Expression of the Arabidopsis Transposable Element *Tag1* Is Targeted to Developing Gametophytes

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ABSTRACT

The Arabidopsis transposon *Tag1* undergoes late excision during vegetative and germinal development in plants containing 35S-*Tag1*-GUS constructs. To determine if transcriptional regulation can account for the developmental control of *Tag1* excision, the transcriptional activity of *Tag1* promoter-GUS fusion constructs of various lengths was examined in transgenic plants. All constructs showed expression in the reproductive organs of developing flowers but no expression in leaves. Expression was restricted to developing gametophytes in both male and female lineages. Quantitative RT-PCR analysis confirmed that *Tag1* expression predominates in the reproductive organs of flower buds. These results are consistent with late germinal excision of *Tag1*, but they cannot explain the vegetative excision activity of *Tag1* observed with 35S-*Tag1*-GUS constructs. To resolve this issue, *Tag1* excision was reexamined using elements with no adjacent 35S promoter sequences. *Tag1* excision in this context is restricted to germinal events with no detectable vegetative excision. If a 35S enhancer sequence is placed next to *Tag1*, vegetative excision is restored. These results indicate that the intrinsic activity of *Tag1* is restricted to germinal excision due to targeted expression of the *Tag1* transposase to developing gametophytes and that this activity is altered by the presence of adjacent enhancers or promoters.

THE movement of transposable elements can be regulated by host genes and by sequences within the element itself (reviewed by Labrador and Corces 1997; Fedoroff 2002; Kidwell and Lisch 2002; Labrador and Corces 2002). One form of regulation is selective transposition in germinal *vs.* somatic lineages. Such regulation has been described in *Drosophila melanogaster* where *P* elements specifically excise in the germline but not in the soma (reviewed by Rio 2002). This regulation is the result of alternative splicing that produces an 87-kD transposase in the germinal cells but a 66-kD repressor protein in the somatic cells. The Drosophila transposon *hobo* also displays germline specificity, which is due to transcriptional regulation of *hobo* transposase expression (Calvi and Gelbart 1994). In *Caenorhabditis elegans*, a contrasting pattern of tissue-specific control is observed for the *Tc1* element. The Bristol N2 strain shows transposition in the soma but complete silencing in the germline while the Bergerac BO strain shows germinal transposition (reviewed by Plasterk and van Luenen 2002). Germinal transposition is associated with *mut* genes, which are either *Tc1* elements active in the germline or nonmobile genes required for suppression of germline transposition and containing a transposon (reviewed by Plasterk and van Luenen 2002). Recently, suppression of germline transposition in *C. elegans* has also been linked to RNA interference (Ketting *et al.* 1999; Tabara *et al.* 1999). Mutations that release germline suppression at the *mut-7* locus inactivate a protein with a motif found in RNaseD, DNases, and helicases (Ketting *et al.* 1999).

In plants, the maize transposon *Mutator* (*Mu*) shows different excision behavior in vegetative tissues and the lineages that give rise to the gametes (reviewed by Walbot and Rudenko 2002). In vegetative cells, excision is limited to terminally dividing cells producing small revertant sectors. In germinal lineages, replicative insertions are frequent, occurring in premeiotic, meiotic, and postmeiotic cells, while excision events are rare. Several mechanisms that can explain the control of *Mu* excision have been proposed, including regulation of transposase activity, blockage of transposase binding by competing transcription factors, and enhanced gap repair early in development (Walbot and Rudenko 2002). To elucidate these mechanisms, various *Mu* constructs have been tested in maize. These studies have shown that the *MuDR* terminal inverted repeat (TIR) promoters are developmentally regulated in both somatic and germinal tissues (Raizada *et al.* 2001). Additionally, a 35S-*mudrA* cDNA construct results in the same late developmental excision pattern observed with endogenous transposons, suggesting post-translational con-

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trol of the transposase in vegetative cells (Raizada and Walbot 2000).

Another example of germinal regulation has been described for the maize element *Activator* (*Ac*), for which somatic and germinal reversion rates are usually correlated. Elements that show high frequencies of somatic excisions generally produce many germinal revertants and vice versa (reviewed by Kunze 1996; Kunze and Weil 2002). However, Giedt and Weil have recently described a host-encoded factor termed *LAG1-O*, which uncouples somatic and germinal excision rates without affecting *Ac* transposase mRNA levels in reproductive organs (Eisses *et al.* 1997; Giedt and Weil 2000). Another regulated element is *Tag1* from *Arabidopsis thaliana*. *Tag1* is a 3.3-kb DNA transposon that is a member of the *hAT* superfamily, which includes *Ac* from maize (Calvi *et al.* 1991; Liu and Crawford 1998b and references therein). *Tag1* produces one major (2.3 kb) and several minor (between 1.0 and 1.2 kb) transcripts in both Arabidopsis and rice (Liu and Crawford 1998b; Liu *et al.* 1999). The major transcript is thought to encode the transposase, which contains an N-terminal DNA-binding domain that contains a C_2HC zinc finger (Mack and Crawford 2001). This domain interacts specifically with the subterminal AAACCC repeats near the 5′ end and the subterminal TGACCC repeats near the 3′ end of *Tag1* (Mack and Crawford 2001). *Tag1* shows developmental control in that excision events are typically late. Using a 35S-*Tag1*-GUS marker construct, it was found that *Tag1* is capable of both vegetative and germinal excision (Frank *et al.* 1997; Liu and Crawford 1998a). Vegetative excision typically results in the appearance of small blue sectors in leaves, roots, stems, petals, and siliques, indicating that transposition occurs preferentially in terminally dividing cells (Liu and Crawford 1998a). This pattern is reminiscent of the late vegetative excision behavior of *Mu* described above. The delayed excision of *Tag1* is also found in the germinal lineages and leads to independent germinal revertants that appear at frequencies from 0 to 27% (Liu and Crawford 1998a). To determine how *Tag1* transposition is regulated, various deletions of the 35S-*Tag1*-GUS construct were made. When the *Tag1* promoter was deleted (*i.e.*, replaced by the 35S promoter), somatic excision patterns and frequencies remained essentially the same as for 35S-*Tag1*-GUS but germinal excision was lost (Liu *et al.* 2001b). This result indicated that the *Tag1* promoter is needed for germinal excision. To investigate the properties of the *Tag1* promoter, *Tag1*-GUS fusion constructs were made and examined for expression in Arabidopsis plants. The results are described below. They show that expression is targeted to postmeiotic germinal lineages (*i.e.*, the developing gametophyte) and is very weak in vegetative tissues. These results led us to reexamine the vegetative excision activity of *Tag1* in the absence of any nearby 35S promoter. We found that *Tag1*, by itself, shows excision that is restricted to germinal lineages.

MATERIALS AND METHODS

Plant material and transformation: Plants were grown in peat soil with 18 hr light and 6 hr dark. DNA constructs were transformed into *Agrobacterium tumefaciens* strain C58 and then transformed into Arabidopsis plants by the floral dip method (Clough and Bent 1998).

Plasmid construction: *Tag1* promoter-GUS expression plasmids were constructed as follows. The pTPG2 and pTPG3 plasmids were generated by PCR amplifying 1- to 262-bp and 1- to 548-bp fragments, respectively, from the 5′ end of *Tag1* into the *Xba*I and *Bam*HI restriction sites of the pBI101.3 GUS expression vector (accession no. U12640). pTPG4 was made by ligating a *Xba*I-*Sca*I *Tag1* fragment (1–1251 bp of *Tag1*) from the pBT1 plasmid (Liu *et al.* 2001a), which contains *Tag1* in the BlueScript vector (Stratagene, La Jolla, CA), into the *Xba*I-*Sma*I sites of pBI101.3 to generate an in-frame fusion between the *Tag1* transposase and GUS. pTPG5 was made by ligating a *Xba*I-*Nde*I 5′ *Tag1* fragment (1–1950 bp of *Tag1* in pBT1) into the *Xba*I and *BamH*I sites of pBI101.1 (accession no. U12639) using DNA adapters, to produce an in-frame fusion. pTPG6 was made by ligating a *Xba*I-*Ear*I 5′ *Tag*1 fragment (1–2938 bp of *Tag1* in pBT1) to the *Xba*I and *Bam*HI sites of pBI101.1 using DNA adaptors. *Tag1* plasmids containing no 35S promoter were made as follows. For pTAG-G, the 3.3-kb *Tag1* element from pBT1 was inserted into the *Xba*I and *Bam*HI sites of pBI101.1. For pTAG, the 1.87-kb GUS gene was first removed from pBI101.1 by digestion with *Sma*I and *Sac*I and treated with Klenow followed by religation. The 3.3-kb *Tag1* element was then inserted into the *Xba*I and *Bam*HI sites of this modified pBI101.1 vector. *Tag1* plasmids containing a 35S enhancer DNA were made as follows. A 35S enhancer fragment was generated by PCR amplifying a portion of the 35S cauliflower mosaic virus (CaMV) promoter (−50 to −805) in pBI121 (accession no. AF485783) using primers of sequence 5′-GCGGTCGACGGTCTGCTGACCCAC-3′ and 5′-CCGTCTA GAGAAGGATAGTGGGATTG-3′. For making pENT, this same fragment was cloned into the *Sal*I and *Xba*I sites of pTAG described above. To create pENTΔ5′, the 3.3-kb *Tag1* element from pENT was replaced with a 3.0-kb *Tag1* clone from pTGΔ5′ [containing the entire *Tag1* element minus the 245-bp 5′ intron (Liu *et al.* 2001b)].

