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Promoter sequences from a maize pollen-specific gene direct tissue-specific transcription in tobacco

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Summary. A set of 5′ promoter deletions from Zmg13, a genomic clone of a pollen-specific gene of maize, has been transcriptionally fused to a β-glucuronidase (GUS) reporter gene in the binary vector pBI101. Tobacco leaf disks were transformed and mature plants analyzed for GUS activity directed by the Zmg13 promoter constructs. Transgenic plants containing the 375 bp Zmg13 sequence from −314 to +61 relative to the transcription start site transcribed GUS RNA and expressed active GUS enzyme in mature pollen but not in leaves. Plants transformed with a 35S CaMV promoter-GUS transcriptional fusion expressed GUS RNA in leaves but not in pollen. Neither GUS RNA or active enzyme could be detected in pollen or leaves from plants containing a 124 bp Zmg13-GUS transcriptional fusion missing the putative Zmg13 TATA box. No GUS RNA or enzyme expression was not detected in non-transformed tobacco. RNA and GUS histochemical analysis of the T_1 generation confirmed that the temporal expression pattern of Zmg13-GUS transcription in tobacco followed that of the native gene in maize and that the Zmg13 promoter sequences from the maize gene are able correctly to direct genetically stable, tissue-specific gene expression in transgenic tobacco plants.

Key words: Maize – Pollen development – Tobacco – Promoter – β-Glucuronidase

Introduction

Development of plant anthers and the male gametophyte (pollen) requires expression of genes in both gametophytic and sporophytic tissues (Goldberg 1988; Mascarenhas 1989). Molecular genetic studies have characterized sets of genes expressed in a specific manner in tissues involved in pollen development. Several cDNA clones from libraries made from poly(A) RNA from mature pollen of maize and *Tradescantia paludosa* represent genes that are expressed in a pollen-specific pattern. The RNAs complementary to several of these clones are transcribed after microspore mitosis and accumulate thereafter, reaching their maximum concentrations in mature pollen (Mascarenhas et al. 1985; Stinson et al. 1987). Smith et al. (1987) used *in situ* RNA hybridizations to demonstrate tapetal-specific gene expression in tomato. Ursin et al. (1989) isolated and characterized a set of cDNA clones whose RNAs are expressed in specific cells of the sporophyte and developing gametophyte of tomato. One of these cDNAs and its corresponding gene, LAT52, was further characterized by Twell et al. (1989b) and found to be expressed in pollen, petals and anther walls. In maize, studies of RNA reassociation kinetics have shown that approximately 24000 genes are expressed during pollen development (Willing et al. 1988), 10% of which are pollen-specific (Stinson et al. 1987).

A study of pollen-specific regulation of maize gene expression has resulted in the isolation and characterization of a pollen-specific cDNA, Zmc13 (Hanson et al. 1989) and the corresponding genomic clone, Zmg13 (Hamilton et al. 1989). In this study, were report the characterization of the putative 5′ promoter region of Zmg13 by its ability to direct pollen-specific β-glucuronidase (GUS) gene expression in transgenic tobacco plants. A set of 5′ deletion fragments from the Zmg13 promoter was transcriptionally fused to the GUS coding region of the binary vector pBI101. Using *Agrobacterium tumefaciens*-mediated gene transfer, tobacco leaf disks were transformed with the various Zmg13 promoter-GUS constructs. Analysis of RNA isolated from transgenic tobacco plant tissues revealed that Zmg13 promoter sequences can direct pollen-specific GUS transcription. Additionally, GUS histochemical assays showed that active GUS enzyme is present in pollen from plants transformed with the Zmg13-GUS chimeric gene. Plants transformed with a transcriptional fusion between the 35S CaMV promoter and the GUS coding region expressed GUS RNA in leaves but not pollen and GUS histochemical assays did not reveal active GUS enzyme

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in pollen. Progeny analysis revealed that the introduced DNA was stably integrated into the tobacco genome and continued to be expressed in a pollen-specific manner. Nontransformed tobacco plants did not express GUS RNA in leaves or pollen and no active enzyme was found in pollen.

Materials and methods

Plasmid constructions. The genomic clone Zmg13 has been described (Hamilton et al. 1989). pCIB384 was cloned directly into the *Bam*HI site of pBI101. The promoter fragments corresponding to pCIB386–390 were initially subcloned into the *Sma*I site of Bluescript (Stratagene, San Diego, Calif.) and plasmid orientation and sequence fidelity were verified by dideoxy sequencing using Sequenase (US Biochemicals, Cleveland, Ohio). Following verification, restriction fragments from the Bluescript constructs containing the various Zmg13 promoter fragments were subcloned into the *Bam*HI (pCIB386) or *Hin*dIII-*Xba*I (pCIB387–390) polylinker sites of pBI101 (Clontech, Palo Alto, Calif.); the new constructs were used to transform MC1022 cells and each transcriptional fusion verified by restriction mapping. The plasmid pBI101 contains a promoter-less GUS (Jefferson et al. 1986) cassette fused to the nopaline synthase polyadenylation region in the binary vector pBIN19 (Bevan 1984) and a low copy number RK2 origin of replication.

