Preferential Expression of an $\alpha$-Tubulin Gene of Arabidopsis in Pollen

Jeffrey L. Carpenter, a Sara E. Ploense, b D. Peter Snustad,a,c,1 and Carolyn D. Silflow a,b,c

a Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minnesota 55108-1095
b Department of Plant Biology, University of Minnesota, St. Paul, Minnesota 55108-1095
c Plant Molecular Genetics Institute, University of Minnesota, St. Paul, Minnesota 55108-1095

The pool of tubulin protein in tissues of Arabidopsis is provided by the expression of multiple $\alpha$-tubulin ($TUA$) and $\beta$-tubulin ($TUB$) genes. Whereas most tubulin genes are expressed in many tissues, previous evidence suggested that the $TUA1$ gene might be expressed primarily in pollen. We now report a detailed analysis of $TUA1$ expression during Arabidopsis development. In RNA from tissues of dissected flowers, $TUA1$ transcripts were detected only in stamens and mature pollen. Chimeric genes containing $TUA1$ 5' flanking DNA fused to the $\beta$-glucuronidase (GUS) coding region were used to create transgenic Arabidopsis plants. Plants containing a chimeric gene with 533 bp of 5' flanking sequence were analyzed by histochemical assay to localize GUS expression within the plant. The blue product of GUS enzyme activity accumulated very rapidly in postmitotic pollen grains. Much lower levels of GUS activity were detected in anthers with uninucleate pollen grains, in flower receptacles, and in a few vegetative tissues. Analysis of 5' deletions of the $TUA1$ promoter suggested that 97 bp of 5' flanking DNA is sufficient to drive GUS expression in pollen and young anthers, whereas at least 380 bp is required to detect GUS expression in the receptacle. Examination of the $TUA1$ promoter sequence revealed several motifs that are repeated within the $TUA1$ promoter and are similar to sequences in other pollen-specific promoters.

INTRODUCTION

Tubulin genes are often referred to as "housekeeping" genes. In plants, however, a more accurate label would be "housebuilding" genes, which reflects the importance of various microtubule arrays in establishing patterns of cell division and cell elongation. Because rigid walls preclude cell migration, adjustments in cell division and cell elongation patterns become the only means to effect morphogenesis in a growing plant apex. The four microtubule arrays that function in the division and elongation of most plant cells are the preprophase band, mitotic spindle, phragmoplast, and cortical array (reviewed by Hepler and Palevitz, 1974; Gunning and Hardham, 1982; Lloyd, 1987). In addition to these arrays, unusual microtubule arrays are found in cells undergoing tip growth such as pollen tubes and root hairs, and in other specialized cells such as sperm cells (reviewed by Derksen and Emons, 1990; Pierison and Cresti, 1992). Although significant progress has been made to localize microtubules, the means by which they perform their many duties remain obscure.

We are investigating the complex functions of microtubules in plants, initially focusing on roles of individual tubulin gene products in different microtubule arrays. Toward this end, we have characterized the $\alpha$-tubulin ($TUA$) and $\beta$-tubulin ($TUB$) gene families of Arabidopsis, and have analyzed tubulin transcript accumulation in organs of mature plants. Six $TUA$ and nine $TUB$ genes have been identified (Marks et al., 1987; Silflow et al., 1987; Kopczak et al., 1992; Snustad et al., 1992). Most Arabidopsis tubulin genes are expressed in several different tissues (Ludwig et al., 1987; Oppenheimer et al., 1988; Kopczak et al., 1992; Snustad et al., 1992; J. L. Carpenter, unpublished results). A striking exception is the $TUA1$ gene, which encodes the most divergent $\alpha$-tubulin isotype in Arabidopsis (Kopczak et al., 1992). Ludwig et al. (1988) found that $TUA1$ transcript accumulates in flowers, but not in leaves or roots of mature plants, and that transcript levels peak at anthesis, suggesting a possible role for the $TUA1$ isotype in pollen. Carpenter et al. (1990) showed that the $TUA1$ promoter is active in pollen grains.

To begin addressing the role of $TUA1$ in Arabidopsis development, we sought more detailed information regarding the spatial and temporal patterns of $TUA1$ gene expression. To that end, we examined $TUA1$ transcript accumulation in Arabidopsis flower organs. In addition, the expression of a chimeric gene constructed from the putative promoter of $TUA1$ and the $\beta$-glucuronidase (GUS) coding region (Jefferson et al., 1987) was analyzed in transgenic Arabidopsis plants. Localization of GUS activity indicated cells and tissues where the endogenous $TUA1$ gene may be transcribed. Our results indicate that $TUA1$ is expressed preferentially in postmitotic pollen grains, with some expression in young anthers, the receptacle, and a few specific vegetative tissues. Deletion analysis of the $TUA1$
promoter suggested that at least two cis-acting regions control spatial expression of TUA1. Several sequences were identified that are conserved between the TUA1 promoter and promoters of other "pollen-specific" genes.

RESULTS

TUA1 Transcript Accumulates Primarily in Male Reproductive Tissues

TUA1 transcript accumulation in organs of mature flowers was analyzed using RNA gel blots. Flowers just prior to anthesis were dissected into perianth (petals and sepals), pistil, and stamen fractions. In addition, pollen was collected from flowers that had reached anthesis. Total nucleic acids were isolated from these tissues, fractionated on denaturing agarose gels, and blotted onto a nylon membrane. Figure 1A shows the overall pattern of α-tubulin transcript accumulation. As expected, α-tubulin transcripts are present in all tissues. The same membrane was hybridized with a gene-specific probe prepared from the 3' noncoding region of the TUA1 gene (Ludwig et al., 1988). TUA1 transcripts were detected in RNA from mature pollen and predehiscent stamens, but not in perianth or pistil RNA (Figure 1B).

Figure 1. α-Tubulin and TUA1 Transcript Accumulation in Floral Organs and Mature Pollen.

Total nucleic acids (4.6 μg per lane) from perianth, pistil, stamens, and mature pollen were fractionated on a denaturing 1.2% agarose gel and transferred to nylon membrane (see Methods).

(A) α-Tubulin transcript accumulation in Arabidopsis flower tissues. The blotted RNA was hybridized with a full-length TUA3 coding sequence probe, and the resulting autoradiograph is shown.

(B) TUA1 transcript accumulation in Arabidopsis flower tissues. RNA on the membrane from (A) was hybridized with a 3' noncoding genespecific probe from the TUA1 gene (Ludwig et al., 1988).