Histochemical GUS staining and analysis: Standard GUS staining was performed using a buffer containing a final concentration of 35 mm $Na_xH_yPO_4$ (pH 7.0), 1 mm X-gluc, 0.5 mm potassium ferrocyanide, 10 mm EDTA, and 0.1% Triton-X 100. Tissue was incubated in this buffer for 10–35 hr at 37° and then destained in 70% EtOH. To enhance low levels of GUS activity in the ovules of pTPG2-, pTPG3-, pTPG4-, and pTPG5-containing plants, the pH of the X-gluc buffer was lowered to pH 6.8. This lower pH results in background GUS expression in the anthers of untransformed plants; thus, care was taken to remove the stamens and stain only the gynoecium at a lower pH. In addition, low levels of potassium ferro/ferricyanide in the buffer can lead to an overestimation of the number of cells expressing GUS protein (Stomp 1992). To clearly localize the cells expressing GUS, a buffer containing 2 mm potassium ferro/ferricyanide was used to stain flowers for photography. For Figures 2 and 3, GUS-stained floral organs were fixed for 20 min in FAA (50% EtOH, 5% acetic acid, 3.7% formaldehyde) and then cleared in Herr's buffer (lactic acid, chloral hydrate, phenol, clove oil, and xylene: 2:2:2:2:1 by weight) for 20 min (Herr 1971) and observed with differential interference contrast microscopy. GUS assays

to determine *Tag1* excision frequencies were performed as described previously (Liu *et al.* 2001b). Flowers were staged for GUS staining as follows on the basis of previous reports (Smyth *et al.* 1990; Bowman 1994; Christensen *et al.* 1997). Stage 9 buds show stalked petals that are well below short stamens and tetrads of microspores. Stage 10 buds have petals that are even with the short stamens. At the beginning of stage 11, stigmatic papillae appear on the gynoecium and the inner and outer integuments are initiated on the developing ovule. By late stage 11, the pistil is ~1 mm long, the anthers and petals are still green, and the integuments extend toward the top of the nucellus. Stage 12 is divided into three substages referred to as 12a, 12b, and 12c. Stage 12a begins when the petals become translucent and are even with the long stamens. During stage 12b the petals begin to turn white and extend just past the long stamens; the anthers are yellow-green. Stage 12c is marked by the anthers turning yellow although dehiscence has not taken place. Dehiscence occurs during stage 13 and pollination occurs during stage 14.

Northern blot hybridization and RT-PCR: Total RNA was extracted from plant tissues using Trizol reagent according to the manufacturer's protocol (GIBCO Life Technologies, Gaithersburg, MD). Poly(A)$^+$ RNA was prepared as previously described (Liu *et al.* 2001b). For RNA blot analysis, 1 μg of poly(A)$^+$ RNA was separated on a formaldehyde gel and transferred to a nylon membrane. Radiolabeled DNA probe (the 1.3-kb internal *Tag1 Eco*RI fragment) was prepared using an oligolabeling kit (Amersham Pharmacia). Hybridizations were performed at 65° for 18 hr in 0.5 M $NaPO_4$, 7% SDS, 1 mM EDTA. After hybridization, filters were washed at 42° twice with 4× SSPE and 0.1% SDS for 30 min and then twice with 0.1× SSPE and 0.1% SDS. For RT-PCR, tissue (leaves from 15-day-old plants and stage 9–13 flowers) was harvested and then ground into a fine powder in a 1.5-ml Eppendorf tube with a sterile plastic pestle and liquid N_2. Total RNA was extracted using Trizol according to the manufacturer's protocol (GIBCO Life Technologies) and then treated with RNase-free DNase (GIBCO Life Technologies) for 15 min at 37°. Total RNA (300 ng) was used for cDNA synthesis following the Superscript first-strand synthesis system protocol (GIBCO Life Technologies). The cDNA synthesis step was carried out at 42° for 50 min with oligo(dT). A portion (10%) of the products was added to the PCR reactions. The PCR reactions (94° for 30 sec, 60° for 30 sec, 72° for 1 min) were performed using primers Tag 6-2a (5′-GAA GGG ATG TAC CGA GCA AG-3′), Tag1798 (5′-CTC CAC CAT TCA TCT GGC TGA GAG G-3′), ubq10s52 (5′-GAA AGC TCT GAC ACC ATC-3′), and ubq10as861 (5′-GTA ATC CGC CAA AGT ACG-3′). The two *Tag1* primers produce a 345-bp cDNA fragment. The ubiquitin primers produce a 1037-bp fragment. The PCR reaction was carried out for 15, 20, and 25 cycles. Ten microliters of the PCR reaction was removed after 15 cycles, separated on a 1.2% agarose gel, denatured, neutralized, and transferred to a nylon membrane. Hybridization with the 1.3-kb *Tag1* internal *Eco*RI probe and the ubiquitin probe was performed following the same protocol described above for Northern hybridization.

Real-time RT-PCR was performed using a LightCycler (Roche, Indianapolis). RNA was extracted from vegetative and reproductive tissue using the QIAGEN (Chatsworth, CA) RNeasy plant kit. cDNA reactions were performed as above for standard RT-PCR. Two microliters of a 10× cDNA reaction dilution was added to each PCR reaction containing 4 mM $MgCl_2$, 1× FastStart DNA Master SYBR green reaction mix (Roche), and 5 μM of the *Tag1* mRNA primers, Tag1776(C) (5′-AGTGCAGATCCCGAACTCA-3′) and Tag2074(D) (5′-AGA TCGTGGCGCAACAT-3′) or the internal control primers, UBQ10F208 (5′-GTCCTCAGGCTCCGTGGTG-3′) and UBQ-10R383 (5′-TGCCATCCTCCAACTGCTTTC-3′). Samples were denatured initially for 10 min followed by 45 cycles of 95° for 0 sec, 67° (*Tag1* primers) or 65° (Ubq primers) for 5 sec, and 72° for 10 sec. All reactions were performed in triplicate and the mean was used to calculate the increase in expression.

Quantitative PCR excision assay: Genomic DNA was extracted from 20-day-old leaves of plants using the QIAGEN DNeasy plant kit. Two microliters (~50 ng) of eluate was added to each reaction as above except that target excision primers were T-DNA2101 (5′-GACGTTTCCGGCCTTGCTAATGG-3′) and T-DNA4489 (5′-CGCAAGACCGGCAACAGGATTC-3′). Internal control primers were LC-NIR-F1394 (5′-CCGGTAGC CAGTTCTGCG-3′) and LC-NIR-R1631 (5′-CCTATTCGTCCC CCGACGT-3′). Real-time PCR was performed as described above with an annealing temperature of 65° for both primer sets.