Plant material and transformations. Six-week-old sterile shoot tip cultures of *Nicotiana tabacum* var. Havana 38 were transformed by a leaf disk *Agrobacterium*-mediated transformation-regeneration method (Horsch et al. 1985). The *A. tumefaciens* strain was A136 (Watson et al. 1975) containing the helper plasmid pCIB542, a version of EHA101 (Hood et al. 1986) modified by replacing the kanamycin drug resistance gene with a spectinomycin resistance gene (G. Helmer, unpublished results). Transformation of *Agrobacterium* was by the procedure of Holsters et al. (1978). Transformation mixes were plated on nutrient agar/kan_{50} (0.8% w/v Difco nutrient broth, 50 mg/l kanamycin) and isolated colonies cultured in YEP/kan_{50} media (1% w/v Difco bacto peptone, 1% w/v Difco yeast extract, 85 mM NaCl). To verify the presence and size of the transforming plasmid, minipreparations were done according to the alkaline lysis protocol in Maniatis et al. (1982) except the cell pellet was digested with 20 mg/ml lysozyme prior to the alkaline-SDS incubation step.

Leaf disks were infected with *Agrobacterium*, plated and grown for 3 days on shoot-inducing medae without selection. Disks were transferred to fresh shooting media containing 500 mg/l carbenicillin, 100 mg/l kanamycin every 3 days for 2 weeks, then once per week thereafter. Shoots formed within 5 weeks, at which time a single shoot per leaf disk was transferred to root-inducing media with antibiotics. Normally roots formed within 2 weeks, at which time the plantlets were transferred into GA-7 containers (Magenta, Chicago, Ill.) with root media plus antibiotics. Once 4–6 leaves formed, the shoot tips were aseptically transferred to new containers to maintain *in vitro* shoot tip cultures of each line and the rest of the plant was potted in soil for growth in the greenhouse. Prior to flower bud opening, each plant's flower stalk was bagged to prevent cross-pollination.

Nucleic acid manipulations. Genomic DNA minipreparations were done on 0.5–1.0 g leaf tissue frozen in liquid N_2 and ground to a powder in a 2.0 ml microcentrifuge tube using a disposable pellet pestle (Kontes, Vineland, NJ). The tissue was incubated for 15–30 min at 60° C in 1 ml extraction buffer (1% hexadecyltrimethyl ammonium bromide (CETAB); 50 mM TRIS-HCl, pH 8.0; 10 mM EDTA; 0.7 M NaCl; 0.5% polyvinylpyrrolidone, mol.wt. 360000 dalton) followed by extraction in chloroform-isoamyl alcohol (24:1). One-tenth volume of CETAB solution (10% CETAB; 0.7 M NaCl) was added and the solution reextracted with chloroform-isoamyl alcohol. One volume of precipitation buffer (1% CETAB; 50 mM TRIS-HCl, pH 8.0; 10 mM EDTA) was added, the solution was gently mixed and then microcentrifuged. The pellet was dissolved in high salt TE (1 M NaCl; 10 mM TRIS-HCl, pH 8.0; 1 mM EDTA) at 65° C then reprecipitated with ethanol. The DNA pellet was resuspended, extracted with phenol-chloroform (1:1) followed by chloroform alone, and precipitated with sodium acetate and ethanol. Gene copy number was determined by blotting digested DNA onto Duralon membrane (Stratagene) using a capillary transfer procedure similar to Maniatis et al. (1982). Nucleic acid was fixed to the membrane by UV-crosslinking followed by drying for 1 h at 80° C. Prehybridizations were for 4–7 h at 68° C in 5× SSPE (SSPE is 0.18 M NaCl, 10 mM sodium phosphate, 1 mM EDTA), 5× Denhardt's solution, 0.5% SDS, 10% dextran sulfate, 0.1 mg/ml sheared calf thymus DNA. Hybridizations were done overnight in fresh solution with $1.5–2 \times 10^6$ cpm/ml of probe synthesized with the BRL random-primer kit (Bethesda Research Laboratories, Bethesda, Md.).

Total RNA from tobacco tissues was isolated by a minipreparation procedure (Verwoerd et al. 1989) and analyzed by formaldehyde gel electrophoresis (1–2 μg total RNA per lane) followed by blotting onto Duralon membrane according to Maniatis et al. (1982). The integrity of the RNA was checked by agarose gel electrophoresis and ethidium bromide staining. The fluorescence of the nucleic acids also served to verify that approximately equal amounts of RNA were loaded in each lane. Hybridization conditions were as described above for genomic DNA analysis except for the progeny analysis shown in Fig. 5. In that case, the prehybridization solution was 1.5× SSPE, 1% SDS, 0.5% BLOTTO (10% w/v non-fat dry milk, 0.2% sodium azide) and 0.1 mg/ml sonicated herring sperm DNA. For hybridization, 5×10^5 cpm/ml was added to fresh buffer. The probe contained the GUS coding region sequences and was isolated by *Xba*I/*Sst*I double digestion of pBI101 followed by low-melting point agarose gel electrophoresis. DNA was extracted from the agarose by melting at 65° C and adding one-tenth volume of buffer (4.6 M

NaCl; 0.1 M TRIS-HCl, pH 8.0; 10 mM EDTA) followed by two phenol extractions, one phenol-chloroform-isoamyl alcohol (25:24:1) extraction and one chloroform-isoamyl alcohol extraction. DNA was recovered by precipitation with sodium acetate and ethanol.

