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Pollen-specific gene expression in transgenic plants: coordinate regulation of two different tomato gene promoters during microsporogenesis

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Summary

To investigate the regulation of gene expression during male gametophyte development, we analyzed the promoter activity of two different genes (LAT52 and LAT59) from tomato, isolated on the basis of their anther-specific expression. In transgenic tomato, tobacco and *Arabidopsis* plants containing the LAT52 promoter region fused to the β-glucuronidase (GUS) gene, GUS activity was restricted to pollen. Transgenic tomato, tobacco and *Arabidopsis* plants containing the LAT59 promoter region fused to GUS also showed very high levels of GUS activity in pollen. However, low levels of expression of the LAT59 promoter construct were also detected in seeds and roots. With both constructs, the appearance of GUS activity in developing anthers was correlated with the onset of microspore mitosis and increased progressively until anthesis (pollen shed). Our results demonstrate co-ordinate regulation of the LAT52 and LAT59 promoters in developing microspores and suggest that the mechanisms that regulate pollen-specific gene expression are evolutionarily conserved.

Key words: *Lycopersicon esculentum*, microsporogenesis, pollen-specific expression, reporter genes, transgenic plants.

Introduction

Male gametogenesis is a highly regulated developmental process that is essential for the reproductive success of both plant and animal species. The differentiation of sporocytes, meiotic cell division and the maturation of the haploid gametes are universal events in both systems, which rely on interactions between diploid and haploid cells. The study of these coordinated events at the molecular level is an important step towards understanding the process of gametogenesis that also provides a valuable system in which to study cell-specific gene expression. In plants, it is well established that the microgametophytic generation (pollen) expresses a number of unique mRNAs, several of which have been cloned (reviewed in Mascarenhas, 1988, 1989). In animal systems, transcriptional and translational regulation of genes expressed specifically during gametogenesis have been demonstrated (Peschon *et al.* 1987; Kuhn *et al.* 1988; Braun *et al.* 1989).

Our interest is in the development of angiosperm pollen, whose role is the production and delivery of two haploid sperm cells to the ovary where double fertilization occurs. The study of pollen development and function has provided insight into several developmental processes unique to male gametophytes, such as the asymmetric cell division of the uninucleate microspore (Sunderland and Huang, 1987), the growth of the pollen tube by apical extension (for review, see Heslop-Harrison, 1987) and cell–cell recognition in pollen–pistil interactions (for review, see Harris *et al.* 1984). Although major cytological and biochemical events that accompany pollen development have been well studied, relatively little information is available concerning the regulation of such events at the molecular level (for reviews see Mascarenhas, 1988, 1989).

The differentiation and development of pollen depends partly upon transcription of the haploid genome following meiosis (reviewed in Mascarenhas, 1988). Although there is extensive overlap (60–90%) between genes expressed in pollen and those in vegetative tissues, a unique set of genes is expressed in pollen (Willing and Mascarenhas, 1984; Willing *et al.* 1988). We and others have used cDNA cloning to characterize mRNAs corresponding to such genes (McCormick *et al.* 1987; Stinson *et al.* 1987; Hanson *et al.* 1989). Recently, we reported the primary structure of an anther-specific gene from tomato (LAT52) that was abundantly expressed during pollen maturation (Twell *et al.* 1989*b*). This gene is single copy in the tomato genome and shows significant sequence homology to a pollen-specific cDNA clone isolated from maize (Hanson *et al.* 1989), although the function of neither gene is known. We have also isolated a different single-copy gene (LAT59) from tomato that corresponds to a second anther-specific cDNA clone. The predicted protein of LAT59 shows significant amino acid similarity with the pectate lyases of the plant pathogen *Erwinia* (Wing *et*

al. 1989). The aim of the present study was to determine whether 5′ flanking DNA sequences alone are sufficient to direct gene expression in an anther-specific manner.

Here we demonstrate that 1.4 kb 5′ flanking DNA of LAT59 and 0.6 kb 5′ flanking DNA of LAT52 are sufficient to direct gene expression in an essentially pollen-specific manner. This is the first report describing the delimitation of *cis*-acting sequences required for specific expression in the male gametophyte of angiosperms. These sequences provide the ability to differentially direct gene products to developing pollen, and facilitate the further analysis of *cis* and putative *trans*-acting factors that mediate pollen-specific expression.

Materials and methods

Plant material and transformation

Tobacco

Leaf discs of *Nicotiana tabacum* cv. Samsun were transformed with *Agrobacterium tumefaciens* essentially according to Horsch *et al.* (1985). Shoots arising from leaf discs were rooted on agar solidified MSO medium (McCormick *et al.* 1986) containing 100 $\mu g\, ml^{-1}$ kanamycin, transferred to soil and grown to maturity under greenhouse conditions.

Tomato

Cotyledons of *Lycopersicon esculentum* cv. VF36 were transformed essentially as described in McCormick *et al.* (1986) with the following modifications: acetosyringone (75 μM) was added to the diluted *A. tumefaciens* culture for the two day co-cultivation period, in place of the tobacco feeder layer; MSO medium containing 1 $mg\, l^{-1}$ zeatin was used for shoot induction and MSO containing 0.1 $mg\, l^{-1}$ zeatin was used for shoot regeneration.

Arabidopsis

Arabidopsis thaliana (landrace No-O) roots were transformed essentially as described by Valvekens *et al.* (1988) and transgenic plants selected on medium containing 50 $\mu g\, ml^{-1}$ kamamycin.