RESULTS

***Tag1* promoter-GUS fusion analysis:** To investigate the activity of the *Tag1* promoter, a series of *Tag1*-GUS fusions with increasing amounts of *Tag1* DNA sequence were constructed (see constructs pTPG2–pTPG6 in Figure 1). These DNAs were transformed into the Columbia ecotype of Arabidopsis, which contains no native *Tag1* elements (Tsay *et al.* 1993). Two constructs (pTPG2 and pTPG3) are transcriptional fusions, and three (pTPG4, pTPG5, and pTPG6) are translational fusions between the *Tag1* transposase and GUS protein. For each construct, five or more transgenic lines were obtained and then stained for GUS activity. Our initial observations showed that the *Tag1* fusion constructs produced no detectable GUS activity in leaves or other vegetative tissues but showed activity in developing anthers and ovules (summarized in Figure 1). This pattern was similar for all constructs tested but the average level of GUS activity differed among the constructs. Lines containing pTPG2 (the smallest construct containing 262 bp from the 5′ end of *Tag1* to the start of transcription) showed barely detectable levels of GUS activity. pTPG3 [which includes the additional 41-bp 5′ untranslated region (UTR) and a 245-bp 5′ intron] showed strong levels of GUS activity in developing anthers but weak levels in ovules, suggesting that sequences downstream of the transcriptional start site are needed for full anther expression. pTPG4 (containing an additional 700 bp of translated sequence) produced dramatically reduced GUS activity levels compared with pTPG3, suggesting the presence of an inhibitory domain in exon 2 of *Tag1*. pTPG5 (containing another 700 bp of downstream sequence) restores GUS activity to levels roughly equal to pTPG3. The strongest ovule expression was found with the longest fusion, pTPG6, which includes 2.9 kb of the 3.3-kb element. This construct ends ~40 bp before the stop codon for the *Tag1* transposase and contains all four introns. The GUS activity levels in the developing anthers of pTPG6 plants were slightly lower than that observed for the pTPG3 construct but equal to the levels observed in pTPG5 lines. All constructs

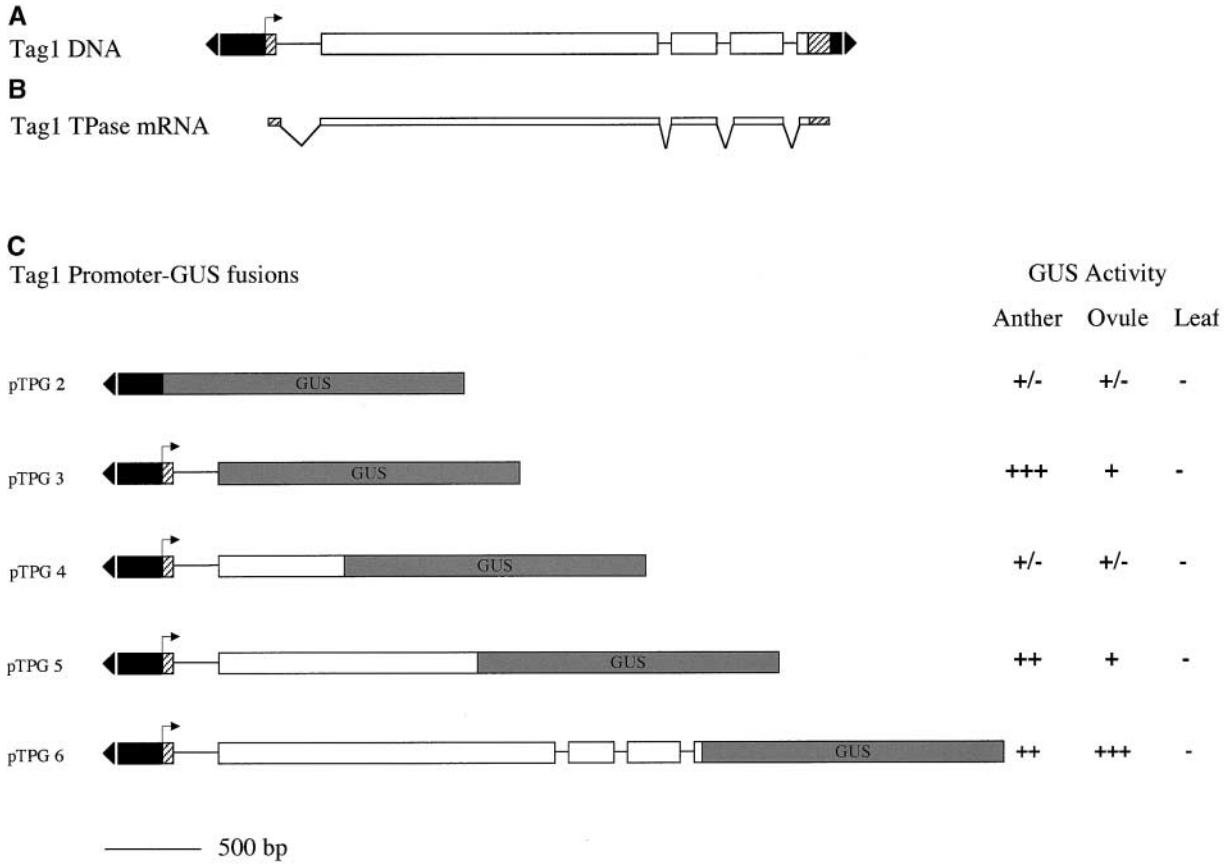

FIGURE 1.—*Tag1*-GUS fusion constructs. (A and B) Schematics of the *Tag1* element and *Tag1* transposase mRNA, respectively. Solid triangles represent terminal inverted repeats, 5′ and 3′ nontranscribed sequences are shown as solid boxes, and boxes with diagonals represent 5′ and 3′ untranslated regions. Introns are shown as straight lines connecting the boxes. Open boxes represent translated sequences. (C) *Tag1*-GUS fusions with corresponding GUS activities on the right. pTPG2 contains only the first 262 bp of the *Tag1* element; pTPG3 includes sequences 1–548 that contain the 5′ UTR and 5′ intron; pTPG4 includes sequences 1–1251; pTPG5 contains sequences 1–1951; and pTPG6 includes all sequences up to 2938, 40 nt upstream of the stop codon. GUS expression in anthers and ovules is strong although no GUS was observed in the leaves of developing plants.

described above displayed very similar spatial and temporal patterns of expression (data not shown); therefore, two constructs (pTPG3 for anther expression and pTPG6 for ovule expression) were selected for more detailed examination as these lines showed the highest levels of GUS activity. Expression from these two constructs was monitored in developing anthers and ovules to determine in which cells and at what stages of flower development expression was occurring. Flower development occurs in 20 defined stages with meiosis occurring at stage 9 in developing anthers and at stage 11 in developing ovules and culminating in fertilization at stage 14 (SMYTH *et al.* 1990; BOWMAN 1994). To assess transposase expression during anther development, GUS activity was examined in pTPG3 lines. Developing flower buds were staged by microscopic examination and then stained for GUS activity as described in MATERIALS AND METHODS. In stage 9 buds, the microspore mother cells undergo meiosis to form tetrads of microspores. During stages 10–12, microgametophyte development occurs to produce the mature pollen grains. At stage 10, the microspores round up and separate. During stages 11 and 12 the mitotic divisions occur, ending with desiccation of the pollen grains (SMYTH *et al.* 1990; BOWMAN 1994). No GUS activity was detected in developing anthers up to stage 10 (Figure 2A). A very low level of GUS activity could be detected during stage 10 (*i.e.*, after meiosis; Figure 2B). Strong GUS activity began at stage 11 and remained high into stage 12 (Figure 2, C–E) but started to disappear near the end of stage 12. GUS activity was almost undetectable at stages 13 and 14 when the anthers dehisce and pollination occurs, respectively (Figure 2F). These experiments indicate that expression in the anthers starts after meiosis, proceeds during gametophyte development, and concludes by dehiscence. To determine when expression occurs during ovule development, pTPG6 lines were examined. The megaspore mother cell undergoes meiosis and forms four megaspores during the late part of stage 11 (BOWMAN 1994; CHRISTENSEN *et al.* 1997). Three of the megaspores degenerate, leaving the chalazal-most spore to become the functional megaspore. Mitotic divisions

