Characterization of the Germinal and Somatic Activity of the Arabidopsis Transposable Element *Tag1*

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ABSTRACT

Tag1 is an autonomous transposon of *Arabidopsis thaliana*. The excision behavior of *Tag1* during reproductive and vegetative development was examined using CaMV 35S-*Tag1*-GUS constructs. Germinal reversion frequencies varied from 0 to 27% and correlated with *Tag1* copy number. Southern blot and somatic sector analyses indicated that each revertant was derived from an independent excision event, and approximately 75% of the revertants had new *Tag1* insertions. Revertants were obtained with similar frequencies from the male and female parents. In flowers, small somatic sectors were observed in siliques, carpels, petals and sepals while stemlike organs (filaments and pedicels) had larger sectors. No sectors encompassing entire flowers or inflorescences were observed, however, indicating that excision occurs late in flower development and rarely in inflorescence meristems. Late excision was also observed during vegetative development with 99.8% of leaves showing small sectors encompassing no more than 20 cells. Roots and cotyledons, however, showed larger sectors that included entire lateral roots and cotyledons. These results indicate that *Tag1* can excise in the embryo and all the organs of the plant with the timing of excision being restricted to late stages of vegetative and reproductive development in the shoot.

AN endogenous transposable element, *Tag1* is found in the plant *Arabidopsis thaliana*. It was discovered in a chlorate resistant mutant that had an insertion in the nitrate transporter gene *CHL1* (Tsay *et al.* 1993). The insertion (*Tag1*) is 3.3 kb in length, has 22-bp inverted repeats at both ends, and duplicates 8 bp of genomic DNA upon insertion. When it excises from the *chl1* locus, it produces chlorate sensitive revertants leaving behind small insertions or "footprints." *Tag1* is found in the Landsberg *erecta* ecotype of Arabidopsis but not in the Columbia or WS ecotypes. Sequence comparisons have revealed that *Tag1* is a member of the *Ac* superfamily of transposons (also called hAT family), which include *Bg* from maize, *Slide* from tobacco, *Tam3* from snapdragon, *Hobo* from Drosophila, and *Hermes* from the housefly (Calvi *et al.* 1991; Warren *et al.* 1994; Grappin *et al.* 1996).

The initial studies of *Tag1* showed that it is active and can excise to produce germinal revertants. To better characterize the transposition behavior of *Tag1,* we introduced it into a 35S-GUS reporter gene (Jefferson 1989), which has been used to follow transposon excision in plants (Finnegan *et al.* 1989; Masson and Fedoroff 1989; Lawson *et al.* 1994). When 35S-*Tag1*-GUS constructs were transformed into tobacco, a heterologous host, *Tag1* excised to produce somatic sectors in leaves (Frank *et al.* 1997). No excision activity was seen when a GUS construct containing a defective *Tag1* element (constructed *in vitro*) was used. We concluded that *Tag1* is an autonomous element capable of independent excision. When *Tag1* was introduced into Arabidopsis ecotypes that contain no *Tag1* elements, it excised to produce small somatic sectors in leaves and both small and large sectors in roots in the four to five transgenic lines examined. Germinal revertants also appeared as completely blue staining progeny.

Having established that *Tag1* is an autonomous element capable of both somatic and germinal excision, we wished to determine the timing and frequency of *Tag1* excision during plant development and search for any consistent pattern that would indicate some form of developmental control. Over 40 independent transgenic plants containing 35S-*Tag1*-GUS insertions located in different regions of the genome were generated for this study. For each line, the somatic and germinal excision behaviors of the introduced *Tag1* elements were examined. Analysis of the location and size of sectors gives us an indication of the developmental timing of excision, which, when it occurs in the cell lineages that give rise to the gametes, determines the number and genetic relationship of the germinal revertants. We also examined the inheritance of revertant alleles from the male and female parents, the frequency with which *Tag1* reinserts in the genome, and the pattern of *Tag1* reinsertion bands in the revertant progeny. The results of these experiments indicate that *Tag1* excision is restricted to late stages of shoot development and produces independent germinal revertants most

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often with new *Tag1* insertions in the Arabidopsis genome.

MATERIALS AND METHODS

Plant growth and histochemical staining: Arabidopsis seeds were sown on GM medium with kanamycin (30 μg/ml) as described (Valvekens *et al.* 1988). Plants were grown under continuous light at 23–25°. Seven days after germination, kanamycin-resistant seedlings were either stained for GUS expression or transferred to soil for further growth. Histochemical assays for GUS expression were performed as described (Jefferson 1989). Samples of plant tissue were directly submerged into solutions containing 50 mm sodium phosphate buffer, pH 7.0, 0.5 mm $KFe(CN)_2$, 0.5 mm $KFe(CN)_3$, 10 mm EDTA, 0.1% Triton X-100, 2 mm 5-bromo-4-chloro-3-indol-glucuronide cyclohexylamine salt (X-Gluc) and incubated at 37° overnight. After incubation, plant samples were cleared by several changes of 70% ethanol and photographed in the same solution.

DNA constructs and transgenic plants: For our DNA constructs, a *Tag1* element was PCR amplified from a genomic clone using two primers that hybridized to genomic flanking sequence 5′-CCCTCTAGATTGATTCCTTACGTCTAAAC-3′, primer 1, and 5′-CCCGGATCCAAATTAATCAATTGTAGTAT-3′, primer 2. PCR reactions were performed as follows: 95°, 1 min; 55°, 1 min; and 72°, 5 min for 30 cycles. The PCR products were digested with *Xba*I and *Bam*HI, and cloned to corresponding sites in the vector pBluescript SK (Stratagene). The PCR products contain the entire 3.3-kb *Tag1* element plus 8 bp of duplicated target sequences at each end along with 12- and 23-bp flanking sequences (excluding 8-bp target sequence) at the 5′ and 3′ ends, respectively. The entire PCR product was resequenced for accuracy. Subsequently, the *Tag1* element was subcloned into the *Xba*I and *Bam*HI sites of the pBI121 expression vector (CLONETECH, Palo Alto, CA). The insertion site was between the CaMV 35S promoter and GUS gene and the *Tag1* element was placed in the same orientation as the 35S-GUS gene. DNA constructs in pBI121 were first transformed into *Agrobacterium tumefaciens* strain C58 AGL-0 (Lazo *et al.* 1991) and then into Arabidopsis plants using vacuum infiltration (Bechtold *et al.* 1993).

Isolation of genomic DNA: Three mature leaves or 0.2 g of progeny seedlings from a single plant were used for genomic DNA isolation. Plant tissues were ground to fine powers in liquid nitrogen and transferred to a Eppendorf tube containing 1 ml extraction mix. Extraction mix consists of 1 volume extraction buffer (0.35 m sorbitol, 0.1 m Tris-base, 2% cetyl trimethyl ammonium bromide, 5mm EDTA, pH 7.5), 1 volume nuclear lysis buffer (0.2 m Tris-base, 50 mm EDTA, 2 m NaCl), and 0.4 volume of 5% sarkosyl. Sodium bisulfite was added to extraction buffer to final concentration of 20 mm before use. After extraction, plant samples were incubated at 65° for 20 min, then 1 ml of chloroform:isoamyl alcohol (24:1) was added to fill the tube and gently shaken for 5 min. Tubes were centrifuged at 10,000 rpm for 5 min, and the top aqueous phase was transferred to a new 2-ml tube. One volume of cold isopropanol was added to precipitate DNA at −20° for 10 min. After precipitation, samples were centrifuged at 10,000 rpm for 5 min, and DNA pellets were washed with 70% ethanol. Dried pellets were resuspended in 50 μl TE buffer. After resuspension, tubes were centrifuged for another 5 min to pellet starch debris. Ten μl of clean supernatant was used for Southern hybridization analysis.