Isolation of maize pollen from different developmental stages and analysis of the corresponding RNA was described in Stinson et al. (1987). Hybridization conditions were similar to those described for the tobacco RNA analysis.

Polymerase chain reaction analysis of genomic DNA minipreps. To verify that individual transformants carried GUS coding sequences, 15mer primers were synthesized and genomic DNA analyzed by PCR carried out using 25–50 µl reactions with *Taq* polymerase as described by Perkin-Elmer Cetus (Norwalk, Conn.). The primer sequences were ACTGGCAGACTATCC and CAACGCTGACATCAC for the 5′ and 3′ primers, respectively. The 5′ primer hybridizes to a sequence approximately 420 bp downstream of the GUS coding region initiator AUG. Typically, 35 PCR cycles were performed using approximately 1 µg of genomic minipreparation DNA with 1 min denaturation at 94° C, 30 s annealing at 46° C, and 30 s polymerization at 72° C. The expected PCR product was approximately 230 bp long and easily visualized by agarose gel electrophoresis and ethidium bromide staining.

Histochemical assays. Intact pollen was collected from developing flower buds and mature flowers and assayed for active GUS enzyme by direct addition to the GUS reaction buffer (100 mM $NaPO_4$; 10 mM EDTA; 0.5 mM $K_3Fe(CN)_6$; 0.5 mM $K_4Fe(CN)_6$; 1 mM X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide); 0.06% Triton X-100). Blue color development was evident after a few hours incubation at 37° C. Other plant tissues assayed histochemically were finely chopped in the GUS reaction buffer to facilitate GUS substrate infiltration. To determine developmental stage, pollen grains of various sized tobacco flower buds were stained with 4′,6-diamidino-2-phenylindole (DAPI). The anther walls were chopped in a few drops of buffer (5 µl of 1 mg/ml DAPI in H_2O added to 1 ml of 45 mM $MgCl_2$; 30 mM sodium citrate; 20 mM MOPS, pH 7.0; 1 mg/ml Triton X-100; 12.5% w/v sucrose) to free individual grains and photographed with a Leitz Orthoplan microscope.

Results

Zmg13-GUS transcriptional fusions and tobacco transformations

A schematic diagram of the Zmg13-GUS transcriptional fusions is shown in Fig. 1. The plasmid pCIB384 contains the entire genomic clone Zmg13 inserted upstream of the pBI101 GUS coding region. pCIB386 was constructed with the 3.1 kb *Bam*HI-*Ppu*MI 5′ end fragment of the Zmg13 genomic clone. The pCIB387, 388, and 389 plasmids were designed as progressive 5′ deletions

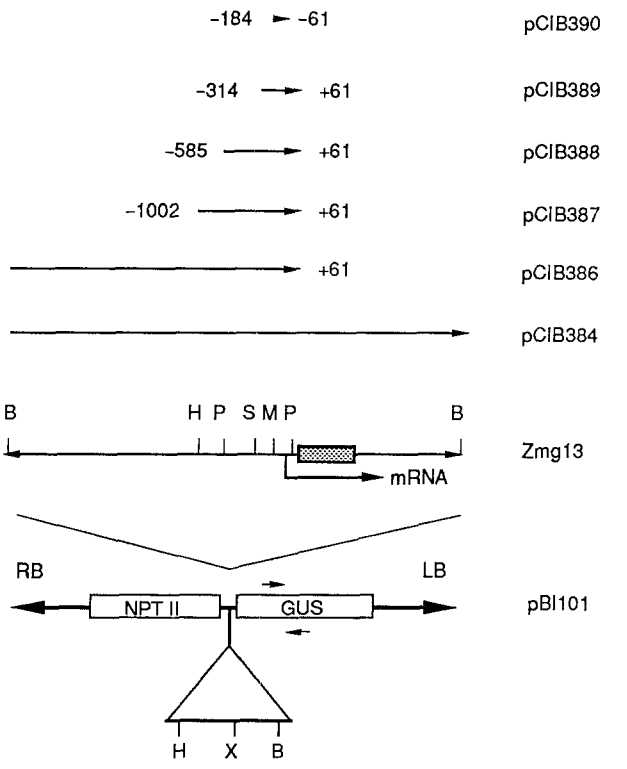

Fig. 1. Construction of Zmg13-GUS transcriptional fusions in pBI101. Plasmids pCIB386–390 were initially cloned into the *Sma*I site of Bluescript plasmid then directionally subcloned into the *Hin*dIII-*Xba*I sites of the pBI101 polylinker. pCIB384 contains the entire Zmg13 genomic clone including the transcribed and coding regions and was directly cloned into the *Bam*HI site of pBI101. pCIB386 contains approximately 3.1 kb from the 5′ region of Zmg13. In the Zmg13 schematic, the *arrow* labelled mRNA locates the transcription start site and direction while the *stippled area* indicates the gene coding region. The approximate locations of the two polymerase chair reaction (PCR) primers are indicated by *arrows* on the the β-glucuronidase (GUS) coding region of pBI101. B, *Bam*HI; H, *Hin*dIII; P, *Ppu*MI; S, ScaI, M, *Mbo*II, X, *Xba*I