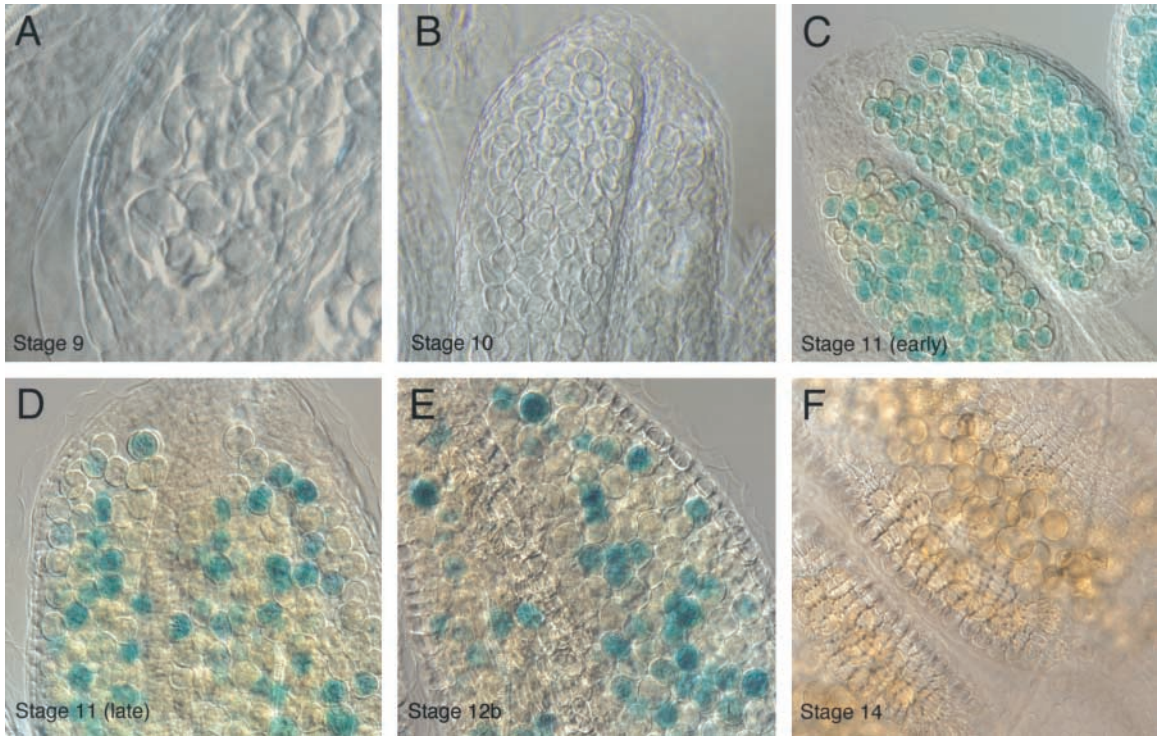

FIGURE 2.—Histochemical analysis of GUS-stained anthers. For this analysis, anthers from line pTPG3-23 were used. (A) Pollen sacs from a stage 9 anther revealing tetrads of microspores with no GUS expression. (B) A stage 10 anther with very low levels of GUS staining. (C) An early stage 11 anther with strong GUS staining. (D) A late stage 11 anther with GUS staining restricted to the developing pollen grains. (E) Stage 12 anther with visible GUS staining. (F) A stage 14 anther with GUS staining no longer evident.

during megagametophyte development occur during stage 12, ending with a mature embryo sac at stage 13. GUS activity was not observed until late stage 11 (Figure 3, A and B). At this stage only ~10% of the ovules within a gynoecium show GUS activity, which appears to be localized near the chalazal end, indicative of the functional megaspore (Figure 3B). GUS expression continued into stage 12a when the functional megaspore is enlarged and uninucleate (Figure 3C). The highest percentage of ovules began to show strong GUS activity at the beginning of stage 12b when the first round of mitosis in the gametophyte takes place (Figure 3D). On the basis of ovule morphology (*i.e.*, location of the integuments), the ovule on the left in Figure 3D is at the early two-nucleate stage where the nuclei are adjacent while the ovule on the right has entered into the late two-nucleate stage or beyond where the nuclei have been separated by a large central vacuole (CHRISTENSEN *et al.* 1997). GUS activity declined during stage 13 and disappeared by stage 14, when fertilization occurs (data not shown). These results indicate that expression in the developing megagametophyte begins either at the time of or just after meiosis and then continues until sometime before fertilization. If GUS expression in developing anthers and ovules is compared (Figure 4), one can see that anther expression occurs first (Figure 4A). As anther expression declines, ovule expression is turned on (Figure 4B) and peaks at stage 12c (Figure 4C). The beginning of these waves of expression correlates with the conclusion of meiosis, which occurs first in developing anthers and then later in developing ovules.

Germline-specific *Tag1* expression in the absence of the 35S promoter: The above results indicate that *Tag1* expression is targeted to the developing gametophyte and is not present in vegetative organs. Previous RNA blot analyses, however, indicated that *Tag1* mRNA is abundant in all organs examined, including vegetative organs (*i.e.*, leaves, roots, stems, flowers, and flower buds; LIU and CRAWFORD 1998b). A possible explanation for this difference is that the earlier experiments were based on constructs containing the *Tag1* element next to a CaMV 35S promoter (in a 35S-GUS marker gene, pT2G2; Figure 5A) while the present experiments use *Tag1*-GUS fusions that lack an adjacent 35S promoter. It is possible that the presence of the 35S promoter next to *Tag1* produces ectopic *Tag1* expression in vegetative organs. To test this idea, *Tag1* expression in the absence of an adjacent 35S promoter was examined by RNA blot analysis. Constructs with a complete *Tag1* element but lacking the 35S promoter (pTAG-G and pTAG, containing or lacking, respectively, the GUS gene; Figure 5B) were transformed into Arabidopsis (Columbia ecotype). A total of 20 lines were generated, and 10 were examined by RNA blot analysis. In agree-

