously established pattern of expression of the *LAT* genes (Twell et al., 1991). 52LSGUS and 59LSGUS plants were analyzed fluorometrically or by histochemical assays of leaves, stems, roots, fruits, seed, and flowers. We observed no changes in the established expression pattern with any of the LS substitutions (data not shown). Expression remained limited to the pollen grain, with the exception of weak expression in the endosperm of immature seed for both promoters and in the root tips for *LAT59*. This demonstrates that the proximal promoter regions (−84 to −47) in *LAT52* and (−98 to −59) in *LAT59* do not contain any repressor binding site and confirms the results obtained by transient expression in cell culture.

### Thirty Base Pairs in the *LAT52* and *LAT59* Proximal Promoter Regions Are Sufficient To Confer High-Level Pollen-Specific Expression on a Heterologous Core Promoter

The previous experiments ruled out a negative mode of regulation directing pollen specificity. Therefore, we used a gain-of-function approach to test whether sequences of the minimal promoters were involved in determining tissue specificity. The results of these transient assays are shown in Figure 3.

*LAT52* and *LAT59* promoter fragments, coined 52PS and 59PS, respectively (for pollen specificity), were fused to the −64 CaMV 35S core promoter. This minimal promoter contains a TATA box followed by a transcription initiation site but no known *cis*-acting element (Benfey and Chua, 1990). The promoter fusion of *LAT59* was designed to maintain the original distance between the putative *cis* elements (corresponding to 59LS123) and the TATA box. The promoter fusion of *LAT52* contained an additional 10 bp (one helix turn) between the putative *cis* elements (corresponding to 52LS123) and the TATA box. These chimeric promoters fused to the *luc* reporter gene were tested in parallel in pollen and cell culture transient assays. The 500-bp CaMV 35S promoter, p35SLUC, directed essentially no LUC expression in pollen but served as a positive control for cell culture, where it is strongly expressed (almost 200-fold above the background level). The pJO64 plasmid, which contains only the −64 CaMV 35S core promoter fused to the *luc* reading frame, was completely inactive in both pollen and cell culture. The *LAT52* promoter sequence between −100 and −55 (52PS1) and the *LAT59* promoter sequence between −113 and −69 (59PS1) were found to enhance expression of the −64 CaMV 35S core promoter in pollen 1300- and 130-fold, respectively, whereas expression in cell culture remained negligible. The same sequences in reverse orientation, 52PS1r and 59PS1r, resulted in 570- and 46-fold, respectively, enhanced expression in pollen. A direct repeat of the *LAT59* PS1 sequence (59PS1PS1) resulted in 165-fold enhancement.

The 52PS1 and 59PS1 regions included the previously identified 52/56 and 56/59 boxes, respectively. To test whether they were essential components for maintaining high-level pollen specificity, we repeated the gain-of-function experiments without the 52/56 and 56/59 boxes. The *LAT52* promoter sequence between −84 and −55 (52PS2) and the *LAT59* promoter sequence between −98 and −69 (59PS2), each corresponding to the LS123 region, were fused to the −64 CaMV 35S promoter as described previously. 52PS2 enhanced expression of the core promoter 560- to 660-fold in pollen irrespective of its orientation, whereas 59PS2 enhanced expression 25-fold in the reverse orientation but 50-fold in the direct orientation. Expression in cell culture remained negligible, attesting to the tissue-specific nature of the enhancement. All together, these data show that the *cis*-acting element(s) controlling pollen-specific expression of *LAT52* and *LAT59* is contained within short sequences of 30 bp, located between −84 and −55 and between −98 and −69, respectively, in the proximal promoters. Neither the 52/56 box nor the 56/59 box located immediately upstream of the 30-bp regions is an essential component for directing tissue specificity.

### Competition Experiments Provide Indirect Evidence for the Interaction of a Common *trans*-Acting Factor(s) with *LAT52* and *LAT59* Proximal Promoter Sequences

Having defined pollen specificity sequences in the functional assay, our next logical step was to examine their possible interactions with *trans*-acting factors. Pollen presents a unique difficulty for footprinting experiments (in vivo and in vitro) because it contains both a generative and a vegetative cell, each presumably with its own specific pattern of expression (reviewed in Mascarenhas, 1990). Indeed, the *LAT52* promoter has been shown to direct vegetative cell–specific gene expression with no detectable levels of expression in the generative cell (Twell, 1992). Gel shift assays have been difficult to pursue due to the limited amount of tissue that one can collect and the difficulty of isolating the fragile vegetative nuclei. Therefore, we decided to use in vivo competition to examine possible interactions between DNA sequences and *trans*-acting factors.