Figure 2. All Kanamycin-Resistant pM533G-Transformed Plant Lines Contain at Least One Copy of the Chimeric TUA1::GUS Gene Insert.

Genomic DNA from untransformed plants and plants transformed using pM533G was isolated, digested with HindIII, fractionated on a 0.8% agarose gel, and blotted onto a nylon membrane as described in Methods. All DNA was isolated from R2 progeny plants except that from line 150.5, which was isolated from R1 plants.

(A) Hybridization with a GUS coding sequence probe reveals the presence of homologous sequences in kanamycin-resistant lines. The transgenic plant lines represented in this panel are indicated at the top of each lane. Numbers to the left indicate the migration of molecular weight standards in kilobases. The two arrows indicate the expected position of fragments derived from inverted (top arrow) and tandem (bottom arrow) repeats. No hybridization was detected in the lane containing untransformed DNA (data not shown).

(B) The blot shown (A) was stripped and hybridized with a 5' genespecific probe to the TUB3 gene. Comparison of hybridization intensity provides an approximation of the relative loading of DNA in each lane.

TUA1 Promoter Activity in Flowers

Our hybridization experiments demonstrated that TUA1 transcript accumulates in mature pollen. To determine more precisely the tissues in which TUA1 is expressed, we analyzed
transgenic Arabidopsis plants that had been transformed using plM533G. This plasmid contains the putative promoter region of TUA1 inserted upstream of the GUS coding sequence in pBl101 (Jefferson et al., 1987). The promoter fragment contains 533 bp of sequence 5' to the TUA1 transcription initiation site, which was mapped by Ludwig et al. (1988), and 56 bp of untranslated leader. Agrobacterium tumefaciens containing plM533G was used to transform sterile Arabidopsis roots (Valvekens et al., 1988). Seventeen independent transgenic plant lines, designated 150.1 to 150.17, were regenerated from kanamycin-resistant callus. As a group, these lines will be referred to as plM533G transformants, in reference to the plasmid that was used for the transformations. From each primary regenerant (R0), kanamycin-resistant R1 progeny were allowed to self, and seeds were collected. Genomic DNA from the resulting R1 progeny (R1 in the case of line 150.5) was used for DNA gel blot analysis. The DNA was hybridized with a GUS coding sequence probe to identify DNA fragments that contain the chimeric gene from plM533G. Results for 10 lines are shown in Figure 2A. These results indicated that most lines contain chimeric gene inserts at one to 10 different locations and that the number of chimeric gene inserts was quite variable between lines, as shown in Table 1. Results of our DNA gel blot analyses also indicated that, in several cases, chimeric genes were inserted as multiple tandem and/or inverted repeats. This conclusion is based on the observation that some hybridizing fragments (see lanes containing 150.3, 150.6, 150.7, and 150.10 DNA in Figure 2) were significantly more intense than other fragments in the same and other lanes. In the case of a tandem insert, the GUS coding region probe should hybridize to a 5.25-kb HindIII fragment, whereas an inverted repeat should produce a 6.4-kb fragment or two fragments longer than 3.25 kb. Relatively strong hybridization signals corresponding to fragments of 5.25 and 6.4 kb (arrows in Figure 2A) indicated that tandem and/or inverted repeats were present in several lines. Intense hybridization to fragments of different sizes in lane 150.10 may be due to specific rearrangements of the chimeric gene. That differences in hybridization intensity were not due to unequal DNA loading is indicated by results in Figure 2B, which shows an autoradiograph of the same membrane after stripping and hybridizing with a gene-specific probe derived from the 5' flanking DNA of TUB3. This blot indicates that the quantity of DNA in most lanes is very similar.

To assess TUA1 promoter activity in flowers, floral apices from R1 and R2 progeny of all 17 plM533G transformants were assayed histochemically to localize GUS activity. The blue reaction product consistently accumulated in three distinct flower tissues, including postmitotic pollen grains, postmeiotic anthers (referred to hereafter as "young anthers"), and the receptacle, beginning in flowers with premeiotic anthers. However, evidence of GUS enzyme activity was consistently detected much more rapidly in postmitotic pollen grains than in either young anthers or the receptacle, suggesting that the TUA1 promoter region in the plM533G construct is most active in pollen.

Table 1. Tissues in Which GUS Activity Was Detected in plM533G-Transformed Plant Lines

<table>
<thead>
<tr>
<th>plM533G Line Copy No.</th>
<th>Po</th>
<th>An</th>
<th>Re</th>
<th>Tr</th>
<th>Ax</th>
<th>Se</th>
<th>Ma</th>
<th>Pe</th>
<th>Ro</th>
<th>Mi</th>
</tr>
</thead>
<tbody>
<tr>
<td>150.5 1-2 + + + NT NT NT NT NT NT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.11 1-2 + + + NT NT NT NT NT NT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.12 1-2 + + + NT NT NT NT NT NT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.16 10+ + + + NT NT NT NT NT NT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.2 10+ + + + NT NT NT NT NT NT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.13 10+ + + NT NT NT NT NT NT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.4 1-2 + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.14 1-2 + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.15 1-2 + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.7 2-3 + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.17 2-3 + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.9 5-10 + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.6 5-10 + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.1 5-10 + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.3 5-10 + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.8 10+ + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150.10 10+ + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For each plM533G-transformed plant line, tissues in which GUS activity was detected are indicated by a (+) and tissues where GUS activity was not detected are indicated by a (-). For the six plant lines at the top of the table, GUS activity in vegetative tissues was not tested (NT). The approximate number of chimeric gene inserts (not loci), as estimated by DNA blot analysis, is indicated in the second column.

Po, postmitotic pollen grains; An, young anthers with uninucleate microspores; Re, receptacle; Tr, trichomes; Ax, axils; Se, developing seeds; Ma, small areas near teeth on leaf margins; Pe, petiole vasculature; Ro, root tip; Mi, micropyle.
Figure 3. Histochemical Localization of GUS Gene Expression in Floral Tissues of Transgenic Plants Containing the Chimeric Gene from plM533G.
The TUA1 Promoter Is Preferentially Active in Postmitotic Pollen

The growth pattern of the Arabidopsis flower stem provides flowers arranged in a developmental profile, with flower primordia at the apex and more mature flowers lower on the stalk. Figure 3A shows the distribution of GUS reaction product in flowers from a plM533G-transformed plant after 21 hr of incubation in assay buffer containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc). The most striking feature that we observed was a strong induction of GUS activity in anthers of young flowers (arrow; see below). In mature anthers, intense blue color derived from GUS activity developed within 15 to 60 min of incubation, although the intensity of staining varied between lines. GUS activity was never observed in sepals, petals, or pistils (Figure 3A), or in mature anthers from untransformed plants or plants transformed with pBI101 (Figure 3B). As shown in Figures 3C and 4F, plants containing the cauliflower mosaic virus (CaMV) 35S promoter::GUS insert from pBI121 (Jefferson et al., 1987) accumulated GUS reaction product in sepals, petals, and pistils, demonstrating that GUS activity is detectable in these tissues under our assay conditions. Several tissues of plants containing the CaMV 35S promoter construct, particularly stems and pistils of older flowers, showed high levels of GUS activity only where substrate penetration was enhanced by cutting the tissue prior to incubation (data not shown).