DNA manipulations and chimeric gene construction

The isolation and partial characterization of EMBL3-derived genomic clone gLAT59 from tomato cv. VF36 is described elsewhere (Wing *et al.* 1989). The 1.4 kb *Sst*I–*Hpa*II DNA fragment from gLAT59 containing 5′ flanking DNA of the LAT59 gene, including 91 nucleotides of 5′ untranslated leader, was ligated into *Sst*I-*Acc*I digested pGEM7zf (Promega). This fragment was excised as a 1.4 kb *Eco*RI-*Pst*I fragment and subcloned into pBluescript (+)KS (Stratagene) to make pLAT59-3. A transcriptional fusion of LAT59 5′ flanking DNA with coding DNA of the *Escherichia coli* β-glucuronidase gene (GUS) was made by ligating the 1.4 kb *Eco*RI-*Pst*I promoter fragment from pLAT59-3 into pGP213 to make pLAT59-12 (Fig. 1B). Plasmid pGP213 contains the GUS coding DNA linked to the nopaline synthase 3′ region (*NOS3′*) on pUC19; the *GUS–NOS3′* fragment is identical to that in pBI101.1 (Jefferson *et al.* 1987). A transcriptional fusion of the LAT59 5′ flanking DNA to coding DNA of the firefly luciferase gene (LUC) was made by ligating the 1.4 kb *Hin*dIII–*Pst*I promoter fragment from pLAT59-3 into pGP214 to make pLAT59-13 (Fig. 1B). Plasmid pGP214 contains the LUC coding DNA linked to *NOS3′* on pUC19.

A transcriptional fusion of the 5′ flanking DNA of LAT52 (Twell *et al.* 1989*b*) to *GUS–NOS3′* was constructed. An *Nco*I restriction site was introduced at the initiator methionine codon of the LAT52 gene by oligonucleotide-directed mutagenesis using single-stranded DNA as template and an Amersham mutagenesis kit according to the manufacturer's instructions. The 5′ flanking DNA including the entire 5′ untranslated leader was excised from this plasmid as a 0.6 kb *Sal*I–*Nco*I fragment and inserted into plasmid pDAT1 to create pLAT52-7 (Fig. 1B). Plasmid pDAT1 was essentially the same as pGP213 except that it contained an *Nco*I site at the initiator methionine codon and was derived from pRAJ275 (Jefferson *et al.* 1986).

The chimeric gene fusions on plasmids pLAT59-12 (*59-GUS–NOS3′*), pLAT59-13 (*59-LUC–NOS3′*) and pLAT52-7 (*52-GUS–NOS3′*) were cloned into binary vector pBIN19 (Bevan, 1984) as follows: a 3.5 kb *Eco*RI fragment containing *59-GUS–NOS3′* was ligated into pBIN19; pLAT59-13 was linearized with *Hin*dIII and ligated into pBIN19, and a 2.7 kb *Sal*I–*Eco*RI fragment containing *52-GUS–NOS3′* was ligated into pBIN19. Binary vectors were transferred to *A. tumefaciens* strain PC2760 (pAL4404) (An *et al.* 1985) by direct transformation of competent *A. tumefaciens* as described by An *et al.* (1988).

RNA isolation and Northern blot analysis

RNA isolation, Northern blotting and hybridization analysis were performed as described in Twell *et al.* (1989*b*).

GUS assays

Histological. Whole root segments, pollen or hand-sections of anthers or seeds cut with a razor blade were placed in the wells of a microtiter dish containing 100–300 μl of reaction buffer. The reaction buffer contained 1 mM X-Glu (5-bromo-4 chloro-3 indolyl-β-D-glucuronic acid, Research Organics) in 0.1 M $NaPO_4$ pH 7.0. Individual wells of the microtiter dish were sealed with parafilm and incubation carried out at room temperature for 4 h (anther sections and pollen) or for 16 h (root segments and seed sections).

Fluorimetric. GUS activity was measured in plant extracts essentially as described by Jefferson *et al.* (1987) with some modifications. Plant tissue was ground (4 $ml\, g^{-1}$ fwt tissue) with a mortar and pestle on ice after addition of GUS buffer (50 mM $NaPO_4$ (pH 7.0), 10 mM 2-mercaptoethanol, 1 mM EDTA and 0.1 % triton X-100). The extract was centrifuged at 13 000 revs min^{-1} for 10 min at 4°C in a microfuge tube and an aliquot (2–100 μl) of the supernatant assayed with 1 mM 4-methyl umbelliferyl glucuronide at 37°C. Fluorescence of the reaction product, 4-methyl umbelliferone (4-MU), was measured with a Sequoia-Turner model 112 digital filter fluorometer using excitation and emission wavelength ranges of 320–390 nm and 430–490 nm, respectively. The specific activity of GUS in each sample was calculated as nmol 4-MU formed $h^{-1}\, mg^{-1}$ protein.

Protein concentration in plant extracts was determined using Bradford reagent (BioRad Laboratories) according to the manufacturers instructions.

Luciferase assays

Plant tissue extracts for luciferase assays were prepared as described above for GUS assays except the extraction buffer was 0.1 M KPO_4 (pH 7.5) containing 1 mM dithiothreitol. Assays were performed essentially as described by Ow *et al.* (1986). Total light emission was measured during 10 s after addition of D-luciferin (Sigma) to the extract using a monolight model 2001 luminometer (Analytical Luminescence

Laboratory Inc.) and luciferase activity calculated as light units emitted s^{-1} μg^{-1} protein.

Results

We have previously described the isolation and anther-specific expression of the gene LAT52 from tomato (Twell *et al.* 1989*b*). LAT52 mRNA accumulates late during anther development and the amount is maximal in mature pollen (Twell *et al.* 1989*b*). LAT52 mRNA is also localized within sporophytic tissues of the anther by *in situ* hybridization but is only detectable after the appearance of the mRNA in maturing pollen grains (Ursin *et al.* 1989). The Northern blot (Wing *et al.* 1989) and *in situ* hybridization (Ursin *et al.* 1989) analyses of the expression of a different gene (LAT59) from tomato gave essentially identical results to those obtained for LAT52. The isolation and characterization of the LAT59 gene and a corresponding cDNA clone is described elsewhere (Wing *et al.* 1989). A partial restriction map of genomic clone gLAT59 showing the organization of the LAT59 gene is presented in Fig. 1A.

To determine whether 5′ flanking sequences of LAT59 and LAT52 were sufficient to direct pollen-specific expression, three transcriptional gene fusions were constructed utilizing genes encoding the reporter enzymes β-glucuronidase (GUS) (Jefferson *et al.* 1986) and luciferase (LUC) (Ow *et al.* 1986). These gene fusions (*59-GUS–NOS3′*, *59-LUC–NOS3′* and *52-GUS–NOS3′*; Fig. 1B) were transferred to a disarmed *A. tumefaciens* strain on a binary vector, used to infect tomato, tobacco and *Arabidopsis* explants and transgenic kanamycin resistant (Km^r) plants regenerated.