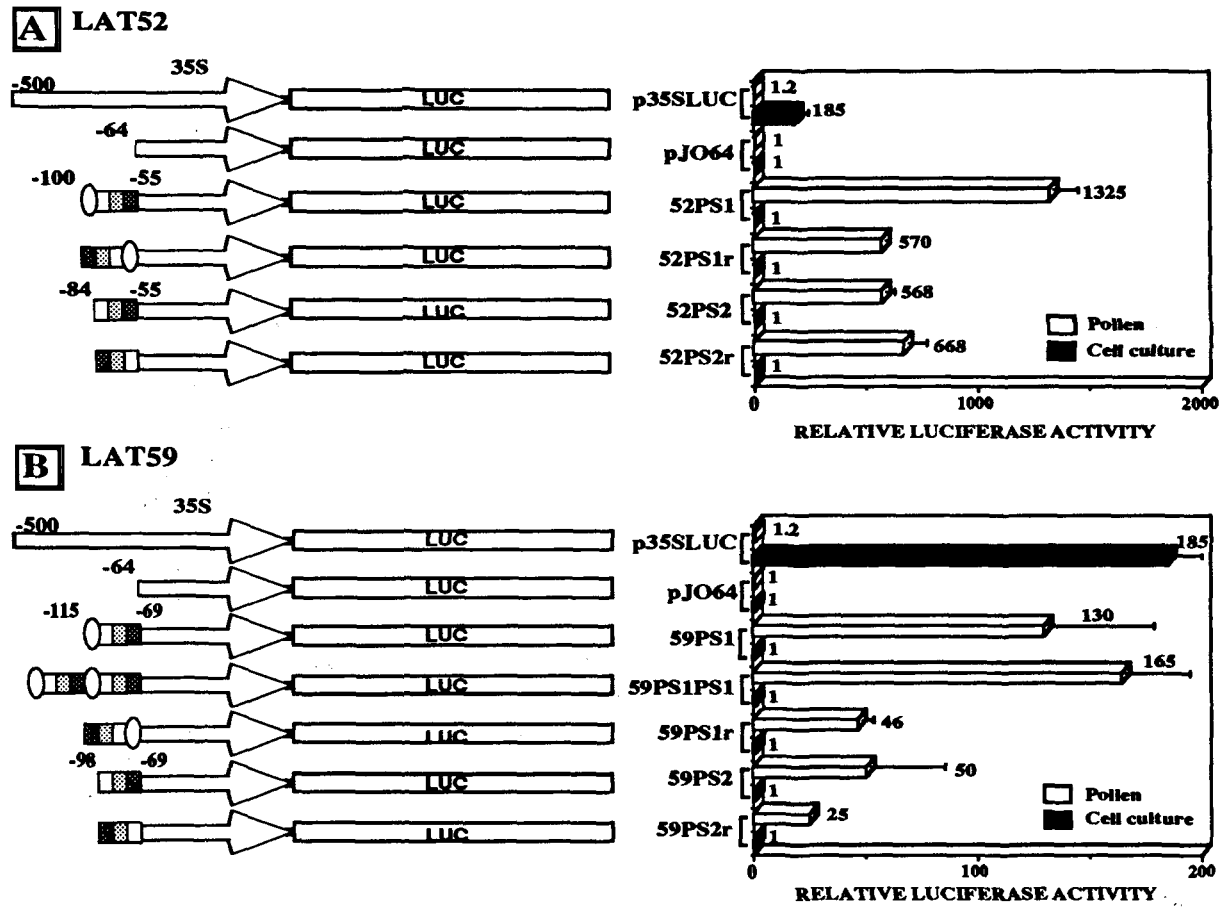


**Figure 3.** Transient Expression Driven by *LAT*-35S Promoter Fusions in Pollen and Cell Culture: Gain-of-Function Approach.

Promoter fusions between the 35S core promoter (−64, +1) and fragments of either the *LAT52* or the *LAT59* proximal promoter region, driving the *luc* reporter gene, were transiently expressed in pollen (white bars) or TXD cell culture (black bars). The (−500 to +1) CaMV 35S promoter served as a positive control for cell culture and a negative control for pollen. The (−64, +1) 35S core promoter (pJO64) served as a negative control for both pollen and cell culture experiments. Each bar represents the mean of at least six independent experiments. Relative LUC activity represents the ratio between the test (LUC) and the reference (GUS) plasmids.