To better illustrate the timing of GUS induction in plM533G-transformed anthers, developmental profiles of anthers (Figure 3D) and individual flowers (Figures 3E to 3H) were prepared. Figure 3D shows anthers obtained from adjacent flowers in one floral cluster from an untransformed plant (top) and from a plant transformed with plM533G (bottom). The rapid induction of GUS activity in anthers from a plM533G-transformed plant is illustrated by comparing stage 7 anthers (no blue product) with stage 8 anthers (intense blue in the plM533G-transformed anther, no blue in the untransformed anther). This induction is shown in the context of whole flowers in Figures 3G and 3H. The most mature flower (Figure 3H) accumulated high levels of the GUS reaction product in all six anthers, whereas the next younger flower (Figure 3G) showed no evidence of GUS activity in any anthers. In some flowers, the four long stamens had blue anthers, whereas the two short stamens (which are slightly less mature) showed little if any GUS activity (Figure 3I, large arrow). Under our growth conditions, the developmental time difference between adjacent flowers was ~10 hr, and the developmental difference between long and short stamens was significantly less, indicating that the TUA1 promoter is induced within a developmental time period of a few hours.

The apparent anther-specific GUS activity in mature flowers was consistent with the hybridization experiments shown in Figure 1. Our next goal was to localize GUS activity within the anther. We typically observed GUS reaction product throughout intact anthers. However, we also observed that individual blue pollen grains were often surrounded by blue clouds of GUS reaction product that stained the surface of any tissue in contact with the pollen grain. To investigate whether staining of anther tissues was due to the GUS reaction product leaking from pollen grains, individual anthers were assayed intact or after removing most of the pollen grains (Figures 3I and 3J). Intact anthers were blue throughout. Anthers from which pollen had been removed were clear, except where isolated pollen grains remained. In these areas, blue pollen was

Figure 3. (continued).

Floral apices from transgenic Arabidopsis plants were assayed for GUS enzyme activity as described in Methods.

(A) Floral apex from a plant containing the chimeric plM533G gene. Arrow indicates GUS product in receptacles of young flower buds.

(B) Floral apex from a plant containing the promoterless GUS gene from pBI101.

(C) Floral apex from a plant containing the chimeric 35S::GUS gene from pBI121.

(D) Developmental series of anthers from an untransformed plant (top) and a representative plM533G-transformed plant (bottom). Intact floral apices were assayed in parallel, and then eight anthers from eight adjacent flowers were removed and aligned from the youngest (1) to the oldest (8). Stage 2 anthers contain tetrads, which become uninucleate microspores by stage 3.

(E) to (H) Developmental series of whole flowers from a plM533G-transformed plant. The floral apex was assayed, then flowers were removed and photographed individually to improve visualization of the GUS product in young anthers (E and F), in the receptacle (E) (see arrow) through (H), and in mature anthers (H). The flower in (E) is at approximately the same developmental stage as the flowers from which stage 3 anthers in (D) were taken. Similarly, flowers in (G), (F), and (H) correspond to stage 5, 7, and 8 anthers, respectively. The flower in (F) is magnified ~2x relative to the other flowers to enhance visibility of GUS activity in the tapetum.

(I) The chimeric gene from plM533G is not coordinately induced in all six anthers. In this flower, the GUS reaction product accumulated rapidly in anthers of the four long stamens (three shown), but little if any product was detected in anthers on the two short stamens (one shown, arrow). One of the long stamens was removed prior to incubation in X-gluc, placed in buffer on a glass slide, and gently squeezed under a cover slip to remove most of the pollen grains. In this anther, the GUS reaction product accumulated only in the immediate vicinity of blue pollen grains (arrow).

(J) Pollen specificity of GUS expression within mature anthers. The squeezed anther and one intact anther shown in (I) were photographed at ~4x higher magnification.

(K) GUS activity is present throughout pollen tubes that grew through stigmas of untransformed plants (described in Methods).
Transgenic Arabidopsis plants were assayed for GUS enzyme activity as described in Methods. 

(A) and (B) Expression of the chimeric gene from plM533G in pollen is induced after mitosis. Pollen grains from a plM533G anther prior to induction of GUS activity were stained with DAPI to detect nuclei. Pollen grains were visualized using bright-field optics (A) and epifluorescence illumination with ultraviolet light (B). Three nuclei were present in each pollen grain, showing that both mitotic divisions were complete before GUS activity was detected.

(C) GUS activity in receptacles was induced early during flower development. A floral apex from a plM533G-transformed plant is shown, demonstrating GUS product accumulation in the receptacle. Six buds are visible in this panel. Receptacles of the three smallest buds (arrows) did not accumulate detectable product.
surrounded by a region of blue anther tissue (arrows). Thus, in mature anthers, the TUA1 promoter appears to be transcriptionally active only in pollen grains. Both blue and white pollen grains were observed in some pLM533G-transformed lines, indicating that the chimeric gene inserts segregated in the haploid pollen grains and were expressed by the haploid genome. In Arabidopsis, the two mitotic divisions that produce sperm cells are completed before anthesis. To determine whether expression of the TUA1 promoter begins before or after these mitotic divisions, anthers from four independent pLM533G-transformed lines were assayed for GUS activity and then stained with 4',6-diamidino-2-phenylindole (DAPI) to detect nuclei. We examined anthers from the oldest flower that showed no GUS activity in pollen (equivalent to the flower in Figure 3G). A bright-field image of DAPI-stained pollen grains is shown in Figure 4A. Illumination of the same pollen grains with ultraviolet light revealed three nuclei in each pollen grain (Figure 4B), indicating that mitosis was complete prior to the induction of GUS activity. Identical results were obtained with pollen from all four lines that were examined. GUS activity was detected throughout pollen tubes (Figure 3K), suggesting that the TUA1 promoter is expressed in the vegetative cells. Our experiments did not examine whether the chimeric gene was also expressed in sperm cells.