Southern hybridization: Genomic DNA was digested with *Hin*dIII and separated on 0.8% agarose gels. After electrophoresis, DNA was denatured and transferred to Hybond-N nylon membrane (Amersham). Membranes were prehybridized in solution containing 50% formamide, 5× Denhardt's, 0.5% SDS, 100 μg/ml salmon sperm DNA, and 6× SSPE at 42° for 4 hr. After prehybridization, 20–40 ng ^{32}P-labeled DNA was added, and membranes were hybridized at same condition for 24 hr. After hybridization, membranes were washed in 2× SSPE, 0.5% SDS two times, 0.1× SSPE, and 0.1% SDS two times. The first three washes were carried out at room temperature, each for 20 min; the final wash was at 42° for 40 min.

RESULTS

Tag1 excision was monitored in transgenic plants using a GUS reporter gene as described previously (Frank *et al.* 1997). Briefly, *Tag1* was inserted between the CaMV 35S promoter and the GUS coding region. *Tag1* blocks GUS gene expression, and upon excision, GUS expression is restored. The 35S promoter is a constitutive promoter allowing us to detect *Tag1* excision in virtually all tissues (Benfey *et al.* 1989). Cells inheriting the excision product are detected histochemically as blue staining sectors. The number of blue sectors is an indication of *Tag1* excision frequency; sector size reflects the timing of *Tag1* excision with small sectors indicating events occurring late in organ development. Progeny that are germinal revertants will stain completely blue. The Arabidopsis ecotype Columbia was the host for these experiments because it contains no endogenous *Tag1* elements as determined by Southern blot analysis (Tsay *et al.* 1993). Forty-four independent transgenic plants containing the 35S-*Tag1*-GUS construct were generated by Agrobacterium-mediated transformation and selected with kanamycin. Progeny from each of these plants (making up a line) were stained for GUS expression.

***Tag1* germinal reversion frequency:** The germinal reversion frequencies (or rate) for each of the 44 transgenic plants were determined and then compared with the number of *Tag1* elements in the genome of each plant. Germinal reversion frequency is defined here as the percentage of germinal revertants (completely blue staining seedlings) among all the kanamycin-resistant progeny, *i.e.*, those containing at least one functional transgene, from a given parental line. *Tag1* copy number for each line was estimated by Southern blot analysis by counting the number of bands produced by *Hin*dIII digestion of genomic DNA probed with a *Tag1* fragment (probe C in Figure 3A).

Germinal reversion frequencies were found to vary from line to line even among those lines that had the same estimated *Tag1* copy number (Figure 1A). For example, the frequency varied from 1.5 to 10% for lines that have six copies of *Tag1*. Among all the lines, the frequency varied from 0 to 10% with lines having more copies of *Tag1* tending to have higher average reversion frequencies. For example, lines with only one *Tag1* element had an average reversion frequency of 0.3% (±0.4%) with four out of eight lines producing no ger-

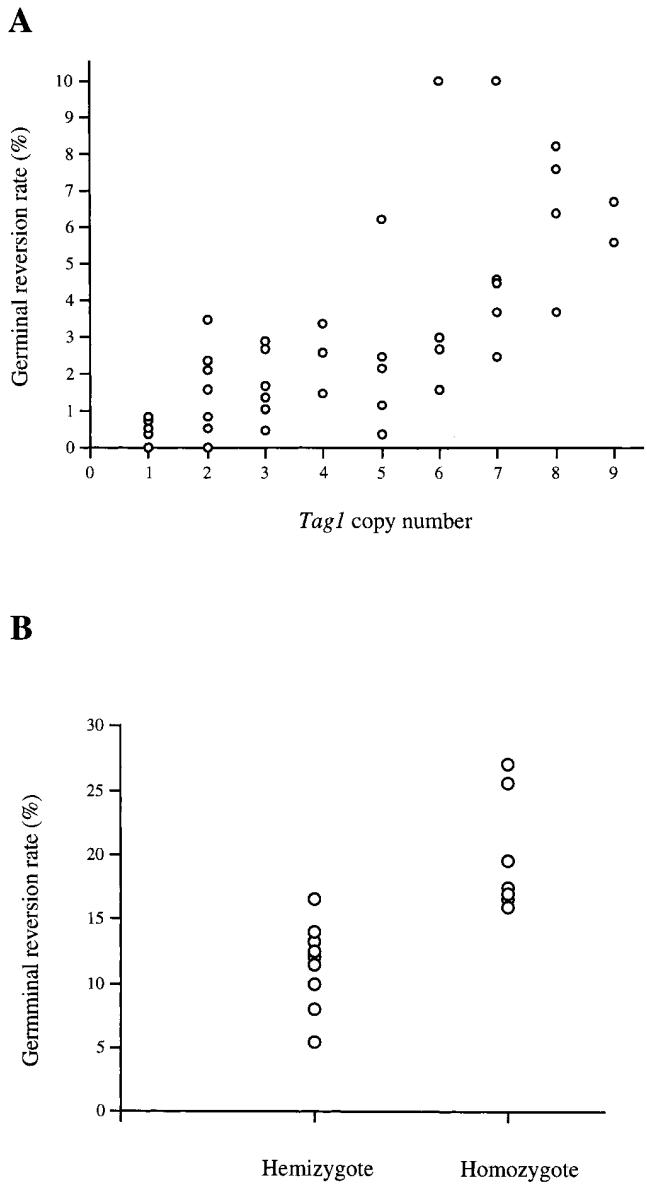

Figure 1.—*Tag1* germinal reversion rate. (A) Percent germinal revertants are given for each transgenic line and plotted based on the estimated number of *Tag1* copies in the primary hemizygous transgenic plants. Kanamycin resistant progeny (approximately 250) from each line were assayed for GUS expression. Completely blue staining seedlings were scored as germinal revertants. Average reversion rates were as follows: 1 copy (8 lines), 0.3 ± 0.4; 2 copies (7 lines), 1.6 ± 1.2; 3 copies (6 lines) 1.7 ± 0.9; 4 copies (3 lines), 2.5 ± 0.9; 5 copies (5 lines), 2.6 ± 2.1; 6 copies (4 lines), 4.3 ± 3.8; 7 copies (5 lines), 5.1 ± 2.8; 8 copies (4 lines), 6.5 ± 2.8; and 9 copies (2 lines), 6.2 ± 0.8. (B) Percent of germinal revertants are given for 9 homozygous and 7 hemizygous progeny from selfed TG-3. Progeny plants were self-pollinated, and seeds were collected from each individual plant. Seeds were plated on nutrient agar with kanamycin, and then resistant seedlings (approximately 250) were assayed for GUS expression. Completely blue staining seedlings were scored as germinal revertants.