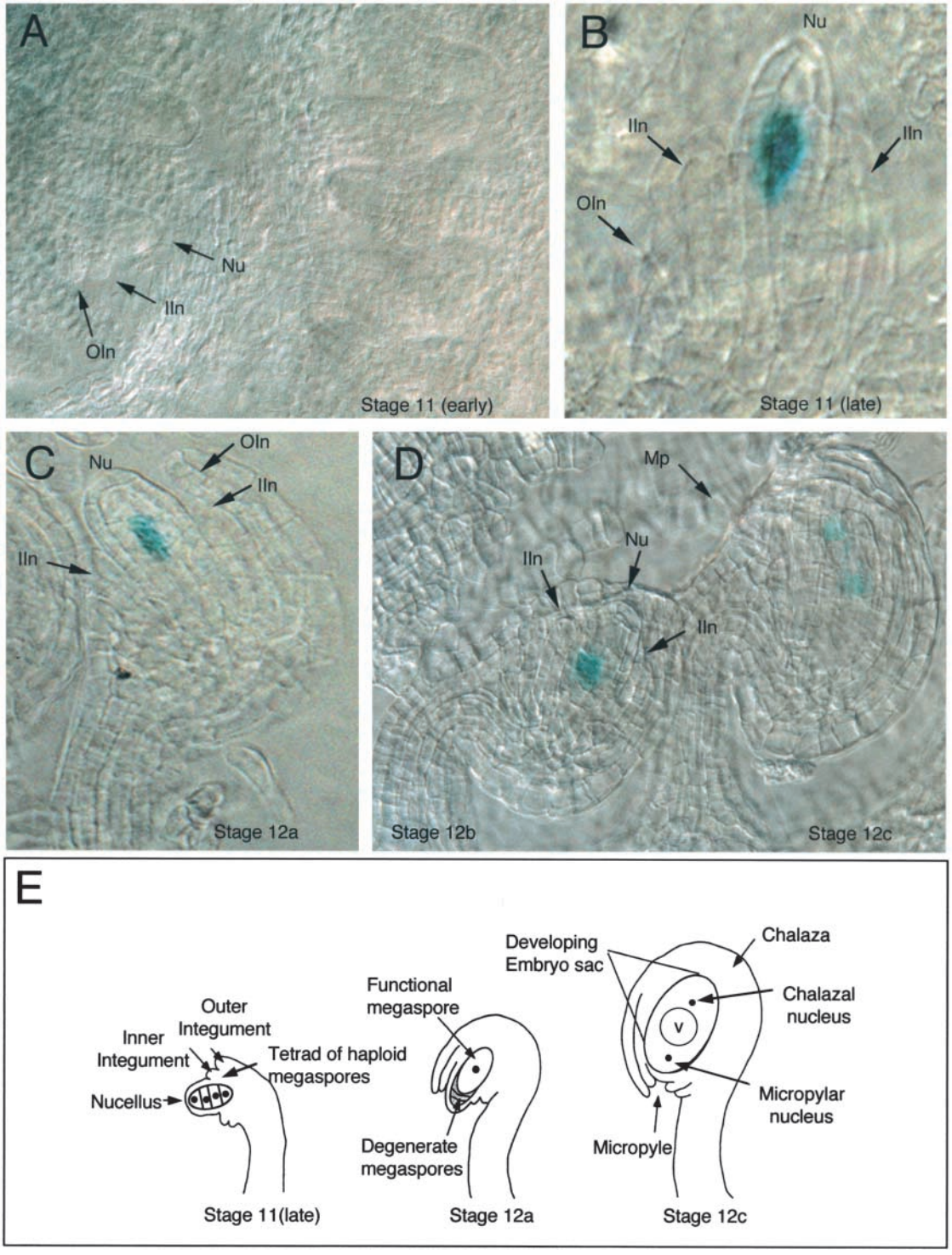

FIGURE 3.—Histochemical analysis of GUS-stained ovules. For this analysis, ovules from line pTPG6-31 were used. (A) Early stage 11 ovule with no visible GUS staining. (B) Ovule at stage 11 (late). At this stage, three of the megaspores are degenerating, leaving the chalazal-most megaspore to develop into the female gametophyte (BOWMAN 1994; CHRISTENSEN *et al.* 1997). GUS expression at this stage is very infrequent (only ~10% of ovules express at this stage). (C) Stage 12a ovule with GUS staining localized to the uninucleate embryo sac. Note that the outer and inner integuments have not yet completely enclosed the nucellus. (D) Stage 12b and stage 12c ovules. The ovule on the left is at the *early* two-nucleate stage, and the ovule on the right has at least entered or progressed beyond the *late* two-nucleate stage. The late two-nucleate stage is recognized by the complete enclosure of the nucellus by the inner and outer integuments and results in the formation of a large vacuole between the two nuclei (CHRISTENSEN *et al.* 1997). (E) Three stages of ovule development (REISER and FISCHER 1993). OIn, outer integuments; IIn, inner integuments; Nu, nucellus; Mp, micropyle.

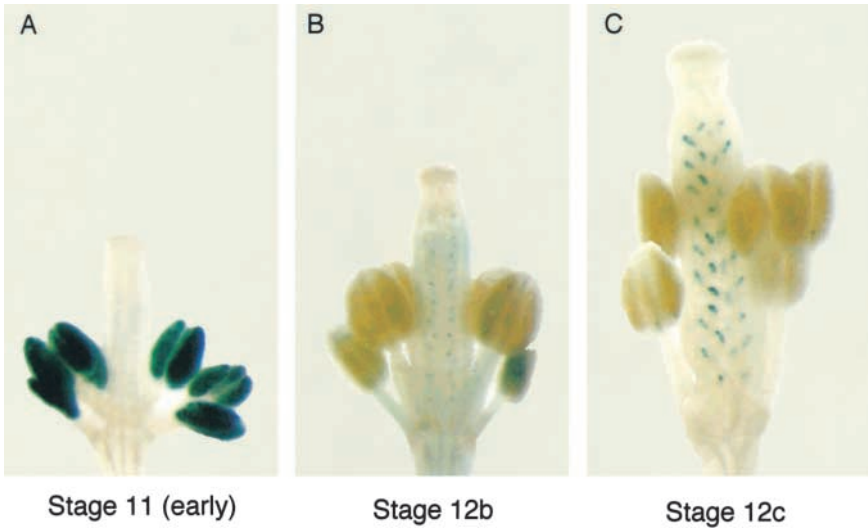

FIGURE 4.—Histochemical analysis of GUS-stained whole flowers. GUS-stained flowers from line pTPG6-25 are shown. Sepals and petals have been removed to reveal the anthers and gynoecium. GUS staining is first observed at stage 10 in developing anthers but does not become strong until the beginning of stage 11 (A). Shortly before GUS staining begins to decline in anthers, GUS activity is observed in developing ovules (B). GUS staining reaches a maximum around stage 12c (C).

ment with our present results, no *Tag1* mRNA was detected in the leaves of any of the lines (Figure 6A; lanes 1–10). To confirm the earlier data, mRNA from a 35S-*Tag1*-GUS line was examined and found to contain a major RNA transposase band and several smaller bands, as previously reported (Figure 6A, lane T2G2). RNA blot analysis was then performed on flower buds, and no *Tag1* signal was detected (data not shown). Attempts to detect *Tag1* expression by *in situ* hybridization were unsuccessful as no signal above background was observed in leaves or flower buds. To increase the sensitivity of our assays, *Tag1* mRNA levels were examined by RT-PCR. RNA was amplified for 15 cycles and then transferred to a blot and hybridized with a *Tag1* probe. *Tag1* mRNA levels from leaves and whole flower buds were compared and found to be much higher in flowers (Figure 6B, lanes 1 and 2). Some mRNA signal was detected in leaf tissue but at a very low level. When floral bud organs were analyzed, *Tag1* mRNA was much higher in the reproductive organs (anthers and pistils, Figure 6B, lane 4) than in the vegetative organs (petals and sepals, Figure 6B, lane 3). These results were verified using six different transgenic lines (data not shown). Primers corresponding to the ubiquitin 10 gene were used as a control to verify equal loading of RNA.

The tissue-specific expression of *Tag1* was analyzed

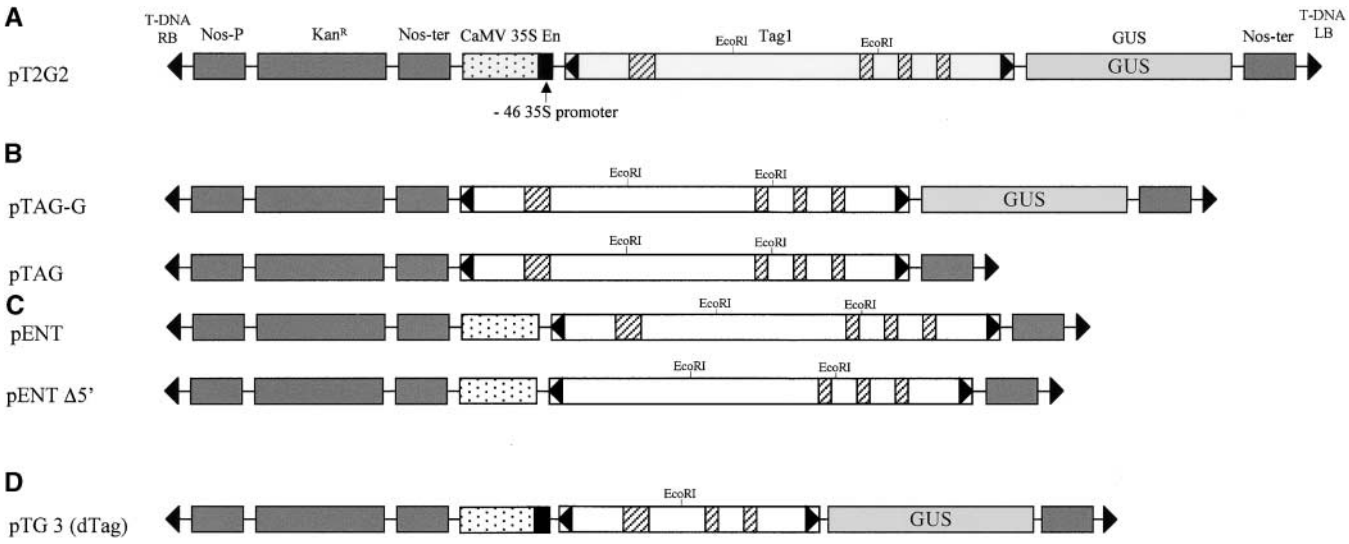

FIGURE 5.— *Tag1* excision and expression constructs. (A) The *Tag1* excision construct in the pBI121 vector. *Tag1* inverted repeats are shown as solid triangles. Hatched boxes represent introns. The CaMV 35S enhancer region is shown as a stippled box while the −46 bp CaMV 35S minimal promoter is shown as a solid box. (B) The *Tag1* expression constructs under the control of the *Tag1* promoter and lacking the 35S promoter. The pTAG-G construct is identical to the pT2G2 construct except that the 35S promoter has been removed. pTAG lacks both the 35S promoter and the GUS gene. (C) The *Tag1* expression constructs, pENT and pENTΔ5, in which the *Tag1* element is adjacent to the 35S enhancer region. For pENTΔ5′ the 5′ intron of *Tag1* is removed. (D) A defective *Tag1* (*dTag*) element missing the internal 1.3-kb *Eco*RI fragment of *Tag1* in a 35S-GUS excision marker construct. Kan^R, kanamycin resistance gene; Nos-P, nopaline synthase promoter; Nos-ter, nopaline synthase terminator; T-DNA RB, right border; T-DNA LB, left border; CaMV, cauliflower mosaic virus.