Low Levels of TUA1 Promoter Activity in Postmitotic Anthers

"Young anthers" of all 17 pLM533G-transformed lines accumulated low levels of GUS reaction product during histochemical assays. Results of DAPI staining indicated that nearly all of these anthers contained uninucleate microspores; a few anthers contained tetrads (Table I). Detection of GUS activity in young anthers typically required 16 to 24 hr of incubation in assay buffer, and staining intensity varied noticeably between lines and between individual plants within lines. This analysis was complicated by background GUS activity that was detected in young anthers of lines transformed with pBI101 and in untransformed Arabidopsis plants. However, in parallel histochemical assays, GUS expression in young anthers was seen in 20 of 21 (95%) pLM533G-transformed plants that expressed GUS in pollen, but in only four of seven (57%) control plants. These results suggested that evidence of GUS enzyme activity was more readily detected in young anthers of pLM533G-transformed lines than in control plants and that this GUS activity was at least partially due to expression of the chimeric gene from pLM533G.

Figure 3D illustrates the developmental timing of GUS expression in young anthers of an untransformed plant (top) and a pLM533G-transformed plant (bottom). In both cases, GUS activity was detected as early as the tetrad stage (stage 2 anthers). In pLM533G-transformed lines, the blue product of GUS activity was typically more apparent and persisted longer (expression seen in the stage 5 anther) than in control plants (expression not detected in the stage 4 anther). Detectable GUS expression in young anthers of pLM533G-transformed lines disappeared completely prior to the rapid induction of GUS activity in postmitotic pollen (see Figure 3D and compare Figure 3F to 3H). Examination of resin sections using dark-field optics showed that GUS product was detectable throughout young anthers of pLM533G-transformed lines, but was most concentrated in the tapetum (Figures 4D and 4E). Using plants transformed with the 35S promoter::GUS construct, we showed that GUS activity was detectable in all tissues of flowers with uninucleate microspores (Figure 4F). Relatively weak GUS activity was detected in anthers of pBI101-transformed lines and appeared to be most concentrated in the tapetum (Figure 4G).

Low Levels of TUA1 Promoter Activity in Receptacles

The receptacle was the third flower tissue to which GUS reaction product localized in pLM533G-transformed lines. In some lines, GUS activity persisted through anthesis, but the intensity of staining decreased as flowers matured (Figures 3A and 3E to 3H, see arrows in 3A and 3E). As in young anthers, GUS activity in receptacles was detectable only after relatively long incubations in X-gluc (generally between 16 and 24 hr), and staining intensity varied between and within lines. GUS activity was never detected in receptacles of lines 150.2 and 150.13 (Table I). In both of these lines, GUS expression in young anthers was quite obvious, suggesting that the lack of expression in receptacle tissue was not due to a general reduction in expression levels. Figure 4C shows a pLM533G-transformed floral...
two-leaf seedlings were also examined. The results of these assays are summarized in Table 1. In general, GUS activity was either undetectable in nonflower tissues or was detected in vegetative tissues. GUS expression in reproductive tissues was due to "position effects" seen in sterile roots. The exception was line 150.1, in which the receptacle varied among the PM533G-transformed lines.

The youngest bud showing GUS activity had premeiotic anthers (data not shown). It is likely that GUS expression could have been detected if present.

**Analysis of the TUA1 Promoter Region**

To begin localizing cis-acting DNA elements responsible for TUA1 promoter activity in flowers, eight chimeric genes (including PM533G) were constructed with TUA1 promoter fragments fused to GUS coding sequence. These chimeric genes were essentially identical except for their 5' end points, as shown in Figure 5A. GUS activity in transgenic Arabidopsis plants (four to 10 R1 lines for each construct and pBI101; see Figure 5A) was analyzed using histochemical and fluorometric assays.

For each independent line of each deletion, one to three (usually three) floral apices were assayed histochemically to examine GUS activity in mature pollen, young anthers, and receptacles. The results of these assays are summarized in Figure 5B. All transgenic plants containing the -97 promoter deletion (from PM97G) accumulated the GUS reaction product in pollen, whereas plants containing the -39 promoter deletion (from PM39G) produced no detectable GUS activity in pollen. TUA1 promoter activity in young anthers appeared to follow the same pattern although this expression was more difficult to quantify due to background expression. GUS activity in the receptacle was detected only in plant lines that were transformed using pM1500G and PM533G.

GUS activity was quantitated using the substrate 4-methylumbelliferyl glucuronide. Flowers were removed by excising just above the receptacle to ensure that these assays measured GUS activity in pollen only. The results of fluorometric assays (Figure 5C) support observations made using histochemical assays. Extracts from flowers of all four PM39G-transformed lines produced background levels of the fluorescent reaction product 4-methylumbellifere, whereas extracts from flowers of PM97G-transformed lines exhibited approximately fivefold higher GUS activity. This fivefold difference is probably an underestimate of the true difference in the transcriptional activity directed by the PM39G and PM97G promoter constructs. A more accurate estimate would have been possible if GUS activity had been measured in extracts derived from pollen rather than extracts from whole flowers, but collection of sufficient quantities of pollen to do these assays was not feasible.

Comparisons of relative levels of fluorescence suggest that elements controlling the quantity of GUS expression in pollen may be present between -216 and -279 and between -533 and -1500, but the significance of the observed differences is not clear (see Discussion). Comparisons of the TUA1 promoter with promoters from other genes showing preferential expression in pollen revealed several conserved motifs (reviewed by McCormick, 1991; also discussed in Twell et al., 1991). For example, two sequences are highlighted.
were found that are similar to the "56/59 box," which Twell et al. (1991) identified in the promoters of the pollen-preferential LAT56 and LAT59 genes of tomato (Figure 5D). One of these boxes lies between -87 and -79 bp upstream of the TUA1 transcript initiation site, and contains the important GTGA core motif. As noted above, deletion of DNA containing this box was correlated with a complete loss of TUA1 promoter activity in pollen. The second 56/59 box lies between -315 and -307. Deletion of this box had no apparent effect on GUS activity. In addition to the 56/59 motifs, the TUA1 promoter contains five highly conserved 10-bp motifs and two 12-bp perfect repeats (-304 to -293 and -439 to -428), 5 bp of which are similar to the "PB core motif" (Twell et al., 1991) that is present in several plant promoters. Computer alignment identified two 10-bp sequences that are conserved between the TUA1 promoter and the pollen-specific chalcone flavonone isomerase (Chi) gene A P2 promoter (van Tunen et al., 1989). One of these motifs contains one mismatch (at -123 in TUA1), whereas the second is a perfect match (at -233 in TUA1). None of these upstream elements is essential for GUS expression in pollen, but some may have quantitative effects, serve as redundant elements, or promote expression in other tissues.