of the *Bam*HI-*Ppu*MI 5′ end fragment, and contain 1063, 646 and 375 bp of sequence upstream from the indicated *Ppu*MI site. In all these cases, the *Ppu*MI site which forms the 3′ end of the fragment is located 61 bp downstream of the transcriptional start site of Zmg13 RNA and 66 bp upstream of the translational AUG initiator (Hanson et al. 1989). The pCIB390 construction resulted from the fortuitous isolation of a 124 bp fragment which starts and ends 184 and 61 bp upstream of the transcriptional start site, respectively. This fragment does not contain the putative Zmg13 −34 TATA box described by Hamilton et al. (1989). The 15.25 kb plasmid pCIB907 containing a kanamycin resistance gene and a 1 kb *Bgl*II-*Bam*HI CaMV 35S promoter fragment (from pCIB710, Rothstein et al. 1987) transcriptionally fused to the GUS coding region was used as a known GUS positive control for the tobacco leaf disk transformation experiments.

Shoot tip cultures of *N. tabacum* var. Havana 38 were transformed with each transcriptional fusion as described in Materials and methods. Once rooted, plants were transferred to greenhouse pots, genomic DNA, was

Table 1. Polymerase chain reaction and pollen β-glucuronidase (GUS) assays of kanamycin-selected shoots

pCIB plasmid (fragment)	Number of positives		
	Kanamycin selection	PCR	GUS assay
Nontransformed control	–	0	0
907 (35S-GUS)	14	12	0
384 (entire Zmg13 clone)	7	6	0
386 (*Bam*HI-*Ppu*MI)[a]	15	10	11
387 (*Hin*dIII-*Ppu*MI)	17	17	17
388 (*Ppu*MI-*Ppu*MI)[b]	16	12	11
389 (*Sca*I-*Ppu*MI)	20	17	17
390 (−184 to −61)	13	13	0

[a] Individual 386–42 gave a positive GUS assay but a negative PCR result

[b] Individual 388–22 gave a positive GUS assay but a negative PCR result. Individuals 388–10 and −37 gave negative GUS assays but positive PCR results

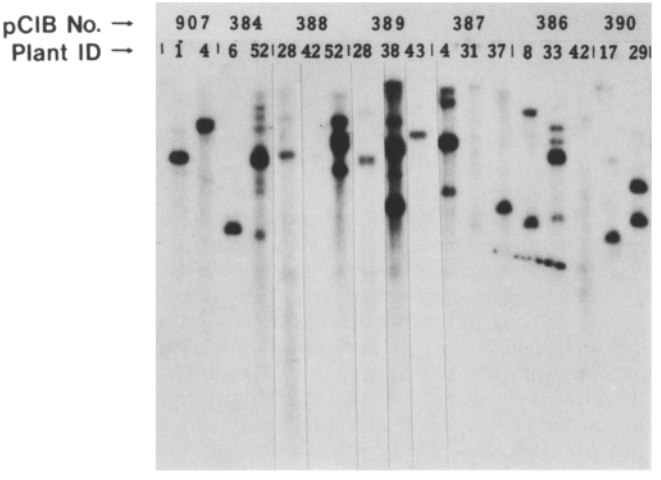

Fig. 2. Genomic GUS copy number analysis of transformants. Genomic DNA was isolated from mature transformed greenhouse plants and digested with *Bam*HI. DNA blots were probed with radiolabelled GUS coding sequence DNA. pCIB No. denotes the construct used in the transformation experiment while Plant ID denotes the number assigned to the individual Kanamycin selected host

isolated from a portion of a leaf and analyzed by PCR to verify the presence of GUS coding sequences in the plant. Most of the shoots which survived kanamycin selection gave a positive result in the PCR analysis. The results from the experiments with each construct are shown in Table 1. To determine the GUS copy number in selected transformants, genomic DNA was digested to completion with *Bam*HI and analyzed by agarose gel electrophoresis and Southern blotting using a probe from a *Xba*I-*Sst*I restriction fragment of pBI101 which contains the entire GUS coding region sequence. Since *Bam*HI digests outside the region to which the probe hybridizes, the number of radioactive bands in Fig. 2 directly indicates that the GUS copy number varied from one to several copies in individual transformants. A longer exposure of the Southern blot verified the copy numbers deduced from Fig. 2 (data not shown).

Pollen analysis for GUS activity

Pollen from mature flowers was analyzed by *in situ* staining for GUS activity. These results are summarized in Table 1 and compared to results from the PCR screening experiments. In the GUS-positive plants, generally the entire pollen grain was blue, although some individuals from the pCIB388 and 389 transformations had both yellow and blue areas on the same grain. At this point we cannot explain this differential staining although it could be due to variations of promoter expression within the pollen cell. No GUS-positive pollen grains were obtained from Havana 38 or Petite Havana SR1 nontransformed controls or from plants transformed with pCIB907 (35S-GUS), pCIB384 (entire Zmg13 clone-GUS), or pCIB390 (124 bp promoter fragment minus TATA-GUS). It should be noted that Benfey and Chua (1989) reported CaMV 35S promoter activity in transgenic petunia pollen, perhaps reflecting differences in promoter activity between tobacco and petunia. In addition, Twell et al. (1989a) showed that in transient gene expression assays of tobacco pollen transformed by bombardment with microprojectiles coated with DNA, a lowel level of 35S promoter activity was detected shortly after bombardment, although GUS expression dropped to background levels within 2 days. Figure 3 shows a light micrograph of pollen from a nontransformed Havana 38 plant and pollen from two plants transformed with pCIB387. The transformed individuals depicted in Fig. 3 are from the T_1 generation which is described in more detail below.