minal revertants. In contrast, the average reversion frequencies in lines with eight or nine *Tag1* elements were 20-fold higher. Overall, the average germinal reversion frequency was found to increase fairly linearly with *Tag1* copy number (see Figure 1A legend), indicating that the effect is additive and not synergistic so that the frequency per element is approximately the same. This hypothesis would predict that a homozygous plant should have approximately two times the reversion frequency compared with a hemizygous sibling. To test this, reversion frequencies were determined for 17 individual progeny from a hemizygous plant (designated TG-3). TG-3 has an estimated seven copies of *Tag1* at a single locus (segregating kanamycin-resistant to -sensitive progeny at a ratio of 3:1) and a reversion frequency of 10%. Hemizygous offspring from TG-3 had an average reversion frequency of 11.5% (±3.5%) while homozygous offspring had 20% (±4.5%) (Figure 1B). Some of the homozygous offspring had the highest reversion frequency (over 25%) we have yet observed.

The stability of the reversion frequencies from one generation to the next was assessed next by following *Tag1* reversion frequencies for five consecutive generations in two different lines. Line TG-3 began with a reversion frequency of 10% and line TG-1 with a frequency of 5%. The primary transformants for each line were both hemizygous and showed a 3:1 segregation of kanamycin-resistant progeny. Reversion frequencies were determined for only hemizygous plants for each generation. The data show (Figure 2) that the average reversion frequencies do not vary much from generation to generation and are maintained for at least five

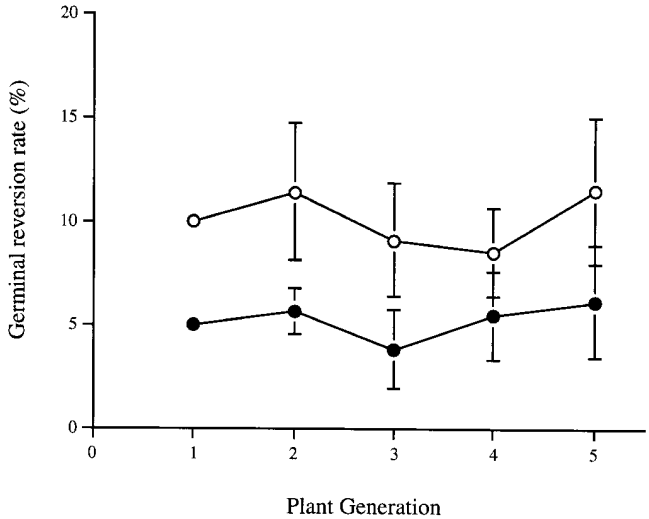

Figure 2.—Inheritance of germinal reversion activity. Germinal reversion rates for eight plants were determined in each of five generations and for the primary transformant (generation 1). All plants tested were hemizygous. Data are average values with standard deviation. (●), Line TG-1; (○), Line TG-3.

generations. We conclude that *Tag1* reversion frequencies are relatively stable from generation to generation.

We also examined the inheritance of revertant alleles from the male and the female organs of a plant. Differences in the frequency or transmission of excision events in the cell lineages that produce male or female gametes can skew the number of revertants that are inherited from the male or female parent. To test this, reciprocal crosses between untransformed, wild-type plants and transgenic plants that carried the 35S-*Tag1*-GUS constructs were performed. To determine the contribution of revertants from the male lineage, pollen from four plants of line TG-3 (two hemizygotes and two homozygotes) were applied to flowers of untransformed plants. Kanamycin-resistant seedlings derived from F1 hybrid seeds were stained for GUS expression. Reciprocal crosses where the female contained the 35S-*Tag1*-GUS construct were performed to assess the contribution from the female parent. The four transgenic plants were also self-pollinated, and their progeny was stained for GUS expression.

The data show that the germinal reversion frequencies were approximately the same for both reciprocal crosses (Table 1). Approximately the same fraction of germinal revertants were observed from crosses where the male contributed the 35S-*Tag1*-GUS construct and from crosses where the female contributed the 35S-*Tag1*-GUS construct. We conclude that there is no bias in the inheritance of the germinal revertant alleles.

Examination of the data (Table 1) shows that the germinal reversion rates in progeny produced from selfing the transgenic plants is a little higher than the sum of the rates from the pairwise crosses. Assuming no segregation distortion, the frequency of germinal revertants from selfed plants should be slightly lower than the sum of the pairwise crosses because of masking of some of the revertant alleles in homozygous progeny. Our data show the opposite; the frequency is slightly higher among the progeny of selfed plants. One explanation for this result is that some excision events are occurring in the zygote, and, when a zygote is homozygous for the *Tag1* transgene, the excision rate is higher. As noted above, genetic dosage does influence the rate of germinal reversion.

Once an element excises, it can either reinsert or be lost from the genome. The value of *Tag1* as an insertional mutagen depends in part on its probability of reinsertion. Previously, we showed that new insertion bands could be observed on Southern blots of DNA from chlorate-sensitive plants derived from *chl1::Tag1* mutants (Tsay *et al.* 1993). In the present study, we examined the fraction of revertant progeny from a single transgenic plant that showed new *Tag1* insertion bands. Genomic DNA was analyzed by Southern blot analysis. *Hin*dIII digestion cuts *Tag1* in the 35S-*Tag1*-GUS construct into three fragments: a central 260-bp fragment, a 2.3-kb left fragment containing the 0.8-kb 35S promoter, and a right fragment whose size depends on the position of a *Hin*dIII site in the flanking genomic sequence (Figure 3A). If the element excises and reinserts, additional bands should be observed with distinct flanking sequences on both sides. Two probes were used for this analysis; probe A is a fragment from the left side of *Tag1*, and probe C is from the right side (Figure 3A).

Forty-seven germinal revertants from the primary transformant TG-3 were examined (Figure 3 and data not shown). Genomic DNA from TG-3 produced seven

TABLE 1

Germinal reversion rates for progeny of line TG-3[a] crossed with WT plants[b]

Crosses		Kan^R progeny	Kan^S progeny	Germinal revertant	Germinal reversion rate (%)
Male	Female				
Selfed	TG-3.8[c]	397	144	32	8.2
TG-3.8 ×	WT	210	195	8	4.1
WT ×	TG-3.8	156	160	6	3.7
Selfed	TG-3.5[c]	338	123	31	9.1
TG-3.5 ×	WT	150	132	5	3.7
WT ×	TG-3.5	195	213	7	3.2
Selfed	TG-3.24[d]	253	0	24	9.4
TG-3.24 ×	WT	178	0	9	5.0
WT ×	TG-3.24	219	0	6	3.7
Selfed	TG-3.25[d]	197	0	31	15.7
TG-3.25 ×	WT	150	0	9	6.0
WT ×	TG-3.25	162	0	11	6.8

[a] Primary transformant TG-3 is T_1 generation. Direct progeny plant from TG-3 is defined as T_2 generation, and so on.