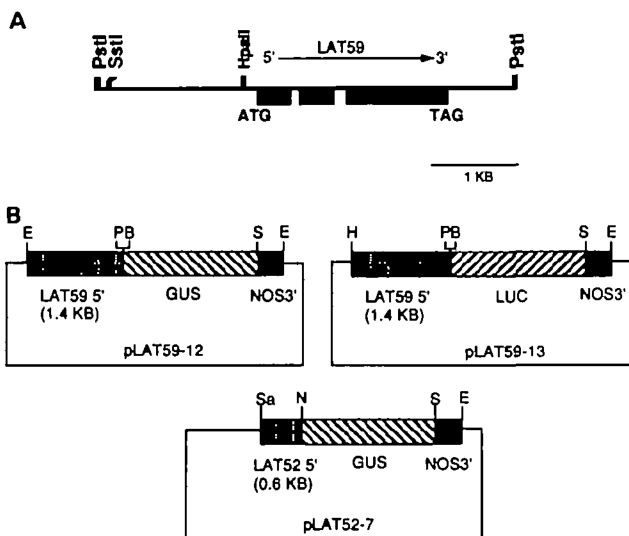

Fig. 1. Diagram of LAT59 and gene fusion constructs. (A) Partial restriction map of the LAT59 gene showing coding DNA regions (solid boxes) and introns (gaps in boxes). Translational start and stop codons are indicated as ATG and TAG respectively. Restriction enzyme sites are shown above the line. (B) Gene fusions *59-GUS–NOS3′* (pLAT59-12), *59-LUC–NOS3′* (pLAT59-13) and *52-GUS–NOS3′* (pLAT52-7) introduced into binary vector pBIN19. GUS and LUC indicate the coding DNAs of the β-glucuronidase and luciferase genes, respectively. NOS3′ indicates 3′ flanking DNA of the nopaline synthase gene. Further details are described in Materials and methods. B=*Bam*HI, E=*Eco*RI, H=*Hin*dIII, *N*=*Nco*I, P=*Pst*I, Sa=*Sal*I and S=*Sst*I.

Two independent assays were used to localize GUS activity in tissues of transgenic plants: (1) a histochemical assay of anther sections incubated in the indigogenic substrate X-Glu and (2) a quantitative (fluorimetric) assay of crude extracts (Jefferson *et al.* 1987). The use of both assays was important because Plegt and Bino (1989) have reported blue staining with X-Glu in untransformed anthers and pollen of several species. Under our assay conditions (1 mM X-Glu at room temperature for 4 h), no histochemical staining of pollen from untransformed tobacco, tomato and *Arabidopsis* plants was detected, whereas transgenic pollen stained bright blue within one hour. However, incubation of untransformed tomato pollen for up to 16 h gave a faint blue staining reaction which was not detectable using the fluorimetric assay (data not shown). In seeds from untransformed tomato plants, a higher background staining reaction was evident after overnight incubation in X-Glu (see below). This apparent GUS activity was clearly detectable in untransformed seeds using the fluorimetric assay, but the fluorometric assay could be used to detect additional activity due to the introduced GUS construct (Fig. 2D).

Tissue specificity of the LAT52 and LAT59 promoters

(1) *LAT52 promoter*

Five Km^r tomato plants resulting from transformation with *A. tumefaciens* containing the gene fusion *52-GUS–NOS3′* were analyzed for GUS activity. These plants showed high levels of GUS activity in extracts from mature anthers, with essentially no GUS activity in other vegetative or floral organs, including fruit pericarp and seeds (data not shown). The results of GUS assays performed on a single representative transgenic tomato plant (VF52G-435) are presented in Fig. 2A. Eight Km^r tobacco plants containing the *52-GUS–NOS3′* gene fusion showed similar tissue specificity; the results of GUS assays performed on a single representative transgenic tobacco plant (ST52G-8) are presented in Fig. 2B. Four *Arabidopsis* plants containing the *52-GUS–NOS3′* gene fusion were assayed histochemically for GUS activity. Blue staining was detected only in pollen (Fig. 3K), with no staining in other tissues (not shown).

(2) *LAT59 promoter*

Eight transgenic tomato plants containing from one to four copies of T-DNA (data not shown) harboring the *59-GUS–NOS3′* fusion were analyzed for GUS activity. High levels of GUS activity were detected in mature anthers of all of these plants, with approximately 30-fold variation between independent transformants. There was no correlation between the level of GUS activity and T-DNA copy number among the eight transformants (data not shown). GUS activity in all other organs except roots and seeds was only slightly