**(A)** *LAT52 promoter fusions.* The fragment PS1 corresponds to the (−100 to −55) sequence of the *LAT52* proximal promoter containing the 52/56 box (ovals) and the wild-type regions corresponding to 52LS1 (white boxes), 52LS2 (lightly stippled boxes), and 52LS3 (heavily stippled boxes). The PS1 fragment was fused in the wild-type orientation in 52PS1 and in the opposite direction in 52PS1r. The PS2 fragment (−84 to −55) corresponds to the PS1 fragment in which the 52/56 box has been removed. PS2 is present in the wild-type orientation in 52PS2 and in the reverse orientation in 52PS2r.

**(B)** *LAT59* promoter fusions. The fragment PS1 corresponds to the (−115 to −69) sequence of the *LAT59* proximal promoter containing the 56/59 box (ovals) and the wild-type regions corresponding to 59LS1 (white boxes), 59LS2 (lightly stippled boxes), and 59LS3 (heavily stippled boxes). The PS1 fragment was fused in the wild-type orientation in 59PS1 and in 59PS1PS1, where it is present as a dimer, and in the opposite direction in 59PS1r. The PS2 fragment (−98 to −69) corresponds to the PS1 fragment in which the 56/59 box has been removed. PS2 is present in the wild-type orientation in 59PS2 and in the reverse orientation in 59PS2r.

We opted for transient assays because they have been previously described (Schöler and Gruss, 1984) and because they are free of the complications of position effects prevalent in transgenic plants. These in vivo competition experiments are based on the assumption that a *trans*-acting factor present in limiting quantities can be quenched by excess binding sites. When excess putative binding sites are cointroduced into the cell in the context of our standard transient functional assay, competition for *trans*-acting factor binding might consequently affect promoter activity.

We tested the ability of the 52PS2 30-bp sequence (−84 to −55; corresponding to LS123) to compete with the activity of the *LAT52* promoter. A multimer containing 12 copies of this sequence in direct repeat, coined C1, was used as the

competitor. We initially tried to cobombard the competitor sequence, cloned or uncloned, with the *LAT52–luc* fusion but were unsuccessful in obtaining competition using this approach. It is conceivable that conditions in which the test plasmid and competitor plasmid can be introduced into the same cell are not possible with particle bombardment because only 0.1% of the pollen grains exhibit expression (Twell et al., 1989b). We overcame this problem by combining the two sequences on the same plasmid. The competitor multimer (C1) was inserted downstream of the *LAT52–luc* gene fusion in sense and antisense orientations (p52C1.S and p52C1.AS). Plasmids containing a mutant multimer, coined M1, inserted in the same site served as a negative control in the competition experiments (p52M1.S and p52M1.AS). Figure 4A shows that wild-type competitor C1 (p52C1.S and p52C1.AS) caused a fourfold reduction in LUC activity, whereas the mutant competitor M1 (p52M1.S and p52M1.AS) did not lead to a significant change. Moreover, the competition levels were equivalent regardless of the orientation of C1 in the plasmid. We concluded that the wild-type *LAT52* LS123 region (52PS2) can compete with *LAT52* promoter activity, presumably by binding to at least one *trans*-acting factor in a sequence-specific manner. The orientation-independent results of this experiment add credence to our interpretation of the data.

The possibility that the same *trans*-acting factor(s) might be involved in the regulation of expression of *LAT59* was examined by competing *LAT59* promoter activity with multimers of 52PS2. The *LAT52* C1 and mutant M1 multimers were inserted downstream of the *LAT59–luc* fusion in p59ΔILUC in the antisense orientation (p59C1.AS and p59M1.AS). The construct containing the *LAT52* competitor (p59C1.AS) resulted in a threefold reduction in LUC activity relative to p59ΔILUC, whereas the construct containing the mutant competitor (p59M1.AS) did not lead to a significant change in activity (Figure 4B). This result suggests that the *LAT52* and *LAT59* proximal promoter regions are the target for a common *trans*-acting factor. A different interpretation of the data is that the C1 multimer negatively affects pollen expression of any gene cloned upstream of it. We therefore tested the specificity of the competition by competing the same C1 multimer with another pollen-specific promoter, *LAT56* (Twell et al., 1991). We observed no significant reduction in *LAT56* promoter activity with either C1 or M1 competitors (p56C1.AS and p56M1.AS, respectively; Figure 4C), suggesting that the competition we observed with the *LAT52* and *LAT59* promoters is indeed specific.