[b] Wild-type, untransformed plants.

[c] Hemizygous plants (T_2 generation).

[d] Homozygous plants (T_3 generation).

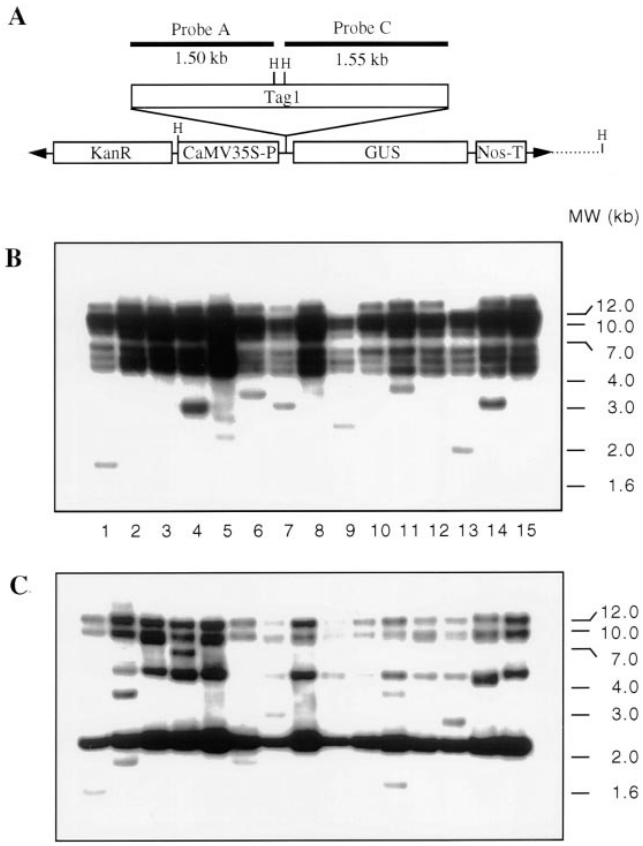

Figure 3.—Southern blot analysis of germinal revertants. (A) Diagram of chimeric gene construct used in this study. Kan^R, Kanamycin resistance gene; CaMV 35S-P, Cauliflower mosaic virus 35S promoter; GUS, β-glucuronidase gene; Nos-T, nopaline synthase gene terminator sequence; H, *Hin*dIII enzyme site. Arrows at both ends refer to T-DNA left and right borders. Regions on *Tag1* element covered by probe A and probe C are indicated. (B) Southern blot of Arabidopsis genomic DNA from the progeny of individual revertant plants obtained from a single plant in line TG-3 digested with *Hin*dIII and hybridized with probe C. (C) Same blot as in B except hybridization was performed with probe A. Lane 1–14, revertant lines; lane 15, primary transformant.

bands after *Hin*dIII digestion and hybridization with probe C (Figure 3B, lane 15). There was no significant hybridization to DNA from untransformed plants (data not shown). DNA from 63% of the revertant lines showed new hybridization bands (Figure 3B, lanes 1–14, and data not shown). Because some of the new bands could overlap with those present in the primary transformant, the same blot was rehybridized with probe A. With this probe, only a 2.3-kb band from the 35S-*Tag1*-GUS construct is expected (Figure 3A). We detected three additional bands in the TG-3 transformant indicating that the internal *Hin*dIII site flanking the 35S promoter was altered in some of the integrants (Figure 3C, lane 15). Examination of the revertant lines showed new hybridization bands in 53% of the lines (Figure 3C, lanes 1–14, and data not shown). When data from both blots were combined, new *Tag1* hybridizing bands were detected in 74% of the lines, which we believe is a minimal estimate. Eleven revertant lines had one new band, twelve lines had two new bands, and one line had three new bands with an average number of new bands in each revertant line being one. In summary, three-fourths of the revertants show new hybridization bands indicating that *Tag1* has a high probability of reinsertion.

Another important conclusion can be made from the Southern blot data. If excision events are occurring early during the development of a plant, large sectors will be produced that contain many identical revertants. If excision events occur late, most of the revertants will arise from independent excision events. The pattern of hybridization bands for each revertant was found to be unique (Figure 3, B and C, and data not shown). This result indicates that each revertant is independent, arising from unique excision events, and that early excision is not occurring.

***Tag1* somatic excision in siliques and flowers:** To gain more insight into the pattern of *Tag1* excision during plant development, we examined the pattern of somatic sectors in various organs of the plant. If excision is occurring late during development in the cell lineages that give rise to the gametes, we should also observe small sectors in flower organs and siliques, which arise from the same floral meristems that give rise to the gametes. Eight to ten fully developed green siliques 6–8 days after pollination, and two whole inflorescences containing 8–12 flowers at different developmental stages (from buds to fully opened flowers) were examined for each plant.

Siliques from all 44 primary transformants and 20 progeny plants from each of four individually selected lines (TG-1, TG-2, TG-3, and TG-5) were examined. The primary transformants TG-1, TG-2, TG-3, and TG-5 have an estimated 9, 1, 7, and 5 copies of the 35S-*Tag1*-GUS construct, respectively (Figure 3 and data not shown), and show an approximate 3:1 segregation of the kanamycin-resistant phenotype indicating a single locus for the transgene(s). Siliques from plants containing a 35S-GUS construct stained completely blue (Figure 4A), while siliques from untransformed plants showed no staining (Figure 4B). For transgenic plants containing the 35S-*Tag1*-GUS construct, all siliques (over 1000 examined) showed GUS sectors as small blue dots or short strips (Figure 4, C–E). Small sectors were also observed on silique pedicels (Figure 4F). In a few rare cases, the entire pedicel stained blue (Figure 4G), but the GUS staining did not extend into the silique. These results indicate that the observed sectors were because of *Tag1* excisions that occurred late during carpel/silique development. Because no large sectors encompassing entire siliques were found in any of the transgenic plants, we conclude that the late timing of excision was independent of *Tag1* copy number and genetic dosage.

The somatic sectors described above are in the maternal tissues of the silique. By examining the embryos/seeds within the silique, one can gain insight into the pattern of germinal excision events. Blue staining seeds indicative of germinal revertants were detected in siliques (Figure 4H). The maternal tissues surrounding these seeds, the funiculus (Figure 4I) and seed coats (Figure 4J), did not stain blue. The position of the blue staining seeds in a given silique was random. Occasionally, two revertant seeds were found adjacent to each other, but the surrounding maternal tissue showed no staining (Figure 4I). Thus, we find no evidence of sectors that encompass more than a single seed. Because we found no bias in the inheritance of germinal revertants from the male versus the female lineages, approximately half of these revertants were because of excision events in the female lineage. These results suggest that germinal excision events are occurring after ovule founder cells have separated from cells that give rise to the surrounding tissues. We have also examined anthers to check for evidence of early excision events that would give rise to large or "jackpot" sectors encompassing entire anthers. We only found a few pollen grains (less than 1%) staining blue in all anthers examined (see below).