To corroborate the Southern analysis of GUS gene copy number, the ratio of GUS-positive (blue) to GUS-negative (yellow) pollen grains was determined for pCIB386–389 transformants. Five individuals carried single copy insertions according to the Southern analysis, 387–37, 388–28 and 42, and 389–28 and 43. All of these except 388–28 exhibit a 1:1 ratio of GUS^+:GUS^- pollen, indicative of a single copy insertion (data not shown). pCIB388–28 had a 97:3 ratio indicating multiple GUS gene insertions. Since both the GUS coding region and the coding for kanamycin resistance were on the same plasmid vector used in these experiments, we sought to compare the segregation pattern of GUS^+ pollen with kanamycin resistance in 388–28 progeny seedlings. Thus, we plated 50 seeds from 388–28 on kanamycin media to determine the ratio of kanamycin resistant to sensitive seedlings. This test gave 37 resistant to 12 sensitive seedlings (1 seed did not sprout), a 3:1 ratio indicating a single copy insertion of the kanamycin resistance gene. We cannot explain the inconsistency between the GUS assay pollen results and the gene copy number data from the Southern blot and seedling platings on kanamycin.

Tissue specific expression as measured by RNA blot analysis

Total RNA was isolated from mature pollen and leaf tip samples from individual transformants. Northern

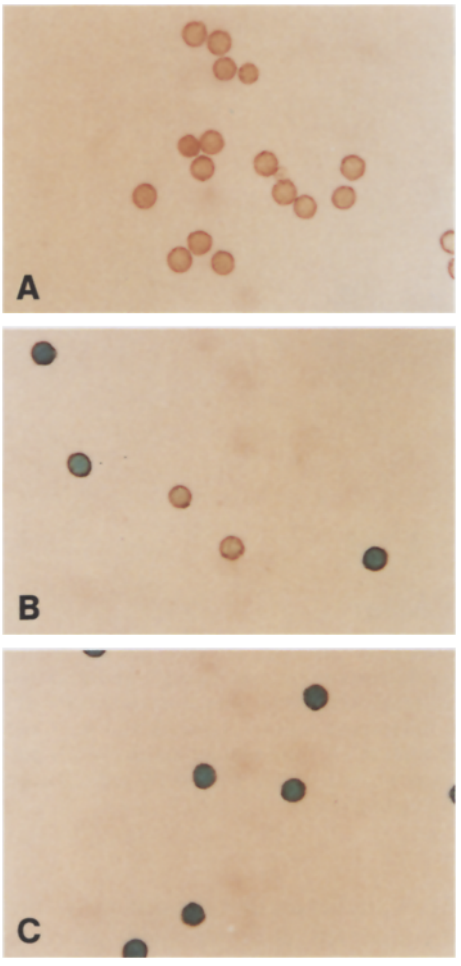

Fig. 3. Light micrograph of pollen analyzed by GUS histochemical assay. Mature pollen from nontransformed Havana 38 and two individuals from the T_1 generation whose line has been transformed with a single copy of the pCIB387 transcriptional fusion was tested by the GUS histochemical assay. The blue pollen in the pCIB387 samples confirm the transcriptional activity of the Zmg13-GUS fusion and its stable transmission into the T_1 generation. A, nontransformed Havana 38 pollen; B, T_1 transformant pCIB–387–37-O heterozygous GUS^+; C, T_1 transformant pCIB387–37-K homozygous GUS^+

blots were probed with radiolabelled GUS-coding sequences and the results shown in Fig. 4 indicate that the 375 bp *Sca*I-*Ppu*MI promoter fragment of Zmg13 is sufficient to direct pollen-specific GUS transcription. A longer exposure of the blot showed that transcribed GUS RNA was not detected in any of the leaf tissue samples except that from the 35S-GUS transformant (data not shown). Conversely, GUS RNA was not detected in pollen from the 35S-GUS plant, however it was detectable in pollen from plants transformed with pCIB386, 387, 388 and 389. GUS RNA was also not detected in pollen RNA isolated from a plant transformed with pCIB384 (containing the entire Zmg13 genomic clone) or pCIB390 (with the 124 bp 5′ fragment minus the TATA box).