### GT-1 Is a Potential *trans*-Acting Factor for the 52/56 Box

The 52/56 box contains a 6-bp GT core motif (Twell et al., 1991) differing by only 1 bp from the transcription factor GT-1a consensus binding site (Gilmartin et al., 1992; Perisic and Lam, 1992). In a wider context of 16 bp, the 52/56 box differs from the tobacco GT-1 binding site by only 4 bp. To test the possibility that a GT-1 transcription factor binds the 52/56 box, we

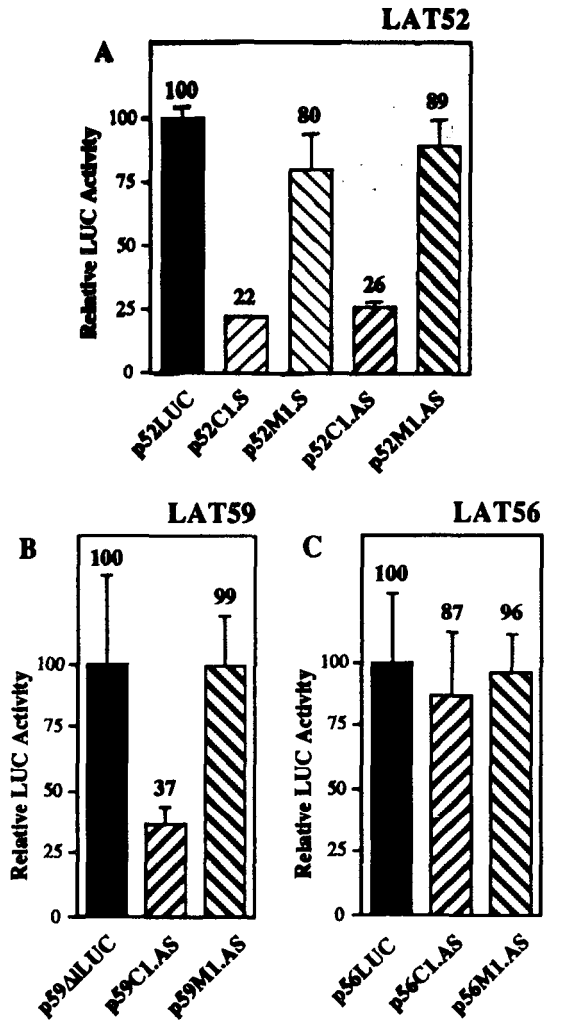


***Figure 4.*** *In Vivo Competition Analyzed in Transient Assay Experiments in Pollen.*

**(A)** Competition of the *LAT52* promoter with 52PS2 multimers. Transient LUC expression was obtained with plasmids containing multimers of the wild-type (C1) or mutant (M1) 52PS2 competitor sequences inserted in the sense (S) or antisense (AS) orientation downstream of the p52LUC fusion (p52C1.S, p52C1.AS, p52M1.S, and p52M1.AS, respectively).

**(B)** Competition of the *LAT59* promoter with 52PS2 multimers. Transient LUC expression was obtained with plasmids containing multimers of the wild-type (C1) or mutant (M1) 52PS2 competitor sequences inserted in an antisense orientation (AS) downstream of the p59LUC fusion (p59C1.AS and p59M1.AS, respectively).

**(C)** Competition of the *LAT56* promoter with 52PS2 multimers. Transient LUC expression was obtained with plasmids containing multimers of the wild-type (C1) or mutant (M1) 52PS2 competitor sequences inserted in an antisense orientation (AS) downstream of the p56LUC fusion (p56C1.AS and p56M1.AS, respectively).

Plasmids lacking competitor sequences (p52LUC, p59ΔILUC, and p56LUC) were used as positive controls (black bars). Results represent the mean of at least six independent transformations. Relative LUC activity (numbers above bars) represents the ratio between the test (*LUC*) and the reference (*GUS*) plasmids.