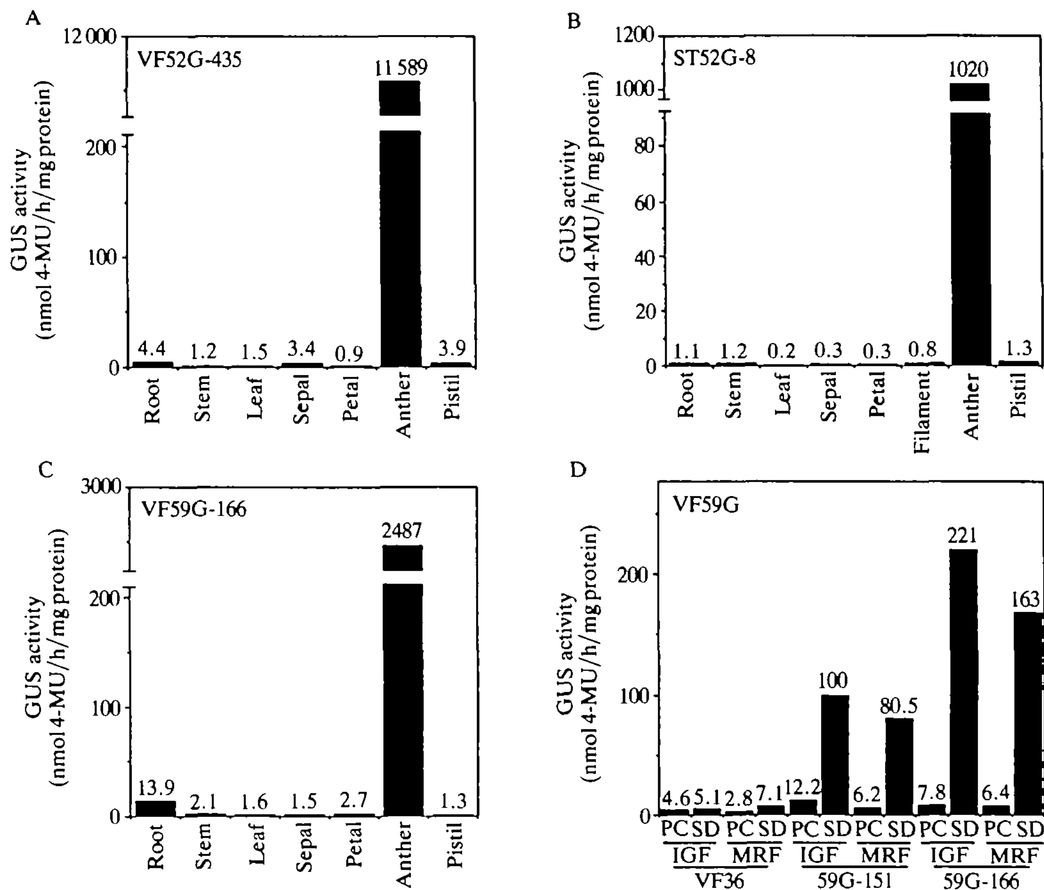

Fig. 2. GUS activity in vegetative and reproductive organs of transgenic plants. Assays were performed on extracts of mature organs of R_0 plants except for roots, where extracts were made from R_1 Km^r seedlings 3 weeks after germination. The results represent the mean activity from at least 3 independent assays of the same tissue (S.D. between experiments was <26%). In panels A–C, background GUS activity in extracts from untransformed plants was subtracted from GUS activity in transgenic plants. (A) GUS activity in transgenic tomato plant VF52G-435 containing gene fusion *52-GUS–NOS3'*. (B) GUS activity in transgenic tobacco ST52G-8 containing gene fusion *52-GUS–NOS3'*. (C) GUS activity in transgenic tomato VF59G-166 containing gene fusion *59-GUS–NOS3'*. (D) GUS activity in immature green fruit (IGF) and mature red fruit (MRF) of transgenic tomato plants VF59G- 151 and VF59G-166, and in untransformed tomato (VF36). Pericarp (PC) and seeds (SD) were assayed separately.

elevated (2- to 3-fold) above the background levels detected in extracts of untransformed tomato plants. In roots harvested from R_1 (progeny from selfed primary (R_0) transformants) Km^r seedlings, low but significant levels of GUS activity were detected. The activity in roots was 100- to 500-fold lower than that detected in mature pollen. These data are summarized in Fig. 2C, which shows the results of GUS measurements for a representative transgenic tomato plant (VF59G-166) containing the *59-GUS–NOS3'* fusion. A transgenic tobacco plant containing a single copy of the *59-GUS–NOS3'* fusion showed a similar pattern of GUS activity (data not shown). GUS activity was detected in roots of R_1 Km^r seedlings derived from this plant, at approximately 50-fold lower levels than in mature pollen. Four *Arabidopsis* plants containing the *59-GUS–NOS3'* fusion were assayed histochemically for GUS activity. All of these plants showed intense blue staining in pollen, weak staining in roots (see below) but no staining in any other tissues (data not shown). Similarily, ten Km^r tobacco plants transformed with *A. tumefaciens* containing the gene fusion *59-LUC–NOS3'* showed high levels of LUC activity in mature anthers (data not shown). LUC activity in roots harvested from R_1 Km^r seedlings was approximately 60-fold lower than in mature pollen; LUC activity in extracts from other vegetative and floral organs was only slightly above background levels in untransformed tobacco plants (data not shown). The results obtained with both the GUS and LUC reporter genes show that coding DNA did not influence the tissue specificity of the LAT59 promoter.

GUS activity was assayed in extracts of pericarp and seeds from immature green fruit (IGF) and mature red fruit (MRF) of transgenic tomato plants VF59G-151 and VF59G-166. Significant levels of GUS activity were detected in immature and mature seeds of both transgenic tomato plants (Fig. 2D), representing approximately 30-fold lower levels than in mature pollen. Only very low levels (2- to 3-fold above background GUS activity levels in untransformed fruit) were detected in pericarp from transgenic IGF and MRF. Similar results