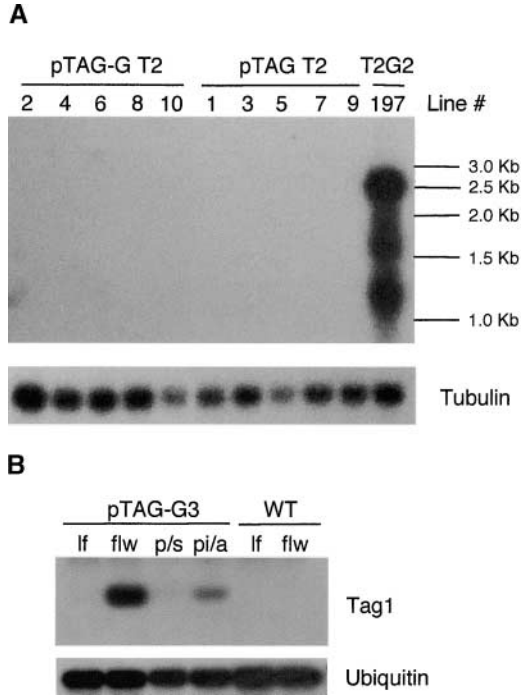

FIGURE 6.—RNA blots for *Tag1* transgenic plants lacking the 35S promoter. (A) RNA blot is shown for poly(A)$^+$ RNA from leaves and flowers of pTAG-G (lines 2, 4, 6, 8, and 10) and pTAG (lines 1, 3, 5, 7, 9) lines. Last lane shows RNA from a 35S-*Tag1*-GUS line, T2G2 (line 197). A 1.3-kb internal fragment from *Tag1* was used as a probe, and tubulin was used as a loading control. (B) DNA blot of RT-PCR products from line pTAG-G3 (first four lanes) and untransformed Columbia (last two lanes). Total RNA was extracted from 2- to 3-week-old leaves (lanes lf), stages 9–13 whole flowers (lanes flw), petals and sepals (lane p/s), and pistils and anthers (lane pi/a). Low levels of *Tag1* cDNA were observed in the leaves and vegetative tissues of developing flowers in longer exposures but at a much lower level than that seen in flowers (data not shown). A 1.3-kb internal fragment from *Tag1* was used as a probe, and a ubiquitin probe was used as a loading control.

further by real-time RT-PCR using three different transgenic lines containing *Tag1* without an adjacent 35S promoter (lines pTAG-7, pTAG-8, and pTAG-10). The levels of *Tag1* mRNA were 10- to >30,000-fold higher in flowers than in leaves (Table 1). Untransformed Landsberg plants, which contain two endogenous *Tag1* elements (FRANK *et al.* 1997), were also examined. The levels of *Tag1* mRNA were 13- to 23-fold higher in flowers than in leaves in two different preparations of RNA (Table 1). *Tag1* expression in specific floral organs was analyzed next. An 18- to 630-fold higher level of *Tag1* mRNA was observed in reproductive organs compared to vegetative organs in the pTAG lines (Table 2). In untransformed Landsberg (*erecta*) plants, a 97-fold difference was seen. These results, showing *Tag1* expression localized primarily to the reproductive organs of developing flower buds, support the findings obtained with *Tag1*-GUS fusions, which showed that expression is restricted to developing gametophytes. In addition, these results indicate that *Tag1* is active in the germinal lineages of untransformed Landsberg plants.

Germline-specific *Tag1* excision activity in the absence of a 35S promoter: In previous experiments we found that *Tag1* undergoes vegetative excision from a 35S-*Tag1*-GUS construct (LIU and CRAWFORD 1998a). Because low levels of *Tag1* expression are observed in vegetative organs in the absence of an adjacent 35S promoter, we wondered if *Tag1* excision would also be diminished in the absence of the 35S promoter. To test the excision activity supported by such elements, transgenic lines containing *Tag1* with no adjacent 35S promoter (constructs pTAG-G and pTAG, which contain or omit the GUS gene, respectively) were crossed to a homozygous line containing an excision construct, 35S-*dTag*-GUS (Figure 5D; see also FRANK *et al.* 1997). When sufficient levels of transposase are supplied, the defective *Tag1* element (*dTag*) excises, producing a functional 35S-GUS gene and the appearance of small blue sectors when the tissue is stained with X-gluc (FRANK *et al.* 1997). The F_1 plants from these crosses showed no GUS sectors in the leaves or other vegetative tissues (Table 3). Seeds from these F_1 plants were collected and pooled, and the F_2 progeny were analyzed for the presence of germinal revertants. For each F_1 plant, ~1000 F_2 whole seedlings were stained for GUS. Germinal excision frequencies were scored as the percentage of completely blue-staining plants from within

TABLE 1

Real-time RT-PCR of *Tag1* mRNA in leaves and flowers

Line	Relative *Tag1* mRNA levels in flowers	Relative *Tag1* mRNA levels in leaves	Ratio (flower/leaf)
pTAG-7	3.9×10^{-3}	ND	$>3 \times 10^{4}$
pTAG-8	3.0×10^{-3}	2.8×10^{-4}	10.7
pTAG-10	2.0×10^{-2}	2.3×10^{-4}	87.0
Landsberg (1)	1.8×10^{-3}	1.4×10^{-4}	12.9
Landsberg (2)	3.4×10^{-3}	1.5×10^{-4}	22.7

Tag1 mRNA levels were determined relative to UBQ10 mRNA levels. Values of mRNA levels were calculated as $2^{\Delta C_T}$, where C_T is cycle threshold and $\Delta C_T = C_T$ UBQ10 $- C_T$ Tag1. Landsberg (1) and (2) refer to independent preparations of Landsberg tissue. ND, none detected after 45 cycles ($<1.3 \times 10^{-7}$).

TABLE 2

Real-time RT-PCR of *Tag1* mRNA in vegetative and reproductive organs

Line	Relative *Tag1* mRNA levels in reproductive organs[a]	Relative *Tag1* mRNA levels in vegetative organs[b]	Ratio (reproductive/ vegetative)
pTAG-7	1.3×10^{-2}	7.0×10^{-5}	185.7
pTAG-8	5.9×10^{-3}	3.2×10^{-4}	18.4
pTAG-10	2.9×10^{-2}	4.6×10^{-5}	630.4
Landsberg	3.0×10^{-3}	3.1×10^{-5}	96.8

Tag1 mRNA levels were determined relative to UBQ10 mRNA levels. Values of mRNA levels were calculated as $2^{\Delta C_T}$, where C_T is cycle threshold and $\Delta C_T = C_T$ UBQ10 $- C_T$ Tag1.

[a] Reproductive organs are pistils and anthers.

[b] Vegetative organs are petals and sepals.

this mixed population of segregating progeny. Examination of the F_2 seedlings revealed the presence of completely blue-staining progeny (at a frequency of 0.3–1.7%, depending on the line), indicating that the *Tag1* element had undergone germinal excision. As a control, ~1000 T_3 seedlings from the 35S-*dTag1*-GUS marker line were stained for germinal revertants. No germinal revertants were found, eliminating the possibility that germinal excision was occurring spontaneously at a low frequency in this line.

To further verify the lack of vegetative excision, DNA was extracted from the leaves of *Tag1*-transgenic plants and assayed for excision of *Tag1* from the pTAG construct by real-time PCR using primers that spanned *Tag1* in the T-DNA construct. No detectable levels of excision products were found in four different transgenic lines (Table 4, lines pTAG-3, pTAG-6, pTAG-18, and pTAG-21). To be sure that these data were meaningful, several controls were performed. Positive controls (pTAG-3- and pTAG-10-germinal revertants in which all cells have inherited an excision event) gave high signals (6–8 $\times 10^{-2}$ relative to a single-copy DNA for the nitrite reductase gene). The negative control (untransformed Columbia plants) gave no signal as expected. Lines that should produce vegetative excision products were also generated by crossing pTAG lines to two 35S-*Tag1*-GUS lines, T2G2197 and T2G2185, which show medium and low levels of GUS sectors, respectively. F_1 plants from these crosses gave readily detectable signals that correlated with the level of vegetative excision observed from the 35S-*Tag1* constructs (1×10^{-4}–6×10^{-4}).