introduced these changes by site-directed mutagenesis and tested their effect on transient expression in pollen. Figure 5A shows that p52GT1, containing the four base pair changes, consistently resulted in a 1.3- to 1.5-fold increase in activity in the transient assays using pollen, whereas p52GG, mutated at the two G residues corresponding to the critical bases in the GT-1 core motif, resulted in a three- to fourfold reduction in activity (Figure 5A; Twell et al., 1991). To establish whether any GT motif could be substituted for the 52/56 box, we used the binding site of transcription factor GT-2 (Dehesh et al., 1990), which is structurally related to GT-1 but has a different binding site specificity. Alteration of the 52/56 box to a GT-2 binding site (p52GT2) resulted in a 2.5- to threefold decrease in promoter activity (Figure 5A), demonstrating that a specific GT motif is important. We also checked whether GT-1 transcripts were present in pollen by using reverse transcriptase–polymerase chain reaction (RT-PCR) with primers designed to amplify the region coding for the first 136 amino acids (Gilmartin et al., 1992). Figure 5B shows that GT-1 transcripts were detected in RNA isolated from mature pollen as well as from immature anthers. Several clones of this RT-PCR product were sequenced and found to correspond to two different but homologous genes, one of which is the previously described GT-1a (Gilmartin et al., 1992). This result demonstrates that GT-1a is part of a small GT-1 gene family. Taken together, these data support a possible involvement of a GT-1 family member in 52/56 box binding.

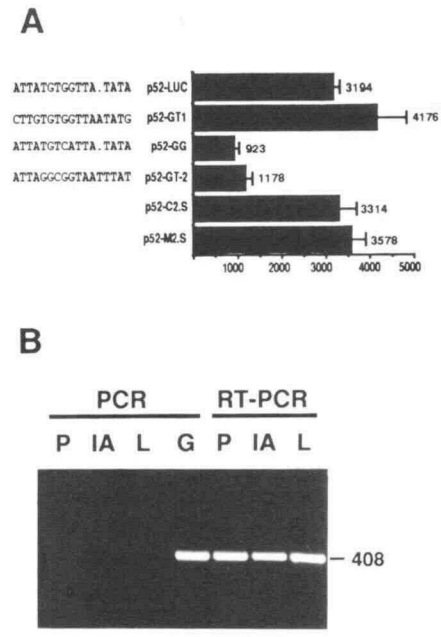


**Figure 5.** Evidence for the Involvement of GT-1 in the Expression of Genes in Pollen.

**(A)** Functional analysis and competition of the 52/56 box. All constructs are based on the wild-type plasmid p52LUC. p52GT1, p52GG, and p52GT2 were generated by site-directed mutagenesis of p52LUC at the 52/56 box region as indicated. p52C2.S and p52M2.S contain multimers (10 copies) of the wild-type 52/56 box (C2) and mutant version (M2), respectively, downstream of the termination/polyadenylation sequence. All constructs were introduced into pollen by particle bombardment, and their transient expression was assayed. Results represent the mean of at least six independent experiments. Relative LUC activity represents the ratio between the test (LUC) and the reference (GUS) plasmids.

**(B)** RT-PCR of GT-1 transcripts. Total RNA (DNA-free) from pollen (P), immature anthers (IA), and leaves (L) was analyzed for the presence of GT-1 transcripts by RT-PCR. Primers designed to yield a 408-bp fragment corresponding to the codons for the first 136 amino acids of GT-1 (Gilmartin et al., 1992) were used. Standard PCR lanes serve as a control for DNA contamination. Lane G represents a standard PCR using tobacco genomic DNA.

We then tested the ability of multimers of the 52/56 box to compete using the in vivo competition approach previously described. Multimers (10 copies) of the 52/56 box (wild type C2 or mutated M2) were inserted downstream of the termination/polyadenylation site in p52LUC (see Methods). Each resulting plasmid had a unidirectional 10mer in the sense orientation (p52C2.S and p52M2.S). When analyzed in a transient functional assay, p52C2.S showed no significant reduction in activity relative to p52LUC (Figure 5A). As expected, the mutated version, p52M2.S, had the same result. We concluded that the 52/56 box was not able to compete by quenching a putative *trans*-acting factor.

## DISCUSSION

To address the mechanism regulating tissue specificity, we compared the function of the various proximal promoter constructs using a transient assay system in both pollen and leaf cell culture. In a similar system, consisting of endosperm cell culture and leaf cell culture, Ueda et al. (1992) established that tissue-specific activation of a zein promoter is mediated by binding of the *opaque2* transcription factor. Both systems provide a rapid method to demonstrate tissue specificity. The data obtained by our analysis as well as results from transgenic plants harboring some of the constructs showed that pollen specificity is not due to transcriptional repression in tissues other than pollen but rather to a positive element(s) occurring in pollen. Similarly, some zein promoters have been found to be positively regulated in the endosperm of maize due to the endosperm-specific expression of the *opaque2* transcription factor (Ueda et al., 1992). By analogy, we would expect to find pollen-specific factors or pollen-specific combinations of factors involved in the regulation of expression of *LAT52* and *LAT59*.