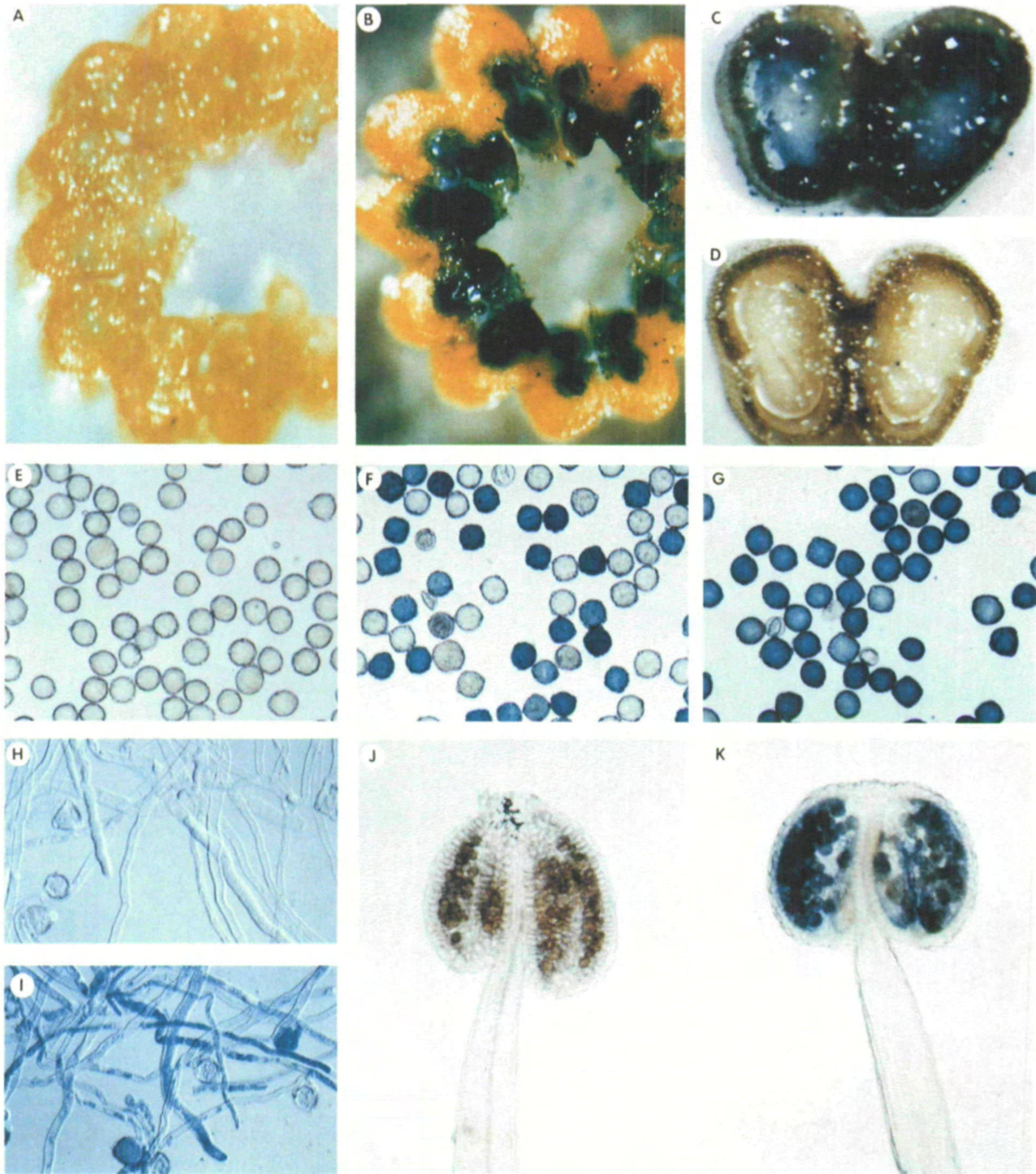

Fig. 3. Localization of GUS activity in transgenic plants. (A) Transverse section of an untransformed tomato anther treated with X-Glu (20×). (B) Transverse section of a mature anther of VF59G-166 stained with X-Glu (20×). (C) Transverse section of a mature anther of ST52G-8 stained with X-Glu (25×). (D) Same as C except pollen was washed out of the anther locules before staining (25×). (E) Pollen from untransformed tobacco treated with X-Glu (80×). (F) X-Glu stained pollen from a heterozygote R_1 plant derived from ST59G-11 (80×). (G) X-Glu stained pollen from a homozygote R_1 plant derived from ST59G-11 (80×). (H) Germinated pollen from untransformed tobacco treated with X-Glu (80×). (I) Germinated pollen from ST59G-11 stained with X-Glu (80×). (J) Stamen of *Arabidopsis* treated with X-Glu (160×). (K) Stamen of transgenic *Arabidopsis* containing gene fusion *52-GUS–NOS3'* stained with X-Glu (160×).

were obtained for two other independent transgenic tomato plants containing the *59-GUS–NOS3'* construct (data not shown).

Sporophytic activity of the LAT59 promoter

The localization of GUS activity in roots of transgenic tomato, tobacco and *Arabidopsis* plants containing the *59-GUS–NOS3'* fusion was examined after incubation in the histochemical substrate X-Glu. Independent transformants showed one of two patterns of staining; one pattern showed blue staining cells in a punctate pattern at sites distal from the tip of primary and lateral roots. The majority of staining cells were located in the root epidermis as evidenced by their elongate shape and the appearance of occasional blue staining root hair cells. No staining was evident in the root cap, root meristem or vascular tissues. The second pattern showed intense blue staining in the root cap cells with a lower intensity in the epidermis of both primary and lateral roots (data not shown). Roots from untransformed tomato, tobacco and *Arabidopsis* plants, control tobacco plants containing the promoterless *GUS–NOS3'* plasmid pBI101.1 (Jefferson *et al.* 1987) or tobacco and *Arabidopsis* plants containing the *52-GUS–NOS3'* fusion showed no blue staining cells after incubation with X-Glu (data not shown). Furthermore, roots of transgenic tomato and tobacco plants containing 0.8 kb of the CaMV35S promoter fused to GUS showed blue staining in all cells of the roots with intense staining in the root meristem (data not shown; also see Benfey *et al.* 1989).

Attempts to localize GUS activity histochemically in transgenic tomato seeds containing the *59-GUS–NOS3'* fusion were unsuccessful due to a high background blue staining in untransformed seeds when incubated in the presence of X-Glu. However, fluorimetric assays of extracts from dissected tissues clearly showed that the GUS activity in transgenic tomato seeds was present in both the embryo and the endosperm at similar levels (data not shown).

Pollen-specific activity of the LAT52 and LAT59 promoters in anthers of transgenic tomato, tobacco and Arabidopsis

Histochemical analysis of anthers containing the *59-GUS–NOS3'* or the *52-GUS–NOS3'* fusions showed blue staining only in pollen, with no staining in the sporophytic tissues of the anther or in other floral organs. (Fig. 3B, 3C, 3K and data not shown). Anthers of ST52G-8 that were washed free of pollen before incubation with X-Glu showed no blue staining except in remnant pollen grains (Fig. 3C, 3D). Quantitative analysis of GUS activity in anthers that were washed free of pollen showed that more than 99 % of the GUS activity in mature anthers was due to pollen (Table 1). The results were similar for transgenic tomato plant VF59G-166, and transgenic tobacco plants ST59G-11 and ST52G-8.