Last, the excision behavior of endogenous *Tag1* elements in Landsberg (*erecta*) plants was monitored by transforming this ecotype with a 35S-*dTag*-GUS construct and assaying for the presence of GUS sectors. No excision was detected in the leaves of 34 T_1 lines (Table

TABLE 3

Excision activity supported by *Tag1* with no adjacent 35S promoter

Tag1 line (T_1) (×35S-*dTag*-GUS)	Kan^R:Kan^S of T_2 plants	Excision frequency in leaves (F_1)	Germinal excision frequency (%)
pTAG-G2	15:1	No activity	ND
pTAG-G3	15:1	No activity	1.4
pTAG-G4	3:1	No activity	0.5
pTAG-G9	3:1	No activity	ND
pTAG-1	1:1	No activity	0.28
pTAG-2	15:1	No activity	1.2
pTAG-3	3:1	No activity	0.74
pTAG-4	3:1	No activity	0.68
pTAG-6	3:1	No activity	0.63
pTAG-7	3:1	No activity	0.59
pTAG-8	15:1	No activity	ND
pTAG-10	3:1	No activity	1.7

Lines containing *Tag1* (transposase source) were crossed to homozygous 35S-*dTag*-GUS lines to determine excision activity promoted by the *Tag1* element. Vegetative excision frequencies in leaves of F_1 plants were determined by counting the number of GUS sectors. Germinal excision frequencies were determined by counting completely blue-staining plants out of ~1000 F_2 seedlings from each line. Lines containing the transposase source were also selfed and 150–200 T_2 progeny were plated on kanamycin plates to determine kanamycin resistance ratios (which indicates the number of loci). ND, no germinal revertants detected.

TABLE 4

Real-time PCR analysis of *Tag1* excision in leaves

Line	Generation	Relative amount of excision product[a]
pTAG-3	T_2	ND
pTAG-6	T_2	ND
pTAG-18	T_1	ND
pTAG-21	T_1	ND
pTAG-3 × 35S-*Tag1*-GUS 197	F_1	1.4×10^{-4}
pTAG-6 × 35S-*Tag1*-GUS 197	F_1	6.0×10^{-4}
pTAG-7 × 35S-*Tag1*-GUS 185	F_1	4.7×10^{-6}
pTAG-7 × pENT-13	F_1	7.6×10^{-6}
pTAG-3 (germinal revertant)	T_2	8.2×10^{-2}
pTAG-10 (germinal revertant)	T_2	6.7×10^{-2}
WT Columbia	—	ND

pTAG lines contain *Tag1* with no adjacent 35S promoter or enhancer. Line 35S-*Tag1*-GUS 197 has a medium sector number (~150 GUS sectors per leaf), line 35S-*Tag1*-GUS 185 has a low sector number (~10–20 GUS sectors per leaf), and line pENT-13 is a 35S enhancer-*Tag1* line with a low sector number (~10–50 GUS sectors per leaf). Germinal revertant indicates a line that inherited a *Tag1* excision event. ND, none detected after 45 cycles ($<1.5 \times 10^{-8}$); WT, wild type.

[a] Relative amount of excision product was determined by normalizing signals to the level of DNA for the nitrite reductase gene (NiR). Values are calculated as $2^{\Delta C_T}$, where C_T is the cycle threshold and $\Delta C_T = C_T$ NiR $- C_T$ Tag.

TABLE 5

Excision activity supported by endogenous *Tag1* in Landsberg

Landsberg lines containing 35S-*dTag*-GUS (T_1)	Excision frequency in leaves (T_1)	Germinal excision frequency (%)
pTG 3-40	No activity	0.85
pTG 3-41	No activity	0.2
pTG 3-42	No activity	0.72
pTG 3-43	No activity	ND
pTG 3-46	No activity	ND
pTG 3-48	No activity	ND
pTG 3-49	No activity	0.34
pTG 3-50	No activity	0.07
pTG 3-51	No activity	0.19
pTG 3-53	No activity	0.15
pTG 3-54	No activity	0.13
pTG 3-56	No activity	0.36
pTG 3-58	No activity	0.06

Landsberg (*erecta*) plants containing an endogenous *Tag1* (transposase source) were transformed with a 35S-*dTag*-GUS construct to determine excision activity promoted by the *Tag1* element. Vegetative excision frequencies in leaves of T_1 plants were determined by counting the number of GUS sectors. Germinal excision frequencies were determined by counting completely blue-staining plants from ~500–2000 T_2 seedlings from each line. ND, no germinal revertants detected.

5 and data not shown). Our previous report of vegetative excision of *dTag* in Landsberg (Liu and Crawford 1998b) could not be repeated and, we believe, is in error. Germinal revertants were observed among T_2 progeny at an average frequency of 0.24% (±0.27%; Table 5). Overall, these results show that the intrinsic excision activity of *Tag1* (*i.e.*, in the absence of an adjacent 35S promoter) is restricted to germinal events.

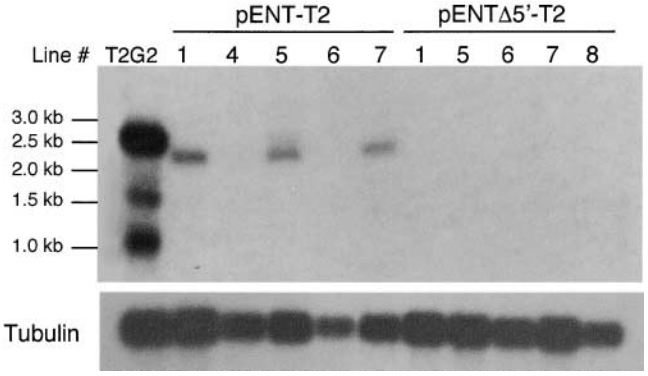

Figure 7.—RNA blot of CaMV 35S enhancer-*Tag1* transgenic plants. Poly(A)$^+$ RNA was prepared from the leaves of 3-week-old T_2 plants. RNA was analyzed from T2G2 197, a line containing the 35S-*Tag1*-GUS construct (line T2G2), from five pENT lines containing the 35S enhancer-*Tag1* constructs (lines 1, 4, 5, 6, and 7), and from five pENTΔ5′ lines missing the *Tag1* 5′ intron (lines 1, 5, 6, 7, and 8). A 1.3-kb internal fragment from *Tag1* was used as a probe, and tubulin was used as a loading control.

Response of *Tag1* to 35S enhancer elements: As described above, *Tag1* is expressed and excises in vegetative tissues in 35S-*Tag1*-GUS constructs. This behavior could be due to 35S enhancers activating the *Tag1* promoter or to the 35S promoter driving transcription through the *Tag1* element. To test the effect of 35S enhancers on *Tag1*, a 750-bp 35S fragment containing sequences upstream of the −46 35S minimal promoter was placed in front of the 3.3-kb *Tag1* element (pENT, Figure 5C). Seven transgenic lines containing this construct were generated, and poly(A)$^+$ RNA was extracted from the leaves of five T_2 lines and analyzed on RNA blots. Three lines showed a single mRNA transcript of ~2.3 kb (Figure 7, pENT-1,5,7). We verified that the 2.3-kb mRNA transcript originated from the *Tag1* promoter by RNase protection assays (data not shown). RNase digestion of probes hybridized to total RNA produced three bands consistent with transcriptional start sites at the following approximate positions within the *Tag1* element: 210, 235, and 260 nucleotides (nt). Thus, the 35S enhancers can activate *Tag1* transcription from the *Tag1* promoter in vegetative tissues.

We also observed that mRNA from the pENT enhancer lines was ~300 bp smaller than the major transcript found in 35S-*Tag1*-GUS lines (lane T2G2 in Figure 7). This result suggests that the predominant transcript in the 35S-*Tag1*-GUS lines is one generated by the 35S CaMV promoter initiating transcription through the entire *Tag1* element. RNase protection assays confirmed this result by showing that the predominant transcript

TABLE 6

Excision activity supported by *Tag1* with an adjacent 35S enhancer element

Tag1 line (T_1) (×35S-*dTag*-GUS)	Kan ^{R}:Kan^S of T_2 plants	Excision frequency in leaves (F_1)	Germinal excision frequency (%)
pENT-1	10:1	Low	0.4
pENT-2	4:1	Very low	0.0
pENT-3	3:1	Low	0.3
pENT-4	1:2	No activity	ND
pENT-5	15:1	Low-medium	0.1
pENT-6	10:1	Very low-low	ND
pENT-7	3:1	Very low	0.8

Lines containing *Tag1* (transposase source) were crossed to homozygous 35S-*dTag*-GUS lines to determine excision activity promoted by the enhancer-*Tag1* element. Vegetative excision frequencies in leaves of F_1 plants were determined by counting the number of GUS sectors and then scoring leaves as 50–300 sectors/leaf, medium; 10–50 sectors/leaf, low; <10 sectors/leaf, very low. Germinal excision frequencies were determined by counting completely blue-staining plants from ~1000 F_2 seedlings from each line. Lines containing the transposase source were also selfed and 150–200 T_2 progeny were plated on kanamycin plates to determine kanamycin resistance ratios (which indicates the number of loci). ND, no germinal revertants detected.

produced in the 35S-*Tag1*-GUS lines was initiated upstream of the *Tag1* element (data not shown). We have yet to determine if this larger transcript, most likely containing extra 5′ sequences of *Tag1*, can produce a functional transposase protein.