We used linker substitution mutations in transient assays and transgenic plants to show that, in the context of the −100 promoter in *LAT52* or the −115 promoter in *LAT59*, short, defined proximal promoter sequences are essential for high-level expression in pollen: −84 to −55 in *LAT52* (corresponding to LS123), and −88 to −69 in *LAT59* (corresponding to LS23).

Furthermore, the sequences corresponding to LS123 of each promoter could confer pollen specificity as well as high levels of expression to a heterologous CaMV 35S core promoter. The pollen-specific expression of these gain-of-function fusions demonstrates that sufficient information for maintaining pollen specificity is contained in a short sequence no longer than 30 bp. The extremely high levels of expression in pollen (more than 500-fold for *LAT52* and more than 50-fold for *LAT59*) further support this conclusion. Such high levels of tissue-specific activation conferred by monomers of a short sequence are among the highest reported in plants. Notably, the 52/56 box and 56/59 box sequences are not included in the minimal 30 bp that confers high-level pollen-specific expression. Thus, as previously noted (Twell et al., 1991), the 52/56 and 56/59 boxes contribute positively to expression levels in pollen but are not essential components for maintaining pollen specificity.

Most tissue-specific promoters studied thus far in plants have been analyzed by a loss-of-function (deletion analysis) approach. As a result, *cis* elements delimited by these experiments could not be positively identified as tissue specificity elements rather than general enhancers. We used a combination of loss-of-function and gain-of-function approaches to identify definitively sequences involved in regulating pollen specificity. Great care must be taken in designing gain-of-function experiments to prevent artifactual interactions. Because the −90 CaMV 35S promoter contains an activation sequence factor-1 (ASF-1) binding site that has been shown to act synergistically with other regulatory elements (Benfey et al., 1990), we opted to use the −64 CaMV 35S promoter because it contains no *cis*-acting elements. Therefore, the expression pattern of the chimeric constructs would reflect the activity of the fused regulatory sequences alone.

The results presented here extend the previously published data showing that tissue-specific elements are maintained in the promoter proximal regions (Twell et al., 1991). Our data do not preclude the possibility that redundant or additional tissue-specific elements occur upstream, although at least some of the upstream elements are involved merely in modulating the level of expression of these genes. One of these elements, the redundant 52/56 box, was previously noted (Twell et al., 1991) to share sequences with the GT-1 transcription factor binding site (Gilmartin et al., 1992). We now know that GT-1 consists of a small family of similar transcription factors (data not shown). The observation that the 52/56 box can be specifically altered to create a GT-1 binding site without reducing promoter activity supports the notion that a GT-1 family member might be involved in *LAT52* expression. Finding GT-1 transcripts in mature pollen and in immature anthers further supports this notion. Whereas the functional assays suggested a positive function for the 52/56 box, in vivo competition experiments did not provide data supporting binding to this sequence. Multimers of the 52/56 box could not compete to reduce the level of pollen-specific expression directed by the −100 promoter of *LAT52*. This finding does not preclude binding of a *trans*-acting factor to the 52/56 box but suggests that the factor is present at nonlimiting levels, that its binding requires the presence of another factor, or that it can function as a transactivator even when bound to a distant downstream location (in the multimer).

We have provided evidence that a *trans*-acting factor can be quenched by multimer competition using the sequence corresponding to LS123 of *LAT52*, indicating that it is present at limited levels. The significance of limiting levels of transcription factors in controlling tissue-specific gene expression has been previously noted in regard to the −90 promoter of the CaMV 35S gene containing the activation sequence-1 (*as-1*) *cis* element (Neuhaus et al., 1994). The *as-1* element has been shown to bind the transcription factor TGA1a, which is present in roots and in leaves, albeit at a 10-fold lower level. Interestingly, the *as-1*–controlled transgene is expressed in roots but not in leaves, suggesting that in leaves this *cis* element cannot compete for binding to limiting levels of TGA1a in the presence of other presumably more favorable binding sites. In roots, TGA1a levels are not limiting, and therefore the *as-1* transgene is expressed. Analogously, it seems that limiting levels of a pollen-expressed transcription factor that binds the pollen specificity region of *LAT52* may be involved in the regulation of a subset of pollen-specific genes.