Was the remaining GUS activity in 'washed anthers' due to expression in the sporophytic tissues of the anther or due to remnant pollen grains? Microscopic examination showed that there were approximately 200 pollen grains remaining in a single tomato anther following washing. Based on this value, mature pollen from transgenic tomato plant VF59G-166 was added to 'washed anthers' of VF59G-166 or untransformed tomato, extracts made and GUS activity determined. The results presented in Fig. 4 show that GUS activity in 200–300 pollen grains was sufficient to account for the residual GUS activity in 'washed anthers' of VF59G-166. Thus, we conclude that the 'washed' sporophytic tissues of the anther do not contain significant levels of GUS activity. In contrast, tomato and tobacco plants containing the CaMV35S promoter fused to the GUS gene exhibit blue staining in sporophytic tissues of the anther when incubated in X-Glu (Plegt and Bino, 1989). This shows that sporophytic tissues of the anther are capable of expressing GUS activity detectable by histochemical staining; further this confirms that the restriction of LAT52- and LAT59-directed GUS activity to pollen is reflective of pollen-specific gene expression.

In situ localization of LAT59 and LAT52 transcripts in mature anthers of untransformed tomato gave significant hybridization signals in both the anther wall and in pollen (Ursin *et al.* 1989). Here, Northern blot analysis was used to quantitate this anther wall signal, and to

Table 1. *Localization of GUS activity in anthers of transgenic plants*

	GUS activity (nmol 4-MU h^{-1} mg^{-1} protein)		
Transgenic plant	Washed anthers	Unwashed anthers	Pollen
VF59G-166	8.3	2487	7307
ST59G-11	7.8	389	726
ST52G-8	24.8	1022	2546

Anthers were washed free of pollen ('washed anthers') and GUS activity determined in these, in 'unwashed anthers' and in pollen washed out from the anther locule.

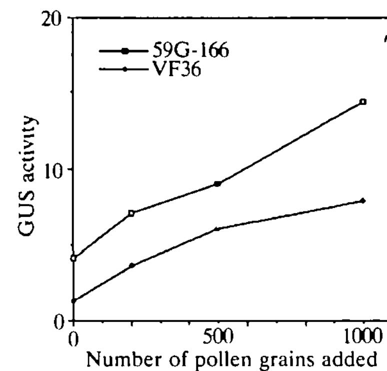

Fig. 4. Determination of pollen grain equivalents of GUS activity remaining in 'washed anthers'. GUS activity in single 'washed anthers' of untransformed tomato and VF59G-166, to which 0 200, 500 and 1000 pollen grains of VF59G-166 were added before extraction. Tomato plant VF59G-166 contained the gene fusion *59-GUS–NOS3'*. GUS activity is shown in fluorescence units per anther (defined as fluorescence/h of the total extract obtained from a single anther).

compare the localization of the endogenous LAT59 transcript with that of the gene fusion *59-GUS–NOS3'*. Total RNA extracted from anthers washed free of pollen ('washed' anthers), and intact ('unwashed') anthers from transgenic plant VF59G-166 was hybridized with probes specific for the GUS, LAT52 and LAT59 transcripts (Fig. 5). The results showed that steady state levels of LAT59 and LAT52 transcripts in 'washed' anthers were approximately 20-fold lower than in 'unwashed' anthers. In contrast, GUS transcript levels were undetectable in RNA isolated from 'washed' anthers of VF59G-166. These results indicate that the spatial pattern of expression of the introduced *59-GUS–NOS'* gene differs from the endogenous LAT59 gene specifically in the sporophytic tissues of the anther.

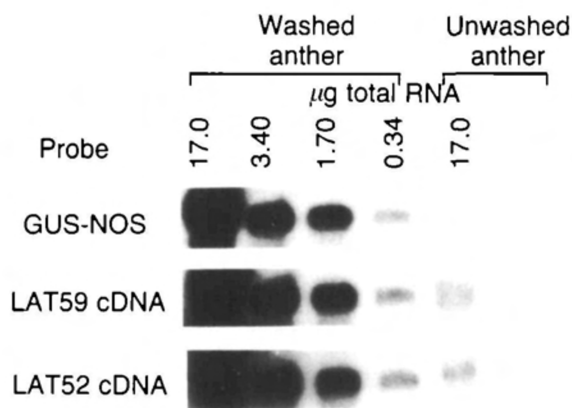

Fig. 5. Northern blot analysis of RNA in 'washed' and 'unwashed' anthers. Total RNA was extracted from 'washed' and 'unwashed' anthers of transgenic tomato plant VF59G-166. A Northern blot was prepared and probed separately with ^{32}P-labelled DNA fragments of GUS–NOS, LAT59 cDNA or LAT52 cDNA sequences. Comparable autoradiographic exposures are shown.

Co-ordinate regulation of the LAT52 and LAT59 promoters during microsporogenesis

To examine the temporal regulation of the LAT59 and LAT52 promoters during microsporogenesis, GUS activity was measured in extracts of anthers taken from tomato and tobacco flowers at different stages of development. Microscopic observation of anther squashes stained with the DNA fluorochrome DAPI (Coleman and Goff, 1985) allowed identification of the nuclear condition of the developing microgametophytes. Tomato anthers at the immature anther (IA) stage contained only tetrads and mononucleate microspores. At the green petal anther (GPA) stage, approximately 50 % of the microspores had undergone mitosis and were binucleate, and, at the mature anther (MA) stage, all of the pollen grains were binucleate. Tobacco 10 mm flower buds contained tetrads and some free mononucleate microspores, 15 mm buds contained greater than 90 % mononucleate microspores with some binucleate grains, and in 25 mm flower buds greater than 50 % of the microspores were binucleate.