The frequency of revertant sectors was also examined in siliques to assess the variation from line to line. Sector number varied from greater than 300 per silique in one line to less than 20 per silique in others (examples are shown in Figure 4, C–E). Within a plant, there was some variation from silique to silique, but 6–8 out of 10 siliques usually had the same variegation pattern. We examined the number of sectors on the carpels (progenitor to the silique) in a line that had many silique sectors (>300) and found that the carpels showed many fewer sectors (<30). This result further supports the finding that excision events are occurring late, in this case, after pollination.

The pattern of sectors on flower organs was exam-

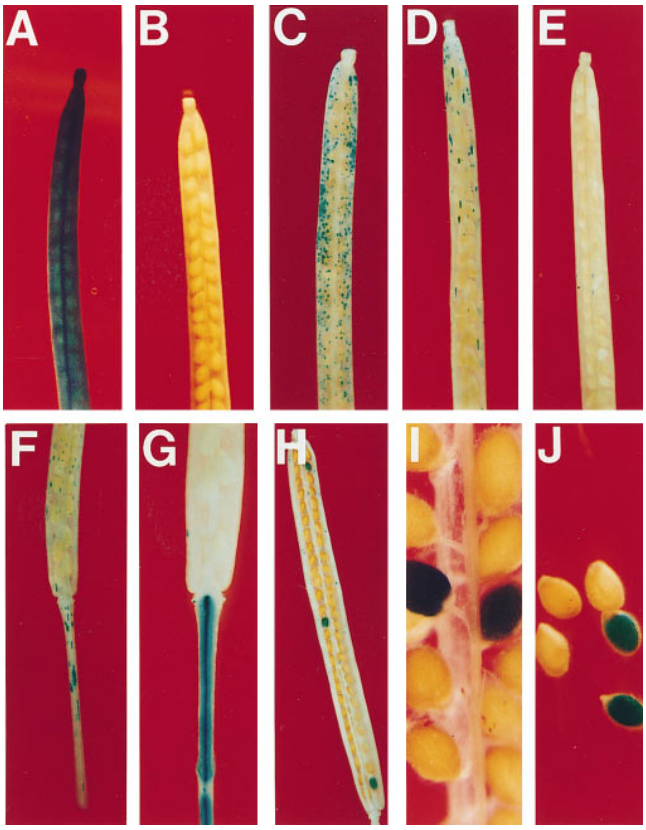

Figure 4.—*Tag1* revertant sectors in siliques. Fully expanded green siliques were stained for GUS expression. (A) Positive control showing a silique from a transgenic plant carrying a CaMV 35S-GUS construct. (B) Negative control showing a silique from untransformed plant. (C-I) Siliques from plants containing CaMV 35S-*Tag1*-GUS constructs. (C) A silique with highly variegated phenotype. (D) A silique with medium variegated phenotype. (E) A silique with lightly variegated phenotype. (F) A silique showing GUS sectors on its pedicel. (G) A silique with a rare completely blue staining pedicel. (H) An opened silique showing randomly positioned germinal revertants. (I) An enlargement of opened silique with two adjacent revertant seeds. (J) An enlargement of individual seeds (note nonstaining seed coats).

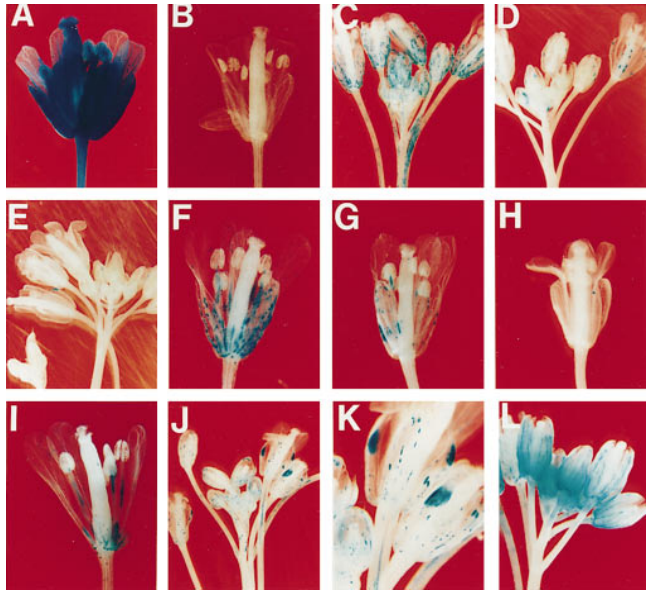

Figure 5.—*Tag1* revertant sectors in flowers. Flowers were stained for GUS expression as described in materials and methods. (A) Positive control showing a flower from a transgenic plant carrying CaMV 35S-GUS construct. (B) Negative control showing a flower from untransformed plant. (C-L) Flowers from plants containing CaMV 35S-*Tag1*-GUS constructs. (C) A highly variegated inflorescence from line TG-1. (D) A medium variegated inflorescence from line TG-3. (E) A lightly variegated inflorescence from line TG-2. (F) A highly variegated flower. (G) A medium variegated single flower. (H) A lightly variegated single flower. (I) A flower from line TG-1 showing large GUS sectors on filaments. Four sepals were removed to expose the inner organs. (J) An inflorescence from line TG-1 with two single flowers showing rare larger sectors. (K) An enlargement of J. (L) An inflorescence from line TG-1 showing uniform blue staining.

ined next. Plants carrying the 35S-GUS construct served as positive controls and stained completely blue (Figure 5A). The intensity of the staining varied from organ to organ (being lighter in petals, anthers, and parts of the carpels) but was detectable in all tissues. Flowers from untransformed plants showed no staining (Figure 5B). When transgenic plants containing the 35S-*Tag1*-GUS construct were examined, blue sectors were found in all four organs of the flower (sepals, petals, stamens, and carpels; Figure 5, C–L).

The size of the sectors (mostly small dots and strips) indicate that the timing of *Tag1* excision also occurs late in flower organ development. This pattern was strictly conserved in sepals, petals, and carpels; over a thousand flowers analyzed had the same pattern with only two flowers showing slightly larger but still localized sectors on the petals (Figure 5, J and K). In filaments and pedicels, the stem-like organs that attach to anthers and flowers/siliques, larger sectors covering one-third to one-half were often observed in addition to the small dots and strips (Figure 5, I and J). In a few rare cases, an entire filament stained blue (data not shown), but a whole blue staining anther was never observed. Examination of the pollen in over 40 individual anthers showed that all had a very low percentage of blue staining pollen (less than 1%; data not shown). No large dark staining sectors encompassing multiple organs and not an entire flower or branch were observed in any of the 44 lines, which contained a distribution of *Tag1* elements at different locations. We conclude that *Tag1* excises late during flower development regardless of its copy number and genetic dosage.

When the number of sectors was examined in the transgenic lines, it was apparent that frequency of somatic sectors varied greatly from line to line. Highly variegated flowers had over 100 sectors (Figure 5, C and F). Flowers with medium variegation had between 10 and 100 sectors (Figure 5, D and G). Lightly variegated flowers had less than 10 sectors on all four sepals (Figure 5, E and H). Within a plant, the excision frequency did not vary significantly between petals and sepals within a flower, among flowers within an inflorescence, nor among inflorescences. Within a line, however, the frequency varied from plant to plant. We observed one exceptional case (Figure 4L) in which half of the progeny from TG-1 had the unusual uniform blue staining pattern, which was restricted to sepals and could be because of splicing of the *Tag1* sequences from the chimeric *Tag1*-GUS mRNA.