The coordinate pattern of expression of the *LAT52* and *LAT59* genes suggests that a common regulatory mechanism might be involved in the transcriptional activation through binding of a common *trans*-acting factor(s). This possibility is supported by the similarity in promoter organization between the two genes in which proximal elements determine tissue specificity. The finding that multimers of the *LAT52* proximal promoter can cross-compete with an *LAT59* promoter suggests that a common *trans*-acting factor is involved. When examining potential binding sites for a common *trans*-acting factor, we noted only limited sequence similarities in *LAT52* and *LAT59* proximal promoter regions, as shown in Figure 6. Most notably, the sequence ACTGT is present at almost exactly the same position relative to the transcription initiation site in *LAT52* (−80) and *LAT59* (−79) and is absent in *LAT56*. This sequence occurs in one of the regions of the promoters shown by LS mutations to have the strongest effect on expression in pollen (LS1 in *LAT52*, and LS2 and LS3 in *LAT59*). The palindromic nature of this sequence could potentially explain the orientation-independent activation we observed in the gain-of-function fusions. Whether this motif is in fact a target for a common *trans*-acting factor remains to be determined. Other common *cis*-acting elements may exist; their functional similarity may be less obvious at the sequence level. Indeed, the recognition of variable *cis* elements has been noted for the maize *opaque2* transcription factor, which binds to completely different sequences in α-zein genes and in a *b-32* albumin gene (Yunes et al., 1994).

We have defined 30 bp in the proximal promoter regions that confer high-expression pollen specificity in *LAT52* and *LAT59* and have shown evidence suggesting that they both interact with a common *trans*-acting factor. We can now pursue *trans*-acting factors, toward the goal of further understanding how tissue specificity is regulated in pollen. We are currently

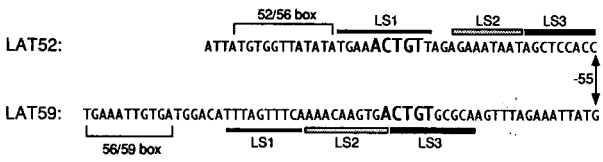


**Figure 6.** Sequence Comparison between the *LAT52* and *LAT59* Proximal Promoter Regions.

The positions of the sequences relative to the transcription start site are shown by the double-headed arrow. Boldface letters indicate sequence elements conserved between the *LAT52* and *LAT59* proximal promoters. The positions of the corresponding LS mutations as well as the 52/56 and 56/59 boxes are indicated.

using a yeast genetic selection system (Wang and Reed, 1993) to screen a pollen cDNA library for genes encoding proteins that are able to bind specifically to the *LAT52* and *LAT59* pollen specificity regions.

## METHODS

### Construction of Plasmids

p52LUC, which contains 100 bp of promoter sequence and the entire 5′ untranslated region (5′ UTR; −100 to +110), was created by a translational fusion at the translation initiation codon (via an NcoI site) of the luciferase (*luc*) open reading frame that was fused to a cauliflower mosaic virus (CaMV) 35S termination/polyadenylation sequence. p59ΔILUC contains 115 bp of the promoter sequence and only 10 bp of the 5′ UTR (−115 to +10) fused at the translation initiation codon (via an NcoI site) to the *luc* open reading frame and the CaMV 35S termination/polyadenylation sequence. p56LUC, which contains 164 bp of the promoter sequence and the entire 5′ UTR (−164 to +191), was created by a translational fusion at the translation initiation codon (via an NcoI site) of the *luc* open reading frame that was fused to a CaMV 35S termination/polyadenylation sequence. p35SLUC contains 500 bp of the CaMV 35S promoter sequence (−500 to +1) transcriptionally fused to the *luc* gene and a CaMV 35S termination/polyadenylation sequence. All of these translational fusions were cloned into pBluescript KS+ (Stratagene).

Linker substitution mutations for transient assays were created by site-directed mutagenesis (Kunkel et al., 1987) followed by replacing the wild-type late anther tomato *LAT52* sequence in p52LUC or the *LAT59* sequence in p59ΔILUC with the mutated sequence. Linker substitutions for plant transformation were created by replacing the *luc* reading frame in the aforementioned plasmids with an *Escherichia coli uidA* reading frame and subcloning the new translational fusion into pBin19 (Bevan, 1984).