Figure 5. Analysis of Chimeric TUA1::GUS Constructs in Transgenic Plants.

Histochemical and fluorometric GUS assays were performed as described in Methods. For each TUA1 promoter deletion, four to 10 independent transgenic plant lines were analyzed.

(A) Successive 5' deletions of the TUA1 5' flanking DNA were inserted upstream from the GUS coding sequence in pB101, and the resulting chimeric genes were used to create transgenic plants. The deletions are drawn to scale, except in the case of plM1500G. The number in the name of each construct indicates the amount of sequence upstream from the GUS coding sequence in pB101. Evidence of GUS activity in pollen and young anthers was seen in all plant lines that were transformed with chimeric genes containing TUA1 promoter fragments with 97 bp or more of 5' upstream sequence. GUS activity in the receptacle was not seen with promoter fragments containing 380 bp or less of TUA1 5' upstream sequence. The 5' deletion end point of each construct is indicated along the top. Evidence of GUS activity in pollen is indicated by a (+), and undetected activity is indicated by a (-). For young anthers and the receptacle, the numerators indicate the number of plants in which GUS activity was detected out of the total number of assayed plants containing blue pollen (denominator). For plant lines transformed using plM39G and pB101, the denominator indicates the total number of plants that were examined. (B) Results of histochemical assays on floral apices from four to 10 independent lines (A) representing each TUA1 promoter deletion and pB101. Evidence of GUS activity in pollen and young anthers was seen in all plant lines that were transformed with chimeric genes containing TUA1 promoter fragments with 97 bp or more of 5' upstream sequence. GUS activity in the receptacle was not seen with promoter fragments containing 380 bp or less of TUA1 5' upstream sequence. The 5' deletion end point of each construct is indicated along the top. Evidence of GUS activity in pollen is indicated by a (+), and undetected activity is indicated by a (-). For young anthers and the receptacle, the numerators indicate the number of plants in which GUS activity was detected out of the total number of assayed plants containing blue pollen (denominator). For plant lines transformed using plM39G and pB101, the denominator indicates the total number of plants that were examined. (C) Relative GUS activity in mature flowers representing each deletion and pB101. Mature flowers from four to 10 independent lines representing each TUA1 promoter deletion and pB101 were pooled, and protein extracts were analyzed fluorometrically to quantify GUS activity. Three parallel experiments were performed, as indicated by the black, hatched, and white bars. (D) Nucleotide sequence of the TUA1 5' flanking DNA that was included in the plM533G construct. The 5' endpoints of each deletion are indicated by the vertical line above the sequence. Conserved motifs are indicated as follows: Sequences similar to the 56/59 box are underlined; the five 10-bp repeats are underlined; the two motifs with similarity to sequences in the Chi A P2 promoter are indicated by overlining with a solid line (−123) and with a dashed line (−233). The putative TATA box is in boldface type.
DISCUSSION

**TUA1 Is Preferentially Expressed in Postmitotic Pollen**

We have shown that *TUA1* transcripts accumulate in stamens and mature pollen, but not in perianth or pistils. Analysis of 17 independent transgenic plant lines containing a chimera between the putative *TUA1* promoter and the β-glucuronidase coding sequence (Jefferson et al., 1987) suggested that *TUA1* transcript accumulation is accompanied by increased *TUA1* promoter activity. A much less likely interpretation of the results is that the *TUA1* promoter is active in all tissues, but the 56 bp of the *TUA1* 5' untranslated sequence in the chimeric genes had a dramatic effect on transcript stabilization in mature pollen and a few other tissues.

Several other genes with pollen-preferential expression have been characterized (reviewed by McCormick, 1991). Stinson et al. (1987) identified at least two temporal classes of pollen-specific transcripts from maize and Tradescantia. In one group (class I), transcription was initiated after microspore mitosis, and transcript accumulation increased as pollen matured. The pollen expression exhibited by *TUA1* falls into this class. The second group of transcripts (class II) accumulated soon after meiosis, peaked prior to pollen maturation, and then decreased. Several additional pollen-specific genes have been identified, all of which seem to fall into one of these two classes. Other class I genes include the maize Zm13 gene (Hamilton et al., 1989; Hanson et al., 1989), the petunia chiA gene (P_2 promoter) (van Tunen et al., 1989, 1990), the P2 gene family from *Oenothera* (Brown and Crouch, 1990), and the LAT genes from tomato identified by McCormick et al. (1987). Although LAT transcripts accumulate primarily after mitosis, these genes exhibit low level expression as early as tetrad stage anthers (Twell et al., 1989; Ursin et al., 1989; Wing et al., 1989). Class II genes include the petunia chiB gene (van Tunen et al., 1989, 1990) and several genes from oilseed rape (Albani et al., 1990, 1991; Roberts et al., 1991; Scott et al., 1991).

Expression of these genes can be loosely described as pollen-specific, but like *TUA1*, many "pollen-specific" genes exhibit low levels of expression in other tissues. For example, the petunia chiB promoter is active in the tapetum as well as in microspores (van Tunen et al., 1990). Similarly, all five LAT genes are expressed in at least one tissue in addition to pollen (Twell et al., 1989, 1990, 1991; Ursin et al., 1989; Wing et al., 1989). Although often not the major focus of study, these "minor" expression patterns may provide important clues about the roles of these genes during plant development.

The grouping of pollen-specific genes into two major temporal expression classes almost certainly oversimplifies the actual situation. However, Twell et al. (1991) noted that promoters for at least five class I genes contain a PB core motif, and all but one of these genes contain at least one 56/59 box. Of particular interest here is the 56/59 box, which, with its GTGA core, affects at least quantitative expression of both LAT56 and LAT59. Two motifs that are similar to the 56/59 box are present in the *TUA1* promoter. Deletion of the most conserved element is correlated with complete loss of *TUA1* promoter activity in pollen, based on both histochemical and fluorometric assays. In addition, two 12-bp repeats are present in the *TUA1* upstream region that contain TGTGG, part of the PB core motif. Results of Twell et al. (1991) suggest that the PB core has a quantitative role in the LAT52 and LAT56 promoters, but loss of these sequences from the *TUA1* promoter had no obvious effect.