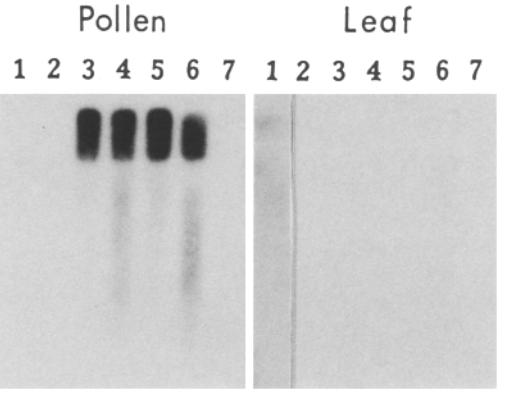

Fig. 4. RNA blot analysis of pollen and leaf RNAs showing pollen-specific GUS expression from the Zmg13-GUS fusions. Total RNA was isolated from leaf tip and pollen from plants transformed with the 35S-GUS pCIB907 or the Zmg13-GUS fusions. A blot containing 1–2 μg RNA per lane was probed with radiolabelled GUS coding sequence DNA. Lane 1, pCIB907; 2, pCIB384; 3, pCIB388; 4, pCIB389; 5, pCIB387; 6, pCIB386; 7, pCIB390

Expression in T_1 individuals

T_0 plants were allowed to self-pollinate and mature pollen from T_1 individuals was histochemically tested for GUS activity. Each selfed parent plant from pCIB386–389 yielded T_1 progeny which expressed active GUS enzyme in mature pollen while nontransformed plants and progeny from lines transformed with pCIB907, 384 or 390 did not express GUS. The segregation pattern of GUS^+ pollen in the T_1 individuals from the single copy T_0 plant pCIB387–37 (1063 bp Zmg13 promoter fragment-GUS) was 5 homozygous GUS^+: 13 heterozygous GUS^+: 6 homozygous GUS^-. This approximates the 1: 2: 1 pattern expected from a parent containing a single insertion of the Zmg13-GUS construct. Figure 3 shows light micrographs of pollen from the T_1 individuals pCIB387–37-O which is heterozygous for GUS activity in pollen, and 387–37-K which is homozygous GUS^+. Additionally, leaf, pistil, anther, stem and root tissue from T_1 individuals 387–37-G and 389–43-A (both homozygous GUS^+ in pollen) were tested with the GUS histochemical assay and found to be negative. A pCIB907 (35S-GUS) positive control plant expressed GUS in leaf, stem and pistil tissue (data not shown).

To verify that the Zmg13 promoter was regulating transcription in the transgenic T_1 tobacco plants in a pattern similar to that in maize, RNA was isolated from mature pollen, petal, pistil, root, sepals, leaf and stem of the T_1 plant 387–37-K. RNA blots were probed with radiolabelled GUS DNA and the results are shown in Fig. 5. Since in maize plants Zmg13 is expressed only in pollen (Hanson et al. 1989), the results from Figs. 4 and 5 and the GUS histochemical assays indicate that the Zmg13 promoter fragments, including the 375 bp region from pCIB389, can direct transcription in transgenic tobacco with an expression pattern similar to that of maize and that this ability is transmitted to progeny.

The temporal expression of Zmg13 during microspore development was examined. Total RNA was iso-

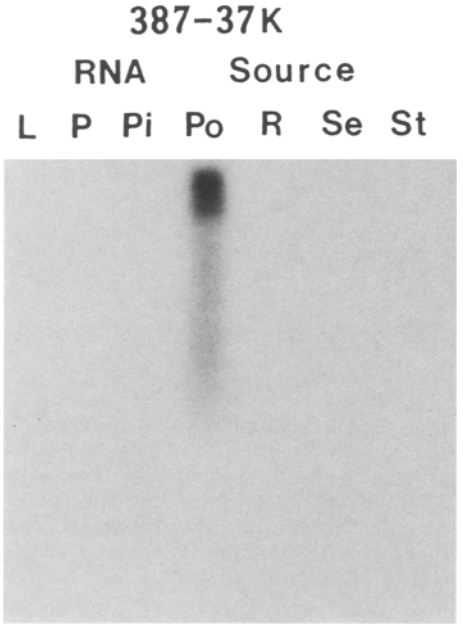

Fig. 5. RNA blot analysis of various tissues from a T_1 individual. Total RNA was isolated from individual tissues of the T_1 transformed line pCIB387–37-K. An RNA blot containing 1–2 μg RNA per lane was probed with radiolabelled GUS coding sequence DNA. L, leaf; P, petal; Pi, pistil; Po, mature pollen; R, root; Se, sepal; St, stem

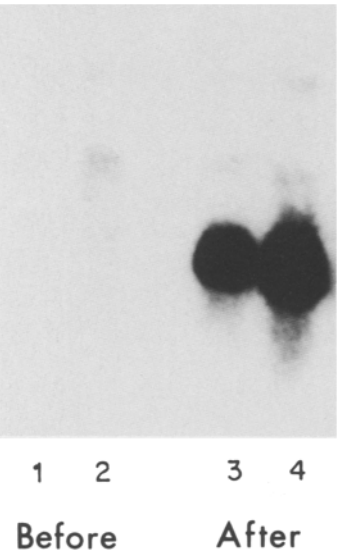

Fig. 6. Analysis of RNA in microspores showing that the Zmg13 gene is expressed after microspore mitosis. Lanes 1 and 2: 5 and 10 μg, respectively, of total RNA from immature maize (W-22) microspores prior to microspore mitosis. Lanes 3 and 4: 5 and 10 μg, respectively, of total RNA from mature maize (W-22) pollen. The RNA blot was probed with a 513 bp by radiolabelled fragment of the Zmg13 coding region