A CaMV 35S core promoter (pJO64) was generated by a 5′ deletion of CaMV 35S promoter sequences up to position −64 in the plasmid pJO62 (Ow et al., 1987), resulting in an XhoI site at this position. Heterologous promoter fusions were created by inserting polymerase chain reaction (PCR) products or synthetic double-strand oligonucleotides into the XhoI site of pJO64.

Competition plasmids and their corresponding control plasmids were created by the directional cloning of multiple copies of the competitor sequence (or its mutated variant) downstream of the terminator/polyadenylation site in p52LUC, p56LUC, or p59ΔILUC in a sense (S) or antisense (AS) orientation. The following oligonucleotide pairs were used to create the multimers: for C1, 5′-CATGAAACTGTTAGAGAAATAATAGCTCCAC-3′ and 5′-CATGGTGGAGCTATTATTTCTCTAACAGTTT-3′; for M1, 5′-CATGAGCAGACCGTCAGCCAGCCTAACTAGC-3′ and 5′-CATGGCTAGTTAGGCTGGCTGACGGTCTGCT-3′; for C2, 5′-CATGATTATGTGGTTATATATGTTATGTGGTTATATATGC-3′ and 5′-CATGGCATATATAACCACATAACATATATAACCACATAAT-3′; for M2, 5′-CATGATTATACATCGATATATGTTATACATCGATATATGC-3′ and 5′-CATGGCATATATCGATGTATAACATATATCGATGTATAAT-3′.

The plasmids p52GT1, p52GG, and p52GT2 are mutated versions of p52LUC. The specific mutations in the 52/56 box were introduced by site-directed mutagenesis (Kunkel et al., 1987).

All the aforementioned constructions were confirmed by sequencing (Sequenase version 2.0 from U.S. Biochemical Corp.).

### Plant Transformation and Analysis of Transgenic Plants

Constructs cloned into pBin19 were introduced into tomato (*Lycopersicon esculentum* cv VF36) by *Agrobacterium tumefaciens* LBA4404 as previously described (McCormick, 1991b). At least 20 independent transformants were obtained for each construct.

For β-glucuronidase (GUS) assays, 5 to 20 μL of pollen, pooled from several flowers of the same plant, was ground directly in Eppendorf tubes in 50 to 100 μL of GUS extraction buffer (Jefferson et al., 1987) using a Teflon-tipped homogenizer driven by a drill. Expression in pollen was measured by fluorometrically assaying GUS activity in supernatants of pollen extracts using 2 mM 4-methylumbelliferyl β-D-glucuronide (Sigma) as substrate (Jefferson et al., 1987). GUS activity was corrected for variation in total protein content using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Expression in leaves, flowers, stems, roots, and seed was tested histochemically by staining with 5-bromo-4-chloro-3-indolyl β-D-glucuronide (Molecular Probes, Eugene, OR) as described previously (Jefferson et al., 1987). Expression in leaves was also analyzed fluorometrically as given previously.

### Transient Transformation of Tobacco Pollen and Cell Culture

Pollen spread out as a monolayer was bombarded essentially as previously described (Twell et al., 1991), except that gold was substituted for tungsten and only 1 μg of test plasmid was used per plate. TXD cell culture (maintained as described by Howard et al. [1992]) was spread out similarly as a monolayer (1 mL of a 50-mL stationary culture per plate) and bombarded as previously described. Between six and 12 independent bombardments were performed for each construct. In each experiment, the test plasmid was cobombarded with a reference plasmid: pBI223 (Clontech, Palo Alto, CA) was used for assays of all constructs in tobacco cell culture; pLAT59-12 (Twell et al., 1990) for assays of *LAT52* and *LAT56* constructs in tobacco pollen; pLAT56-12 (Twell et al., 1990) for assays of *LAT59* constructs in tobacco pollen. Processing of the tissue after ~15 to 17 hr and analysis of GUS and LUC activity were as described previously (Twell et al., 1991). Transient expression was reported as "relative LUC activity," which represents the ratio between the test (LUC) and the reference (GUS) plasmids.

### Reverse Transcriptase–PCR

Total RNA was isolated from tobacco pollen, leaves, and immature anthers as described by Logemann et al. (1987) and treated with DNase

to prevent DNA contamination (Dilworth and McCarrey, 1992). The RNA samples were subjected to reverse transcription followed by PCR using the rTth reverse transcriptase RNA PCR kit (Perkin-Elmer Cetus). Conditions were as described by the supplier. PCR control reactions were done by excluding the 3′ primer at the reverse transcription step and adding it together with the 5′ primer at the PCR step.

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