We next tested the pENT enhancer lines to see if the increased vegetative expression of the transposase mRNA leads to detectable vegetative excision. pENT transgenic lines were crossed to the 35S-*dTag*-GUS (pTG3-7.1) marker line. Leaves from 5 to 20 F_1 progeny from each cross were stained for GUS, and the number of sectors corresponding to independent excisions was counted. Six of the seven lines showed small blue sectors, indicating late excision events in the leaves of the F_1 plants. The number of excision events occurred on average at a low frequency (defined as 10–50 sectors/leaf; Table 6). This frequency is slightly lower than that observed previously for the 35S-*Tag1*-GUS lines, where frequencies varied from very low (<10 sectors/leaf) to high (>300 sectors/leaf) but were on average observed to be about medium (50–300 sectors/leaf; Liu and Crawford 1998a). Seeds from these F_1 plants were pooled, and the F_2 progeny were analyzed for the presence of germinal revertants. For each F_1 plant, ~1000 F_2 whole seedlings were stained for GUS activity. Germinal excision in these enhancer lines occurred at a reduced frequency [average of 0.23% (±0.3%) for 7 lines; Table 6] compared with lines containing only the *Tag1* element with no 35S enhancers [average of 0.65% (±0.56%) for 12 lines; Table 3]. Thus, the 35S enhancer sequence, while activating vegetative excision, does not appear to increase germinal excision of *Tag1*.

Expression of *Tag1* mRNA requires the 5′ intron of *Tag1*: The 5′ untranslated region of *Tag1* contains a 245-bp intron. Previous experiments showed that expression of the *Tag1* transposase by the 35S promoter requires the 5′ intron (Liu *et al.* 2001b). In addition, removal of this intron in the 35S-*Tag1*-GUS construct (pTGΔ5′) results in the loss of somatic excision; however, germinal excision frequencies are roughly equivalent to those observed for the original 35S-*Tag1*-GUS lines (Liu *et al.* 2001b). We tested the requirement of the 5′ intron for vegetative expression and excision in the context of the 35S enhancer-*Tag1* (pENT) constructs. Transgenic Arabidopsis lines containing pENTΔ5′ showed no detectable *Tag1* mRNA in leaf tissue on RNA blots (Figure 7, pENTΔ5′-T_2 lanes). Additionally, no vegetative excision was observed in the F_1 plants generated by crossing to the *dTag* marker line (Table 7). Seeds from F_1 plants were pooled and screened for completely blue-staining progeny. Germinal revertants were observed, but at the lowest frequency observed so far for all the constructs [average of 0.1% (±0.1%); Table 7].

DISCUSSION

The results described above show that *Tag1* expression and excision are targeted to late stages of germinal development in Arabidopsis. Previous experiments using plants transformed with 35S-*Tag1*-GUS constructs led to the conclusion that *Tag1* excision occurred late in both vegetative and germinal lineages. The germinal excision activity was found in both studies, but the vegetative excision results differed. What is different in the present article is that the activity of *Tag1* was tested in the absence of an adjacent 35S promoter, and no evidence for vegetative excision was found. These results indicate that vegetative excision is the product of an adjacent 35S promoter and is not an intrinsic property of *Tag1*.

Examination of *Tag1* expression provided an explanation for the lack of vegetative excision in the *Tag1* lines described here. Transgenic plants containing *Tag1* elements with no adjacent 35S promoter had low levels of

TABLE 7

Excision activity supported by 35S enhancer-*Tag1* minus 5′ intron

Tag1 line (T_1) (×35S-*dTag*-GUS)	Kan ^{R}:Kan ^{S} of T_2 plants	Excision frequency in leaves (F_1)	Germinal excision frequency (%)
pENT Δ5′-1	3:1	No activity	0.2
pENT Δ5′-2	3:1	No activity	ND
pENT Δ5′-3	2:1	No activity	0.1
pENT Δ5′-4	2:1	No activity	ND
pENT Δ5′-5	22:1	No activity	ND
pENT Δ5′-6	3:1	No activity	0.3
pENT Δ5′-7	4:1	No activity	0.2
pENT Δ5′-8	15:1	No activity	ND

Lines containing an enhancer-*Tag1* (transposase source) were crossed to homozygous 35S-*dTag*-GUS lines to determine excision activity promoted by the *Tag1* element. Germinal excision frequencies were determined by counting completely blue-staining plants out of ~1000 F_2 seedlings from each line. Lines containing the transposase source were also selfed and 150–200 T_2 progeny were plated on kanamycin plates to determine kanamycin resistance ratios (which indicates the number of loci). ND, no germinal revertants detected.

Tag1 mRNA in leaves, and there was no expression from *Tag1*-promoter-GUS constructs in vegetative organs. If a 35S enhancer element is placed next to *Tag1*, *Tag1* mRNA is expressed in leaves, and vegetative excision is restored. These results suggest that it is the poor expression of *Tag1* transposase mRNA in vegetative organs that accounts for the absence of vegetative excision.

Further analysis of *Tag1* expression showed a remarkable level of tissue specificity. Histochemical analysis of *Tag1*-promoter-GUS lines showed expression specifically in developing ovules and pollen, becoming active after meiosis (or possibly during meiosis in the case of the megaspore mother cell), and then disappearing by the time of fertilization. These results suggest that transposase expression is targeted specifically to the developing gametophyte and provide an explanation for the late germinal excision behavior of *Tag1*. Previous studies showed that (1) germinal revertant sectors in siliques never included more than one seed and (2) revertants from a single plant had different Southern blot patterns indicative of independent insertion events (Liu and Crawford 1998a). If germinal excision is also restricted to the developing gametophyte due to targeted expression of *Tag1* transposase, then this could explain why germinal reversion events are independent. None of these findings preclude post-transcriptional mechanisms that might control *Tag1* expression and excision, but transcriptional regulation that directs *Tag1* mRNA to the developing gametophyte is a mechanism that can explain the developmental control of *Tag1* excision.

Another interesting result from these studies is that an adjacent enhancer can influence the expression and excision of *Tag1*. The presence of the 35S enhancer region, which contained no core promoter, promotes ecotopic production of *Tag1* mRNA and excision in leaves. This effect of neighboring enhancers is similar to that of *Ac*, which responds to adjacent enhancers (Balcells and Coupland 1994), but not to that of *Spm*, which is resistant (Raina *et al.* 1993). The regulation of *Tag1* expression and excision was analyzed primarily in transgenic Columbia plants containing an introduced *Tag1* element as the source of transposase. To verify that *Tag1* elements native to the Arabidopsis genome behave in a similar fashion, *Tag1* expression and excision were analyzed in plants of the Landsberg (*erecta*) ecotype, which contains two closely linked and identical endogenous elements. In untransformed plants, the same distribution of *Tag1* mRNA was found (*i.e.*, much higher in reproductive organs than in vegetative organs of flower buds using real-time PCR) as that in *Tag1*-transformed Columbia plants. In addition, germinal excision but no vegetative excision was found with a 35S-*dTag*-GUS construct transformed into Landsberg plants. By these criteria, we conclude that *Tag1* excision is specific to the germinal lineages as either a native or an introduced element.

Germline-restricted transposition has the feature that transposon amplification can occur specifically in the cell lineages where new insertions can be inherited without risking damage to the vegetative parts or soma of the host. The germline-specific regulation of the *P* element in Drosophila provides an example. It is speculated that the element has been able to amplify to high-copy numbers since its introduction into the Drosophila genome 200 years ago (Engels 1996). Germline-specific transposition not only occurs naturally, but also has been designed for transposon-tagging purposes. Similar systems of germline-restricted transposition have been artificially engineered using anther-specific promoters to drive transposase expression (Firek *et al.* 1996). This artificially created system may in fact mimic the natural regulation of certain developmentally controlled transposons such as *Tag1*. For example, the *Tag1* promoter region may have acquired *cis*-acting elements from gametophyte-specific genes. This has been proposed for the *Mutator* transposon, which contains several pre-

viously characterized pollen enhancer motifs within the TIR promoter regions (Raizada *et al.* 2001). Sequence analysis and transcription factor binding site database searches did not reveal the presence of any obvious regulatory motifs in the *Tag1* promoter or in the 5′ UTR region. Our goal now is to determine how expression of *Tag1* is restricted to specific stages of gametophyte development.

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