lated from maize microspores prior to microspore mitosis and from mature maize pollen. The RNA was separated by formaldehyde gel electrophoresis and blots probed with a 513 bp fragment of the Zmg13 coding region (positions +401 to +914). The results in Fig. 6 show that Zmg13 is expressed only after microspore mitosis. To study the expression of the Zmg13-GUS construct in T_1 individuals, pairs of anthers were dissected from flower buds of various sizes. Pollen grains from one anther of the pair were stained with DAPI to determine developmental status while grains from the other member of the anther pair were checked for Zmg13 promoter activity with the GUS histochemical assay. The T_1 plant used was pCIB387–37-K, a homozygous GUS^+ individual containing a single insertion of the 1063 bp Zmg13 promoter fragment-GUS coding region construct. Figure 7 compares the results from the DAPI developmental staging (A_1–A_3) with the timing of GUS expression (B_1–B_3). Flower buds were sized according to the length of the pistil, measuring from the tip of the stigma to the junction with the ovary. The photomicrographs are of developing grains from buds having pistils of 4, 8 and 12 mm, respectively. Figure 7 A_1 shows that microspore mitosis has not yet occurred and expression of GUS driven by the Zmg13 promoter has not been initiated (B_1). In fact, no grains were found to possess active GUS enzyme in grains from anthers of this size. In A_2, microspore mitosis has occurred and B_2 shows that, at this stage, GUS expression has begun. Indeed, 30% of the grains from these anthers were expressing significant levels of GUS as evidenced by the blue-colored grains. A_3 indicates that the generative nuclei become more condensed as development proceeds and the GUS histochemical assay shows an increase in GUS expression. In fact, 92% of the grains from this anther were GUS^+. At this stage, the only grains which were not stained blue had a lobed appearance and more closely resembled pre-mitotic than post-mitotic microspores (compare pre-mitotic microspores of B_1 to yellow grains of B_3). Similar results were obtained from plants transformed with the 375 bp Zmg13-GUS construct of pCIB389 (data not shown).

Discussion

In this study, we have shown that the promoter from the pollen-specific maize gene Zmg13 can direct pollen-specific expression in transgenic tobacco. A set of 5′ promoter deletion fragments from a Zmg13 genomic clone was transcriptionally fused to the GUS coding region of the binary vector pBI101 and transformed into tobacco using *Agrobacterium*-mediated leaf disk transformation. Histochemical GUS assays and RNA analysis verified the tissue specificity of expression of the GUS reporter gene. Plants transformed with pCIB386, 387, 388 or 389 expressed GUS enzyme only in pollen; no case was found where a transgenic plant with GUS^+ pollen expressed GUS in any other tissue. The CaMV 35S positive control plants (pCIB907) expressed GUS in leaf, stem and pistil tissues. The GUS histochemical assays and Northern blot analysis with pCIB389 show