Results of fluorometric assays support the findings that at least one cis-acting element between -39 and -97 in the *TUA1* promoter is required for GUS expression in pollen. In addition, these data suggest that quantitative elements may lie between -216 and -271 and between -533 and -1500. However, the apparent increases in GUS activity are difficult to interpret, primarily because of the variability that was observed between different lines containing the same promoter construct. For example, observations of histochemical assays suggest that two of the seven plM1500G-transformed plant lines produced extremely high levels of the GUS reaction product. The intensity of histochemical assay development in the other five plM1500G-transformed lines was not distinctly different from typical plM97G-transformed lines. Because fluorometric assays were performed on extracts from pooled flowers, plant lines with very high expression levels could significantly increase the average expression of the plM1500G-transformed lines. Segregation of loci with high levels of expression could also account for the unusually wide variation between repeat experiments using plM1500G-transformed lines. Less extreme examples of this phenomenon were seen with three plM533G-transformed lines. These results suggest that if GUS activity were quantitated in many independent plant lines containing any one chimeric gene, the data would probably produce a skewed distribution, with a few lines expressing GUS at many times the average level. Because of this, statistical analyses that assume a normal distribution are probably not suitable for these data.

**Possible Roles of TUA1 in Pollen Tube Growth**

Mature, ungerminated pollen grains appear to lack microtubules (Derksen et al., 1985; Heslop-Harrison et al., 1988; Tiwari and Polito, 1988). After germination, pollen tubes expand by tip growth in a process similar to the growth of root hairs and fungal hyphae (reviewed by Steer and Steer, 1989; Derksen and Emons, 1990; Pierson and Cresti, 1992). As the pollen tube grows, the vegetative nucleus and sperm cells travel through the tube, as do numerous vesicles and organelles. Concurrently, an intricate microtubule array assembles in the vegetative cell, forming a network with actin, the endoplasmic reticulum, and the plasma membrane (Lancelle et al., 1987). The identification of a kinesin-like protein in pollen tubes (Moscatelli et al., 1998) suggests that microtubules may play some role in sperm cell, organelle, and/or vesicle transport. Interphase microtubule arrays of most plant cells are nearly
perpendicular to the direction of cell elongation (Lloyd, 1987). In contrast, pollen tube microtubule arrays are roughly parallel to the direction of growth (Derksen et al., 1985; Lancelle et al., 1987; Raudaskoski et al., 1987). These microtubules persist during pollen tube growth, with new tubulin continuously added at nucleation sites near the tube apex (Heslop-Harrison and Heslop-Harrison, 1988). In addition to the vegetative cell microtubules, sperm and generative cells contain microtubule bundles that bound the cell membrane (Derksen et al., 1985; Tiezzi et al., 1988; Cresti et al., 1990), possibly maintaining cell shape during pollen tube growth (Derksen et al., 1985; Heslop-Harrison et al., 1988). Thus, there is a correlation between the timing of TUA1 expression and the assembly of extensive microtubule arrays. The presence of GUS activity throughout pollen tubes suggests that the TUA1 isotype is used in the vegetative cell, but does not rule out a role in sperm cell microtubules.

Could TUA1 Be Functionally Specific in Pollen?

Two ideas have been presented to explain the roles of multiple tubulin genes in multicellular organisms. The “multi-tubulin hypothesis” (Fulton and Simpson, 1976) suggests that differences between the tubulin isotypes produced by different members of tubulin gene families represent functional differences. For instance, isotype-specific interactions with other molecules might alter certain microtubule properties. Cowan et al. (1988) proposed that functional specificity of tissue-specific tubulin isotypes might result from co-evolution with microtubule-associated proteins (MAPs) that have very similar expression patterns. Alternatively, tubulin isotypes might be functionally interchangeable, with multiple tubulin genes expressed as a fine-tuning mechanism of gene regulation (Raff, 1984). Results from several systems have provided evidence in support of both hypotheses (reviewed by Joshi and Cleveland, 1990).

Of the six known Arabidopsis α-tubulin genes, TUA1 seems the most likely candidate to be functionally specific. In addition to its pollen-preferential expression pattern, the gene encodes a divergent α-tubulin that is only 89% and 92% conserved in amino acid sequence with the other two major classes of α-tubulin in Arabidopsis. Much of this divergence occurs near the C terminus and within the N-terminal 70 amino acids (Kopczak et al., 1992). These regions have been implicated in binding to MAPs (Littauer et al., 1986; Maccioni et al., 1988) and calcium (Serrano et al., 1986), which directly affect microtubule assembly and stability.

It should be noted that TUA1 is not expressed in root hairs, which, like pollen tubes, elongate by tip growth. Therefore, the α-tubulin isoform encoded by TUA1 is not required for tip growth per se. If TUA1 is in fact required for proper microtubule function in pollen tubes, it may be due to coevolution with a pollen-specific MAP or to microtubule functions unique to pollen tubes such as shaping of sperm cells or some aspect of the fertilization process. The question of whether the α1 isotype encoded by TUA1 has a unique function in pollen is complicated by the low level expression that was observed in other tissues. The expression of the chimeric TUA1::GUS gene in the tapetum, receptacle, and vegetative tissues seems to be almost negligible when compared to the high level of expression in pollen. However, these “minor” areas of expression may provide important clues about the role of the TUA1 gene product in plant development and about the development of pollen-specific transcription. For example, it is possible that the α1 isotype has a specific function in pollen tube growth and serves in a similar role in the other tissues in which it is expressed. Alternatively, low levels of TUA1 expression in sporophytic tissues may simply be a remnant of expression of a gene that is becoming increasingly pollen specific during evolution.

Although our results answer some questions about the spatial and temporal patterns of TUA1 promoter activity, they also raise some questions. When and where does the α1 isotype accumulate, and how closely does this accumulation match accumulation of GUS reaction product? Is the α1 isotype incorporated into a particular microtubule array in pollen? Does the α1 isotype perform a role that other α-tubulin isotypes cannot fulfill? Many questions remain concerning the specific role of TUA1 in pollen and the other tissues in which it is expressed. Experiments using α1-tubulin-specific antibodies to localize the TUA1 gene product in pollen tubes and antisense RNA constructs to inactivate the TUA1 gene are underway to begin addressing these questions.