Transgenic tomato plants containing the *52-GUS–NOS3'* fusion showed no GUS activity above background levels in anthers at the IA stage; GUS activity was detectable at the GPA stage and increased in anthers at the MA stage (Fig. 6A). Transgenic tomato plants containing the *59-GUS–NOS3'* fusion showed essentially the same pattern of GUS activity at the three stages of anther development examined (Fig. 6B). A more detailed developmental analysis was performed on anthers of transgenic tobacco plants containing the *52-GUS–NOS3'* or the *59-GUS–NOS3'* gene fusions. The results for plants containing either gene fusion were essentially identical (Fig. 6C, 6D). GUS activity was undetectable in anthers from 10 mm flower buds, detectable at low levels in anthers from 15 mm flowers and showed a sharp increase in anthers from 20 mm

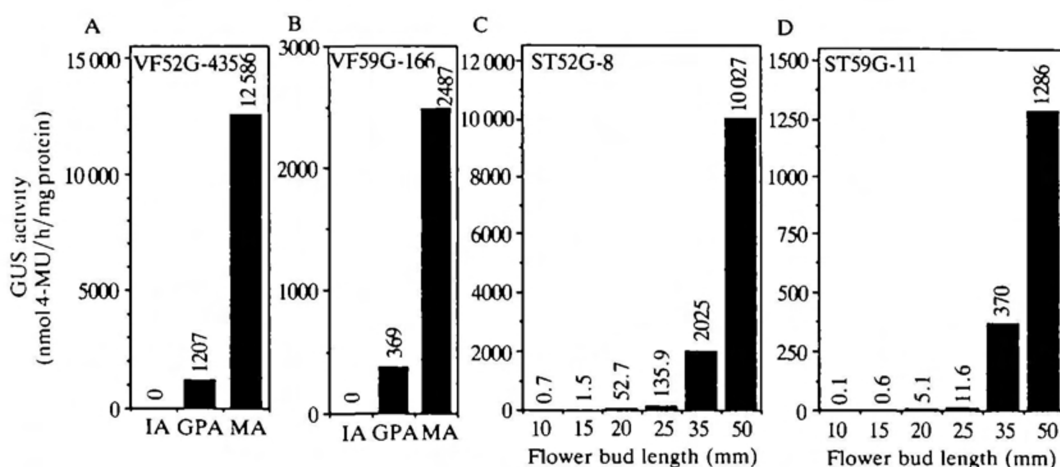

Fig. 6. GUS activity during anther development. (A,B) GUS activity in anthers of transgenic tomato plants VF52G-435 (A) and VF59G-166 (B). Whole anthers were extracted at the immature stage (IA), green petal stage (GPA) or the mature stage (MA). See text for details of microspore condition at each stage. (C,D) GUS activity in anthers of transgenic tobacco plants ST52G-8 (C) and ST59G-11 (D). Whole anthers were extracted from flowers where the bud length (from the top of the pedicel to the tip of the petal) was 10, 15, 20, 25, 35 or 50 mm. Flowers at the 50 mm stage were approximately one day pre-anthesis (pollen dehiscence). The results represent the mean activity from 3 independent assays (S.D. between experiments was <29 %).

flowers. Subsequently GUS activity increased approximately 1000-fold until anther dehiscence (50 mm stage; Fig. 6C, 6D). Using the histochemical substrate X-Glu, blue staining was first detected in GPA stage anthers for tomato, and in anthers at the 35 mm flower stage for tobacco plants containing either the *59-GUS–NOS3'* or the *52-GUS–NOS3'* constructs. This staining was localised to the developing pollen grains with no staining in sporophytic tissues of the anthers (data not shown). These data together with the results presented in Fig. 4, Fig. 5 and Table 1 suggest a strong correlation between the appearance of reporter enzyme activity and microspore mitosis. Furthermore the regulation of the LAT52 and LAT59 promoters (as measured by GUS activity) appears to be co-ordinate during anther development.

Genetic analysis

Five Km^r R_1 individuals derived from transgenic tobacco plant ST59G-11 which contained a single T-DNA copy were grown to maturity and pollen was incubated in the presence of X-Glu. Under our histological conditions (1 mM X-Glu at room temperature for 4 h) pollen from untransformed plants remained colorless. However, initially colorless grains in putative heterozygotes stained blue within 4 h. Mixing experiments suggested that this was due to leakage of the colorless reaction intermediate from GUS positive pollen into the buffer prior to its oxidation to the insoluble indigo. For this reason, heterozygotes were most easily scored at a pollen density of 1500 grains ml^{-1} of 1 mM X-Glu at 37 °C for 1 h. Under these conditions, there was a clear-cut difference between blue and white grains (Fig. 3F). Two plants showed 100 % blue-staining pollen and were considered to be homozygotes (Fig. 3G), while the remaining three plants showed approximately 50 % blue staining pollen and were considered to be heterozygotes (Fig. 3F). These conclusions were confirmed by screening the R_2 seedlings for Km resistance, since the GUS and Km^r markers were linked on the same T-DNA. Putative homozygotes gave 100 % Km^r R_2 seedlings while putative heterozygotes gave a ratio of approximately 3:1 for Km^r:Km^s seedlings. Similar results were obtained for transgenic tomato plant VF59G-151 which contained a single T-DNA locus, and for the *Arabidopsis* transformants (data not shown). These results demonstrate that transcription from the LAT59 promoter occurs post-meiotically and that histochemical screening of pollen can distinguish between heterozygotes and homozygotes for the introduced *59-GUS–NOS3'* gene. The observation that pollen from transgenic tobacco plant ST59G-11 germinated with a similar high efficiency as did untransformed tobacco pollen (Fig. 3H, 3I) indicates that expression of high levels of GUS activity in the mature pollen grain does not affect pollen viability. This is further supported by the normal genetic transmission of the introduced gene. Taken together these results show that the LAT–GUS fusions provide a useful tool to distinguish homozygotes for any introduced gene linked to the LAT59–GUS in the R_1 generation, obviating the need to score R_2 generation seedlings for the linked gene.