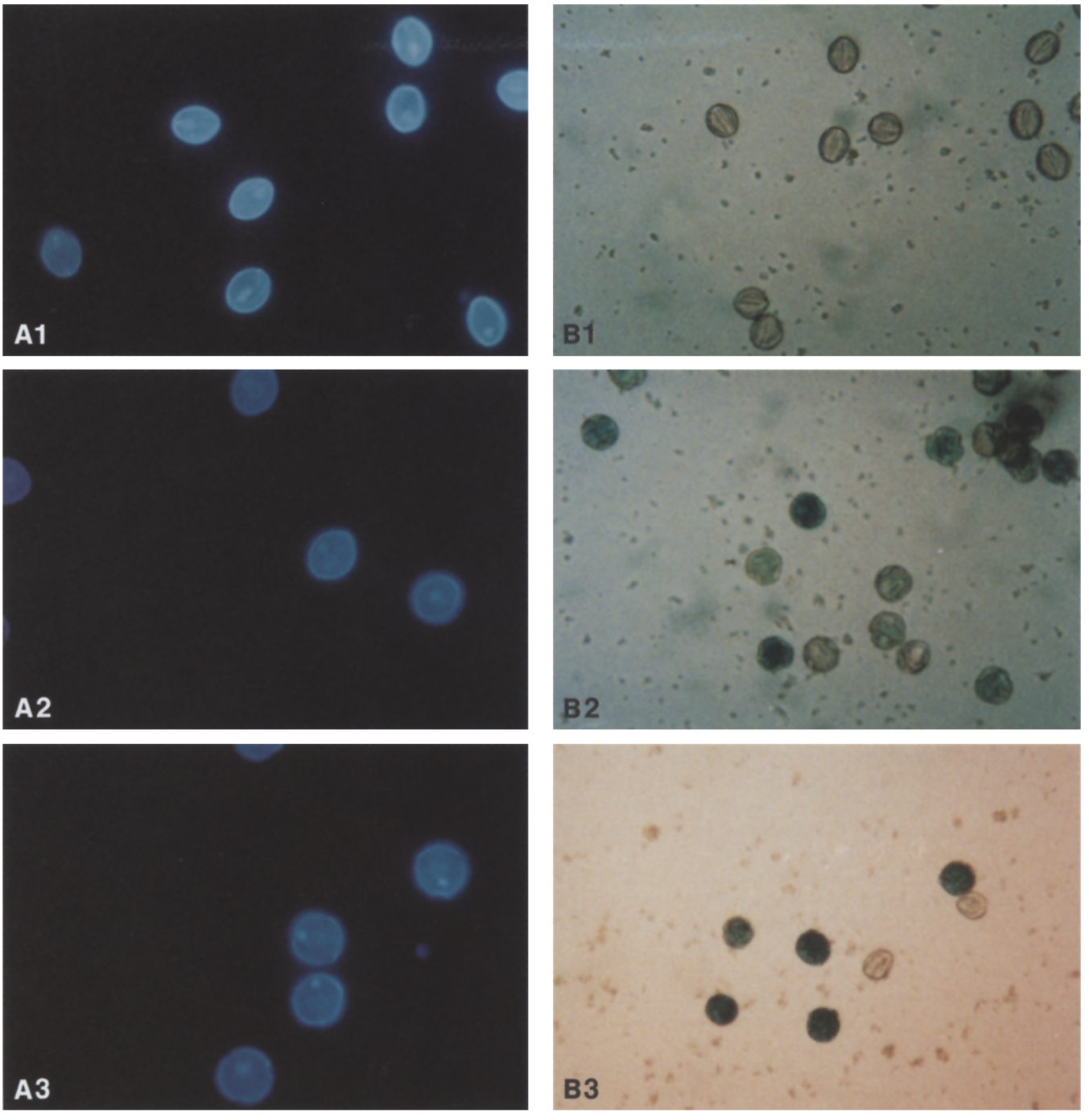

Fig. 7. Zmg13-GUS expression as pollen develops. Flower buds of various lengths were isolated and two anthers dissected from each bud. Grains from one anther were stained with 4′,6-diamidino-2-phenylindole (DAPI) and the other with GUS histochemical assay reagents. Bud development was staged by pistil length measurement. Set A: fluorescence micrographs of developing pollen stained with DAPI. Set B: light micrographs of pollen grains assayed for GUS activity. 1, 2 and 3: grains from buds with 4, 8 and 12 mm pistils, respectively

that sequences within the first 314 bp upstream and 61 bp downstream from the transcriptional start site are sufficient for pollen-specific expression from the Zmg13 promoter. The negative results from pCIB390 transformants containing the 124 bp fragment but without the putative Zmg13 −34 TATA box indicated the importance of sequences between −314 and −184 and/or downstream of the −60 region of the promoter for transcriptional activity. GUS RNA was not detected either in the pollen or leaf samples from pCIB390, which verified the construct was not being transcribed in those tissues. In this 124 bp promoter fragment, there are two TATTT motifs located 16 and 75 bp upstream from the 3′ end, although we have no evidence that either is active as a substitute TATA element.

In their analysis of the Zmg13 promoter for se-

quences possibly involved in pollen gene expression, Hamilton et al. (1989) reported 21 different sequence element homologies with promoters in three other genes expressed in pollen, *Adhl, waxy* and LAT52. Our study should facilitate testing these sequence elements since 9 of the reported elements lie within the 375 bp region tested in pCIB389. Other groups have isolated tissue-specific gene promoter regions which can direct reporter gene transcription in pollen. McCormick et al. (1989) reported that 1.4 kb of 5′ flanking DNA of the anther-specific *lat59* gene of tomato is sufficient to direct pollen-specific expression of a GUS reporter gene in tobacco and tomato. Benfey and Chua (1989) obtained pollen expression of a GUS reporter gene in both transgenic petunia and tobacco. Their transcriptional fusion included the −1800 to −285 promoter region from 5-enolpyruvyl-shikimate-3-phosphate synthase inserted in front of the −90 to +8 region of the CaMV 35S promoter fused to the GUS coding sequence. Our results further delineate the minimum DNA sequence requirements for pollen-specific gene expression. The T_1 generation transcribed the Zmg13-GUS construct in a tissue-specific and temporal fashion similar to the pattern expressed in maize by intact Zmg13. Additionally, the results from our experiments indicate that the components necessary for transcriptional regulation of the maize Zmg13 promoter are present in developing tobacco pollen. Some transformation experiments in which a monocot gene was introduced into a dicot such as tobacco have resulted in low levels of mRNA expression, presumably as a result of difficulty in processing monocot pre-mRNAs within a dicot host. The results from these Zmg13 promoter fusions and the zein gene promoter experiments of Schernthaner et al. (1988) indicate that monocot promoters can be recognized by the transcriptional apparatus of dicots.

In our hands, the GUS histochemical assay provided a convenient test for pollen transcriptional activity of the Zmg13-GUS fusions. Nontransformed tobacco pollen was easily distinguishable from pollen transformed with actively expressing GUS reporter sequences. Other studies have also noted the absence of GUS activity in nontransformed tobacco pollen (Benfey and Chua 1989; McCormick et al. 1989). In contrast, Plegt and Bino (1989) reported that an endogenous GUS gene was expressed in nontransformed SR1 tobacco pollen. This conflict remains puzzling since in no case could we detect blue color among the hundreds of pollen grains examined from nontransformed SR1 or Havana 38 flowers.

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