METHODS

RNA Gel Blot Analysis

Petals plus sepals, pistils, and stamens were excised from predehiscent flowers with the aid of a stereodissecting microscope. Mature pollen was collected from dehiscent flowers. Total nucleic acids were extracted from plant tissues that were frozen in liquid N₂, ground to a fine powder with a motor-driven pestle (CAFRAMO Stirrer Type RZ5), and suspended in RNA extraction buffer (100 mM Tris-HCl, pH 8.0, 1 mM EDTA). Two extractions with phenol/chloroform/isoamyl alcohol (25:24:1) were performed, and nucleic acids were precipitated by the addition of 0.1 volumes of 3 M sodium acetate and 2.4 volumes of 95% ethanol. The nucleic acids were collected by centrifugation, dried under vacuum, and dissolved in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Two extractions with phenol/chloroform/isoamyl alcohol (25:24:1) were performed, and nucleic acids were precipitated by the addition of 0.1 volumes of 3 M sodium acetate and 2.4 volumes of 95% ethanol. The nucleic acids were collected by centrifugation, dried under vacuum, and dissolved in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Nucleic acids were separated on 1.2% agarose-formaldehyde gels, and gel blots were prepared and hybridized as described by Kopczak et al. (1992).

DNA Gel Blot Analysis

DNA gel blot analysis was performed on R₀ progeny (R₁ for line 150.5) of putative TUA1::GUS transgenic plant lines. Genomic DNA was isolated from 2- to 3-week-old rosette leaves (R. Pruitt, personal communication). Ten to 15 plants were ground at room temperature in 1 mL of proteinase K buffer (200 mM Tris-HCl, pH 8.0, 100 mM EDTA,
1% N-lauroylsarcosine, 100 µg/mL proteinase K) in a mortar with a small quantity of glass beads (75 to 150 µm, acid washed). Ground tissue was transferred to a 15-mL Falcon tube, incubated for 48°C for 1 hr, and then centrifuged at 500g in a clinical centrifuge for 10 min. Two 400-µL aliquots of the clear green supernatant were transferred to 1.5-mL microcentrifuge tubes. One hundred microliters of 5 M NaCl was added to each tube. The samples were vortexed and centrifuged for 5 min. Supernatants were transferred to fresh tubes to which 1 mL of 95% ethanol was added. Samples were vortexed, centrifuged for 3 to 5 min, and pellets were resuspended in 400 µL of TE by vortexing. The addition of 100 µL of 5 M NaCl, centrifugation, and ethanol precipitation of the supernatants were repeated, and then pellets were rinsed in 80% ethanol and drained. Pellets were resuspended in 25 µL of TE while they were still moist. Samples from two replicates were combined and passed over a 600-mL spin column of Sepharose CL-6B (Sigma) equilibrated in TE. To prepare the columns, the tip of a 20-gauge needle was inserted through the bottom of a 500-µL microcentrifuge tube. Approximately 20 µL of sterile glass beads (80 to 100 µm) were added to the tube, followed by ~600 µL of the Sepharose CL-6B slurry. Columns were centrifuged for 3 min at 2000 rpm in a clinical centrifuge to remove excess TE (columns were supported in intact 15-mL Falcon tubes). DNA samples were then pipetted onto the columns, which were centrifuged as before but supported in intact 1.5-mL tubes to collect the DNA. Samples were stored at 4°C after addition of 0.1 µg of RNase.

Samples of genomic DNA were digested with HindIII, subjected to gel electrophoresis, and transferred to Nytran filters (Schleicher & Schuell). The hybridization probe was a 1.8-kb fragment of GUS coding sequence DNA labeled by random primer (Feinberg and Vogelstein, 1984). DNA transfer and hybridization were performed following standard protocols (Schleicher & Schuell).

Construction of TUA1 5’ Upstream Deletions

The TUA1 5’ upstream region was isolated from pOCA+ a11, an Arabidopsis thaliana Columbia genomic clone (genomic clone provided by S. D. Kopczak, University of Minnesota, St. Paul; genomic library described by Olszewski et al., 1988) that contains the complete TUA1 gene and ~7 kb of 5’ upstream sequence. From pOCA+ a11, a 1.9-kb HindIII-NruI fragment of TUA1 5’ upstream DNA with 56 bp of untranslated leader sequence was subcloned into pUC18 to make pUPo14. The TUA1 promoter fragments in plM533G and plM271G were isolated using convenient restriction sites. Promoter fragments for all other deletions were created using exonuclease III (Stratagene) and mung bean nuclease, so does not methylate the Bcll site. TUA1 promoter fragments were isolated for subcloning into pBI101 by digesting with Bcll and BamHl and gel purifying through 1% SeaPlaque low gelling temperature agarose. These fragments were ligated into the BamHl site of pBI101 and screened to identify subclones containing the TUA1 promoter in the forward orientation relative to the GUS coding sequence. Plasmids were introduced into Agrobacterium tumefaciens AGL1 (Lazo et al., 1991) by way of electroporation. The plasmid was then isolated (An et al., 1988) and checked for rearrangement by restriction enzyme analysis and analysis of DNA gel blots.

Plant Transformation

A. tumefaciens AGL1 (Lazo et al., 1991), containing the vectors pBI101, pBI121, or one of the plM constructs, was used to transform Arabidopsis, following the root transformation protocol of Valvekens et al. (1988) with minor modifications. Media for regenerating plants were based on the PG1 mixture described by Negrutiu et al. (1975). A. tumefaciens cultures were incubated overnight in Nutrient Broth (Difco) supplemented with 2% glucose, pH 5.4, at 25°C, in the presence of 50 to 100 mM 3’5’-dimethoxy-4’-hydroxy-acetophenone (acetoxyrinigone) (Aldrich) to optimize induction of the A. tumefaciens virulence (vif) genes (Sheikhoslam and Weeks, 1987; Alt-Moerbe et al., 1989). As regenerating shoots began to bolt, they were transferred to soil for seed set.

Plant Growth

Arabidopsis Columbia plants were grown under continuous fluorescent light at 25°C and 75% relative humidity. Sterile plants for histochemical assays and for transformations were grown on the same agar-based medium as used for regenerating plants, but without hormones.

Histochemical GUS Assays

Histochemical assays for GUS activity were carried out essentially as described by Jefferson et al. (1987), with minor modifications designed to improve substrate accessibility to internal flower tissues. Freshly cut tissues were immersed in 50 mM NaH2PO4, pH 7.0, containing 0.1% Triton X-100 (Sigma) as a wetting agent (0.1% Triton X-100 was the lowest concentration that would effectively wet internal flower structures). Closed flower buds were gently squeezed while submerged to remove air pockets. Flowers were then transferred to 50 mM NaH2PO4, pH 7.0, containing 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) (New Jersey Lab Supply, Livingston, NJ), subjected to vacuum for 2 to 3 min, and then incubated at 37°C. After incubation, flowers were rinsed in 50 mM NaH2PO4, pH 7.0, and cleared in two to three changes of 70% ethanol. Tissues used for sectioning were fixed in 0.5% glutaraldehyde for 2 hr before clearing.