***Tag1* somatic excision in vegetative organs:** The pattern of somatic sectors was also examined in leaves, cotyledons, and roots to determine if the late timing of *Tag1* excision observed in flower development was also occurring in vegetative development. The third and fourth true leaves from all 44 primary transformants and whole plants from the progeny of TG-1, TG-2, TG-3, and TG-5 were stained for GUS expression.

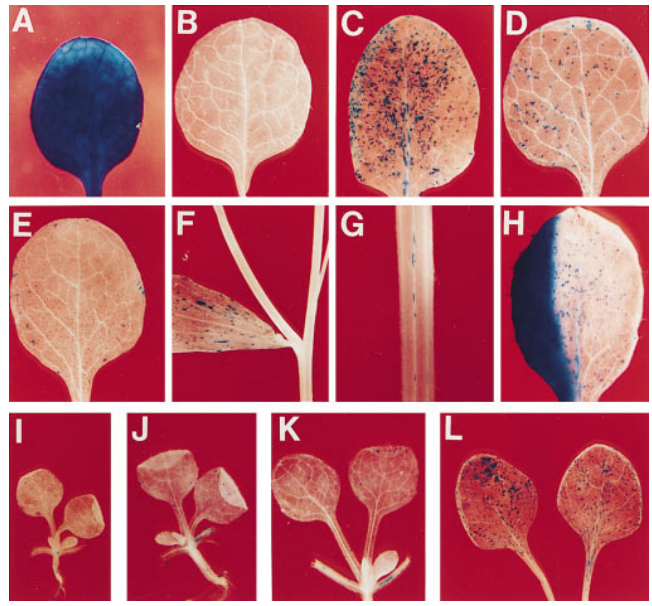

Figure 6.—*Tag1* revertant sectors in leaves. Leaves and stems were stained for GUS expression as described in materials and methods. (A) Positive control showing third true leaf from a transgenic plant carrying CaMV 35S-GUS construct. (B) Negative control showing third leaf from untransformed plant. (C-P) Shows GUS staining of plants containing CaMV 35S-*Tag1*-GUS constructs. (C) Third leaf from line TG-1 showing highly variegated phenotype. (D) Third leaf from line TG-3 showing medium variegated phenotype. (E) Third leaf from line TG-3 showing lightly variegatedphenotype. (F) Cauline leaf showing GUS sectors. (G) Stem showing GUS sectors. (H) Third leaf from line TG-1 at day 16 after seed germination showing a rare large GUS sector. (I)–(L) Leaf 1 and 2 from line TG-1 at consecutive days after emergence starting at day 6 (I) and ending at day 9 (L). Cotyledons were removed.

Leaves were sampled when they had just reached their fully expanded state. As positive and negative controls, leaves from transgenic plant containing a 35S-GUS construct stained completely blue (Figure 6A), and leaves from untransformed plant showed no GUS staining (Figure 6B). Leaves from transgenic plants bearing 35S-*Tag1*-GUS construct displayed a variable number of GUS sectors (Figure 6, C–E), but the size of GUS sectors in all leaves were similar and very small (usually encompassing less than twenty cells and sometimes only one cell). Sectors in other leaves including the cauline leaves and stems also exhibited the same small size (Figure 6, F and G and data not shown). Cross sections of leaf blades showed sectors in all major cell types, including epidermal, mesophyll, vascular, and trichome cells (data not shown). Among the more than 1000 leaves examined, only two had larger sectors. One such sector is shown in Figure 6H. This sector emerged from the leaf petiole and extended into the leaf blade comprising about one-third area of entire leaf. Similar sized sectors have been derived from X-ray irradiated seed used for fate map studies (Irish and Sussex 1992); thus, this rare *Tag1*-induced sector most likely arose

from an excision event in the shoot meristem. Typical small GUS sectors were also present on this exceptional leaf, and other leaves from the same plants and their progeny plants showed only the typical small sectors. These results show that *Tag1* excision almost never occurs in the shoot vegetative meristem but is delayed until late in leaf development after the founder cells leave the meristem to form the leaf primordium. This behavior was found in all 44 lines and is therefore independent of copy number and genomic location.

When the number of sectors per leaf were determined for each line, line TG-1 was found to have an exceptional phenotype. Initially, the number of sectors per leaf was low (less than 50) when the leaves were still small. Then at a specific stage during leaf expansion, a burst of excision activity would occur and continue for as long as the leaves were examined. To quantify this response, the number of sectors present on true leaves 1–4 were compared to the length of the leaf for five plants sampled every day for 23 days. The expansion of leaves 1 and 2 behaved very similarly and were treated as a pair. Figure 7 shows changes in leaf length; leaves 1 and 2 emerged at day 4 after seed germination and reached the maximum length at day 9 (Figure 7A); leaves 3 and 4 appeared at day 7 and 8, and reached full expansion at day 20 (Figure 7B).

A burst of excision activity during a particular interval during leaf expansion was observed (Figure 6, I–L, Figure 7, and Figure 8). For leaves 1 and 2, the burst appeared at day 9 after seed germination when leaves just finished their expansion (Figure 7A). The burst time for leaves 3 and 4 was between days 15 and 16 during the middle of leaf expansion (Figure 7B). When whole plants were stained for GUS expression at day 28 after seed germination (Figure 8), one could observe numerous sectors in leaves 1 through 4, which had reached expansion fully. In contrast, younger leaves still in the expansion stage had very few sectors. Leaf 5 appears caught in the middle of the switch with 80% of the leaf area showing sectors while leaf 6, which is only two days delayed compared with leaf 5, showed only a few sectors. This sudden tran-