Discussion

One of our goals is to specifically direct gene products to the male gametophyte of angiosperms using regulatory sequences from pollen-specific genes. The introduction of the LAT52 and LAT59 promoter fusions into transgenic plants demonstrated that the LAT52 construct used was sufficient to direct expression in an essentially pollen-specific manner, while the LAT59 promoter directed high levels of expression in pollen, with low, but significant activity in some sporophytic tissues (discussed below). The specificity was independent of whether tomato, tobacco or *Arabidopsis* was transformed with these gene fusions. This suggests evolutionary conservation of the mechanisms that regulate the LAT52 and LAT59 promoters.

In general, the tissue-specific pattern of expression observed by Northern and *in situ* hybridization analysis (Twell *et al.* 1989*b*, Ursin *et al.* 1989; Wing *et al.* 1989) for the LAT52 and LAT59 genes was maintained in transgenic plants containing the promoter fusion constructs. However, there are three clear examples where there is an apparent lack of correlation between the expression of the endogenous gene and the introduced gene constructs. First, GUS activity was detected in seeds of transgenic tomato plants containing the *59-GUS–NOS3'* fusion. We were unable to detect the native LAT59 transcript in poly(A)+ RNA from untransformed tomato seeds, where the amount of LAT59 mRNA was at least 50-fold lower than the mature anther level (unpublished results). A similar discrepancy has been noted using the highly sensitive reporter gene β-galactosidase (*lacZ*) in plants (Teeri *et al.* 1989). These authors found that a chlorophyll a/b binding protein (cab) gene promoter fused to *lacZ* showed low levels of expression in the vascular cylinder of the root, when cab mRNA had not previously been detected by Northern analysis in roots. Second, GUS activity was not detected in petals of transgenic tomato or tobacco plants containing the *52-GUS–NOS3'* fusion. However, previous analysis showed that approximately 50-fold lower levels of LAT52 mRNA were present in petals than in anthers of tomato (Twell *et al.* 1989*b*). Finally, GUS mRNA or GUS activity was not detected in the mature anther walls of transgenic tomato or tobacco plants containing either gene fusion, whereas LAT52 and LAT59 mRNA was localized to the mature anther wall of tomato by *in situ* hybridization (Ursin *et al.* 1989); and we have confirmed by Northern analysis (this paper) that the LAT52 and LAT59 mRNAs are present in the mature anther wall of tomato. Several factors that are not mutually exclusive could account for the apparent discrepancies stated above. Sequences required for transcriptional repression in seeds, or activation in petals or anther walls may not be present in the introduced constructs; also differences between the native LAT transcripts and the

chimeric GUS transcripts, such as mRNA stability, processing or translation could account for the observed discrepancies. These possibilities can be addressed more directly when antibodies to the native LAT52 and LAT59 proteins are available and/or by introducing additional gene fusion constructs into transgenic plants.

LAT59 mRNA accumulates to high levels in mature pollen grains (Wing *et al.* 1989). Transcripts from the introduced gene, *59-GUS-NOS3'*, also accumulated to high levels in mature pollen and were translated into active GUS enzyme. Upon pollen germination GUS enzyme moved out of the mature pollen grain with the cytoplasm into the elongating pollen tube. These data may indicate that the endogenous LAT59 mRNA is translated in the maturing pollen grain and perhaps upon germination, and predicts that the LAT59 protein product is present in the elongating pollen tube. Studies with antibodies against the native LAT59 protein will allow confirmation of the time of translation of the LAT59 mRNA.

If indeed LAT59 encodes a functional pectate lyase then its presence in the pollen tube would be consistent with the requirement for pollen tube wall synthesis and solubilization of cell wall material during the emergence and rapid growth of the pollen tube through the pistil. The presence of pectolytic enzyme activity in pollen of several plant species (Pressey and Reger, 1989) is consistent with the notion that LAT59 may encode a pectin-degrading enzyme (Wing *et al.* 1989). Recently it was reported that a pollen-specific cDNA clone from *Oenothera* shows homology with polygalacturonase from tomato (Brown and Crouch, 1990). The corresponding protein was found to be present in the pollen tube and so could have a similar role to that proposed for the LAT59 gene product.

LAT59-GUS transgenic plants showed a low level of GUS expression in roots and seeds. Similar predictions for a requirement for pectin degrading enzymes could be made from the observation of LAT59-directed GUS activity in roots and seeds. It will be interesting to determine whether the GUS and LAT59 proteins show simliar patterns of expression in roots and seeds.

Together with the previously described increase of steady state levels of LAT52 and LAT59 mRNAs during anther development (Twell *et al.* 1989*b*, Wing *et al.* 1989) the data presented here indicate that these genes are regulated at least partly at the level of transcription. The expression from the LAT52 and LAT59 promoters (as measured by GUS activity) was correlated with the appearance of binucleate microspores in developing anthers. Since asymmetric mitotic division of the microspore gives rise to two daughter cells with different developmental fates (Sunderland and Huang, 1987), one model is that unequal partitioning of nuclear and/or cytoplasmic factors acts to regulate transcription factors that control genes (such as LAT52 and LAT59) that may be required for the maturation of the vegetative and/or generative cells. Whether LAT52 and/or LAT59 are transcribed in the vegetative or generative nucleus remains to be determined. The literature suggests that the vegetative nucleus is more transcriptionally active than the generative nucleus (LaFountain and Mascarenhas, 1972), although this needs to be determined for specific pollen expressed genes.

Genes such as LAT52 and LAT59 that show the same spatial and temporal patterns of expression during anther development might be expected to show sequence similarities in their promoter regions. In spite of their coordinate expression, comparison of the 5' flanking DNAs of LAT52 and LAT59 did not reveal any obvious sequence similarities (not shown). This suggests that sequence elements that may be recognized by *trans*-acting regulatory factors in pollen are either degenerate or that these genes respond coordinately, but to different regulatory factors present in pollen. A transient expression system developed for pollen (Twell *et al.* 1989*a*) is now being used to rapidly identify putative shared *cis*-elements that are involved in pollen-specific expression. Since the LAT52 and LAT59 promoters function in *Arabidopsis*, a genetic dissection of the putative *trans*-acting factors is also feasible. The identification of transcription factors involved in the activation of these promoters after microspore mitosis should provide a better understanding of the nature of the unique asymmetric cell division required for the correct development of the pollen grain.

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