Because it is a detergent, Triton X-100 might cause disruption of cell membranes, thereby influencing substrate entry and product exit from affected cells. In addition, the detergent might alter protein activity. We tried to limit these potential problems by using the minimum amount of Triton X-100 that would wet tissue efficiently and by exposing tissues to detergent for as short a time as possible (almost always <1 min). Variations of this technique have been reported previously, with no apparent adverse effects (Koltunow et al., 1990; Toriyama et al., 1991). Although disruption of membranes may have increased the
amount of product transfer between cells, the use of a wetting agent was essential to ensure that all organs were exposed to substrate.

Fluorometric GUS Assays

Fluorometric assays were performed on protein extracts of flowers with mature pollen, essentially as described by Jefferson et al. (1987). Each chimeric TUA1::GUS gene was represented by four to 10 independent lines (Figure 5A). Typically, flowers from at least four plants representing each independent line of each chimeric gene were pooled (20 to 80 flowers for each chimeric gene), with separate pools of flowers for each of the three sets of assays that were performed. For some lines, very few R seeds were available, so only one or two plants were used for each experiment. With very few exceptions, each line was represented by exactly the same number of flowers in the three experiments. All plants were grown in parallel in one incubator, and flowers were collected only from plants that appeared healthy and unstressed. Two flowers were collected from each plant; these were the two youngest flowers that had petals that were longer than the sepals. Flowers were excised just above the receptacle, immediately frozen in liquid nitrogen, and stored at -80°C. By using these procedures, we hoped to minimize variability due to insert segregation, sampling bias, environmental conditions, and developmental differences between flowers.

To extract proteins, frozen tissue and glass beads were ground in liquid nitrogen, which was allowed to evaporate while grinding. One milliliter of extraction buffer (50 mM NaH2PO4, pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% N-lauroylsarcosine, 0.1% Triton X-100) was added to the mortar and ground into a fine powder. When the extraction buffer was just thawed, it was removed to a 1.5-mL microcentrifuge tube, and centrifuged for 3 min at 4°C. The supernatant was transferred to a fresh tube and stored at -80°C until ready to assay. Fluorescence was measured on an SLM 4800C spectrofluorometer. Fluorescence of 100 and 1000 nM 4-methylumbelliferone was transferred to a fresh tube and stored at -80°C until ready to assay. Fluorescence was measured on an SLM 4800C spectrofluorometer. Fluorescence of 100 and 1000 nM 4-methylumbelliferone in 0.2 M sodium carbonate was used for calibration. Protein concentrations in the extracts were measured using the Bradford (1976) assay (Bio-Rad).

Sectioning

After incubating in X-gluc, flowers were rinsed in 50 mM NaH2PO4, pH 7.0, then fixed for 2 hr in the same buffer containing 0.5% glutaraldehyde. Tissue were then dehydrated and embedded in London White Resin (LR White) (Ted Pella, Inc., Tustin, CA) by soaking in the following solutions: 70% ethanol, 20 min, repeat; 95% ethanol, 20 min, repeat; three parts LR White to one part 95% ethanol, 50 min; LR White, 50 min, repeat twice. Tissues were transferred to Beem capsules filled with LR White resin and cured at 60°C, for 20 hr. Sectioning was performed on a Sorvall Porter-Blum MT2-B ultramicrotome using a diamond knife. Three-micron sections were floated onto 1 mL of filtered double-distilled H2O on acid washed glass slides, then dried down on a hot plate at 60°C. A drop of Permunt was added before placing a cover slip over the sections. Sections were viewed with dark-field optics and photographed using a Zeiss Axioskop microscope.

Staining Procedures

Staining of pollen with 4',6-diamidino-2-phenylindole (DAPI) (Sigma) has been described (Coleman and Goff, 1985). Anthers that had been assayed to localize GUS activity were removed from flowers. Isolated anthers were placed on a slide in 5 to 10 mL of 5 μg/mL DAPI. A coverslip was placed on the anther, and used to squeeze pollen out of anthers by lightly touching the coverslip and moving it in small circles. DAPI penetrated pollen within a few minutes, and was examined and photographed within an hour of staining.

Pollen Germination through Excised Style

Pollen germination medium consisted of 15% sucrose, 0.4 mM CaCl2, 0.4 mM H3BO3, 1% agar. After boiling, 2 to 3 μL of medium was pulled into a 200-μL pipette tip. Additional germination medium was pipetted onto glass slides and allowed to cool. The pipette tip was cut in half lengthwise, leaving a trough of exposed agar. A mature, unpollinated pistil from an untransformed plant was placed on the exposed agar in the cut pipette tip, with the ovary in contact with the agar, and the stigma and style extending past the pipette tip. Pollen from an anther of a pM5355-transformed plant was brushed onto the stigma. The stigma and style were excised from the ovary, and placed on germination medium on a glass slide. The tissue was laid on its side, sandwiched between agar on the slide and a small agar block. Slides were placed inside a sealed box at 100% humidity and 25°C. Pollen tubes usually grew through the style and out between the agar layers in 4 to 6 hr, and usually stopped growing shortly after emerging from the cut base of the style.

ACKNOWLEDGMENTS

We thank Neil E. Olszewski, Susan M. Wick, and Robert E. Pruitt for their valuable comments and suggestions. In addition, we thank Robert E. Pruitt for providing the unpublished procedure for isolating Arabidopsis genomic DNA and Steven D. Kopczak for providing the genomic clone containing the TUA1 5' flanking DNA. Excellent technical assistance was provided by Tammy J. Carda. This research was supported by the United States Department of Agriculture (Grant No. USDA-85-CRCR-1-1754) and the National Science Foundation (Grant No. NSF/DDBS-8918547). J.L.C. was supported by the National Institutes of Health Training (Grant No. GM07323) and a University of Minnesota Graduate School Doctoral Dissertation Fellowship.

Received December 27, 1991; accepted March 24, 1992.

REFERENCES


Preferential expression of an alpha-tubulin gene of Arabidopsis in pollen.
J L Carpenter, S E Ploense, D P Snustad and C D Silflow
Plant Cell 1992;4:557-571
DOI 10.1105/tpc.4.5.557

This information is current as of July 9, 2017