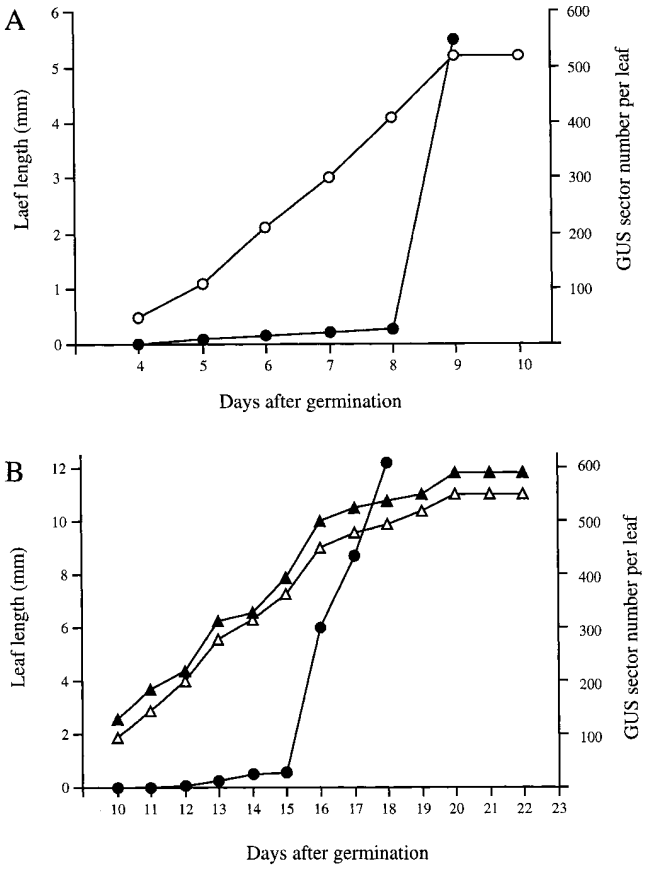

Figure 7.—*Tag1* excision during leaf expansion in line TG-1. Leaf length and GUS sector number were measured every day on leaves 1, 2, 3, and 4 after they emerged. (A) *Tag1* excision pattern during development of leaves 1 and 2: (○) leaf length; (●) GUS sector number. Each point represents average value from 5 pairs of leaves. (B) *Tag1* excision pattern during development of leaves 3 and 4: (▲) length of leaf 3; (△) length of leaf 4; (●) GUS sector number. For leaf length, each point represents an average value from 8 leaves; for sector number, each point represents an average value from three pairs of leaves.

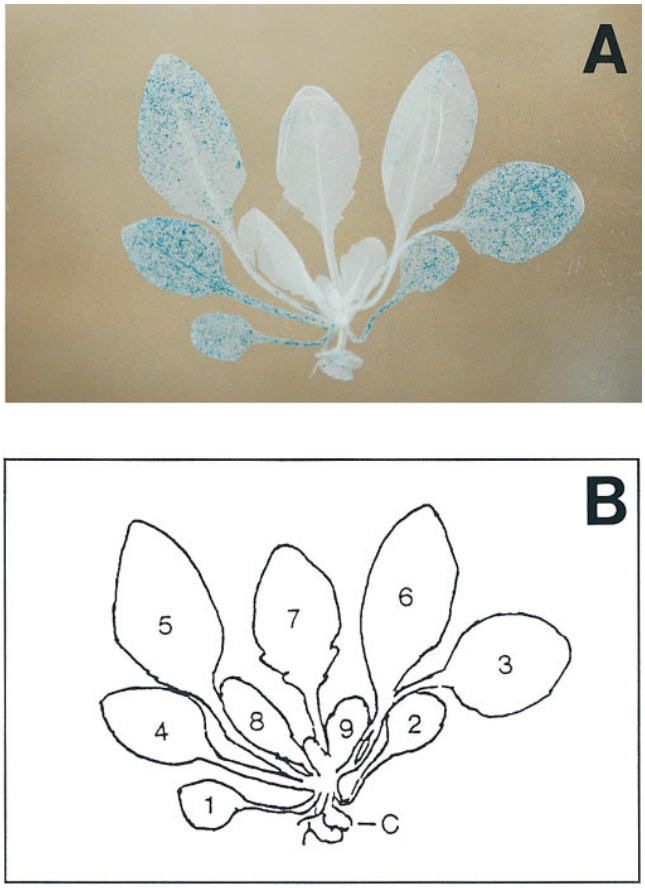

Figure 8.—*Tag1* revertant sectors in a whole plant from line TG-1. (A) Whole GUS staining of a plant at day 28 after seed germination. (B) A diagram indicating leaf number of plant shown in A; C, cotyledon.

sition of *Tag1*-excision activity from leaf 5 to leaf 6 suggests that there is a switch that turns on *Tag1* excision at a particular interval during leaf expansion in this line.

Lastly, *Tag1* somatic sectors were examined in cotyledons and roots in progeny of primary transformants TG-1, TG-2, TG-3, and TG-5 and compared with the sectors in leaves and flowers. Whole seedlings containing 35S-GUS control constructs stained completely blue (Figure 9A), and no GUS staining was found in the seedlings from untransformed plants (Figure 9B). Seedlings from transgenic plants carrying 35S-*Tag1*-GUS constructs exhibited distinct sector types in cotyledons. Lines TG-1 and TG-2 exhibited a mixture of small- to medium-sized sectors (Figure 9, C and D). In line TG-3, sectors were uniformly small consisting of only a single or few cells (Figure 9, E and F). In line TG-5, GUS sectors were medium to large and usually covering one-eighth to one-half area of a single cotyledon (Figure 9, G and H). In a few rare seedlings, two entire cotyledons stained blue indicative of an excision event in the embryo (data not shown). Roots also showed a wide distribution of sector sizes. In lines TG-2 and TG-5, sectors were very small regardless of the pattern in the cotyledons (Figure 9I). However, in lines TG-1 and TG-3, both small and large sectors were observed with some sectors covering one-fourth to one-third area of primary root indicative of excision events in the embryo or root meristem (Figure 9, J and K). In about 2% of seedlings stained from line TG-1, one or two whole lateral roots stained blue (Figure 9L). In rare cases, the primary root stained blue with the shoot part showing variegation (Figure 9M). These results show that the timing of excision in roots, cotyledons, and the embryo is variable and line-dependent.

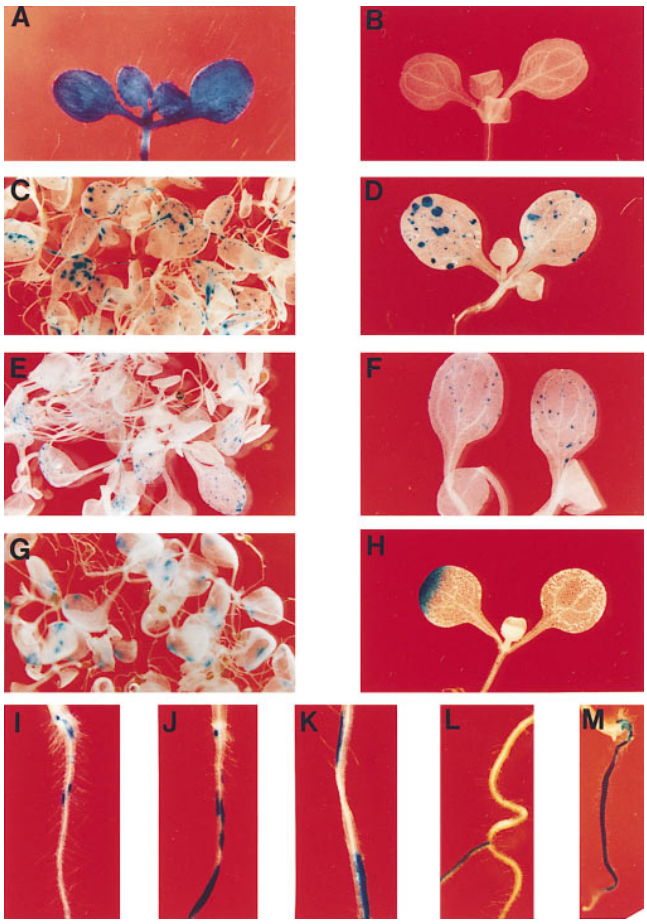

Figure 9.—*Tag1* revertant sectors in cotyledons and roots. Seeds from primary transformants were sown on kanamycin containing medium. At day 8 after seed germination, kanamycin resistant seedlings were stained for GUS expression. (A) Positive control showing a seedling from a transgenic plant carrying a CaMV 35S-GUS construct. (B) Negative control showing a seedling from an untransformed plant. (C-M) Shoots and roots from plants containing CaMV 35S-*Tag1*-GUS constructs. (C) A population of seedlings from line TG-1. (D) A single seedling from line TG-1. (E) A population of seedlings from line TG-3. (F) A single seedling from line TG-3. (G) A population of seedlings from line TG-5. (H) A single seedling from line TG-5. (I) A root showing only small sectors. (J) A root showing both small and large sectors. (K) A root showing only large sectors. (L) A whole blue staining lateral root. (M) A whole staining primary root.

Sector number for cotyledons also showed considerable variation between and within lines. The average number of GUS sectors per cotyledon in lines TG-1, TG-2, TG-3, and TG-5 were approximately 19, 1, 39, and 2, respectively. In roots, sector numbers were low regardless of their frequency in cotyledons. In our most active line TG-1, the average sector number in the whole root system was less than six (for seedlings that were 8-days old after germination; data not shown). Many seedlings from these four lines did not show any GUS sectors in roots.

In all the transgenic lines described above, the sequences immediately adjacent to *Tag1* (the 35S promoter and GUS reporter gene) and the genetic background, *i.e.*, Columbia ecotype, were all the same. To determine if the *Tag1* excision behavior that we were observing is specific to the Columbia ecotype or is dependent on the sequences immediately adjacent to the transposase source, we examined *Tag1* excision in a different ecotype and in a different construct. First, the 35S-*Tag1*-GUS construct was transformed into Landsberg *erecta* plants. Eight independent transgenic lines were produced. All displayed the same pattern of sectors in leaves, roots, flowers, and siliques seen in the Columbia ecotype (data not shown). Second, a defective *Tag1* element (*dTag1*), constructed by deleting an internal 1.4-kb *Eco*RI fragment, was introduced into Landsberg *erecta* plants in the 35S-GUS reporter construct. This *dTag1* has already been shown to be mobile in Landsberg (Frank *et al.* 1997). In these plants, the autonomous *Tag1* elements, which are providing the transposase function, are not adjacent to the 35S promoter but reside next to native sequences on chromosome *1* of the Arabidopsis genome. Five independent transgenic lines were produced and all had the same pattern of sectors observed in the Columbia lines (data not shown). These results indicate that timing of exci-

sion is not ecotype-specific nor dependent on the 35S promoter being adjacent to the transposase source.

DISCUSSION

Tag1 is a transposable element whose mobility has been demonstrated in both its host Arabidopsis and in a heterologous plant, tobacco (Tsay *et al.* 1993; Frank *et al.* 1997). Compared to other plant transposons, such as *Ac*, *Spm*, and *Mutator* in maize and *Tam3* in snapdragon, the genetic and molecular properties of *Tag1* are less well characterized. The original study of *Tag1* relied on an insertion allele of the *CHL1* gene to monitor *Tag1* excision and showed that *Tag1* can excise from the *chl1* locus to produce chlorate-sensitive revertants (Tsay *et al.* 1993). Subsequently, we demonstrated that *Tag1* is an autonomous element that excises during vegetative growth to produce somatic sectors in leaves and roots using a CaMV 35S-GUS reporter construct (Frank *et al.* 1997). In the study reported here, we characterized the timing and frequency of *Tag1* excision in both reproductive and vegetative development to determine if *Tag1* excision activity is developmentally controlled and if *Tag1* had properties that would make it useful for insertional mutagenesis.

After analyzing over 40 independent transgenic lines of the Columbia ecotype, we found evidence of excision activity in all organs of the plant: roots, cotyledons, stem, leaves, and flowers. Examination of the pattern of sectors revealed that the timing of excision is restricted during shoot vegetative and reproductive development but is variable during root and cotyledon development. The restriction of excision activity in the shoot also applies to the cell lineages that give rise to the gametes indicating that germinal excision events are occurring very late in flower development. Because late timing of excision in shoots was observed in all lines, which, because of their independent derivation, should have *Tag1* elements located in different regions of the Arabidopsis genome (although no mapping experiments have been performed), we infer that excision timing is independent of the genomic location of the *Tag1* elements.

The sector patterns in leaves and flowers suggest that *Tag1* excision is rarely occurring in the shoot meristem during all three stages of development: vegetative, inflorescence, and floral. Meristematic events would be expected to give rise to large sectors encompassing major portions or entire organs and branches or stripes along the stem between nodes. Examples of sectors that arise from meristem events from X-ray-irradiated seed and transgenic plants containing a modified *Ac* element (with a 35S promoter driving the transposase expression in a GUS reporter gene) have been published (Furner and Pumfrey 1992; Irish and Sussex 1992; Bossinger and Smyth 1996; Goldsbrough *et al.* 1996). These studies show sectors that include portions or all of a leaf that runs the length of the organ, multiple organs, and entire branches. These type of sectors are rarely if ever produced by *Tag1* in the 35S-GUS reporter construct. The one exception is the stem-like organs of the inflorescence (filaments and pedicels), which showed a distribution of sector sizes including those that encompassed the entire stem. The excision events that gave rise to these large sectors may have occurred in the floral meristem.

The patterns observed in leaves and flowers differed from those observed in roots and cotyledons where both early and late excision events were occurring. Sector sizes were also quite variable even among progeny from a single plant. This behavior suggests that the control of excision timing is not as strict in the embryo, root, and cotyledons as it is in the shoot. The frequency of somatic excision was also found to be quite variable. However, a correlation was found between somatic excision frequency in flowers/siliques and germinal reversion frequency (data not shown), suggesting some overlap in control mechanisms.

It is interesting to compare the excision activities of *Tag1* with those of other transposable elements. The timing of *Tag1* excision appears to be most similar to *Mutator*, which shows both germinal and somatic reversion activity late in maize development (Robertson 1981; Levy *et al.* 1989; Levy 1990; Bennetzen *et al.* 1993; Lisch *et al.* 1995). This delay in *Mutator* excision during development correlates with a reduction in MURB levels, one of the *Mutator*-encoded proteins that is expressed less abundantly in the cells that are not actively dividing (Donlin *et al.* 1995). The late timing of *Mutator* activity is not usually affected by dosage of *Mutator* elements, genetic background, or relative transposition activity (reviewed in Bennetzen 1996). Germinal reversion events usually produce very small clusters of revertant seeds in the ear (most often single-seed sectors), similar to the behavior of *Tag1* in Arabidopsis, but the rate of germinal reversion is low (often about $1/10^4$) (Brown *et al.* 1989; Levy *et al.* 1989; Walbot 1992). Most new *Mutator*-induced mutations, which can occur at high frequency in *Mutator* lines and after meiosis in both the male and female lineages (Robertson and Stinard 1993), also are represented by small clusters of seeds (from 1–11 seeds) on the ear (Robertson 1980; Robertson 1981). A "big spot" line of maize has been identified that has an altered timing pattern for *Mutator* and a higher frequency of germinal reversion (Walbot 1992).

The relationship between copy number and germinal reversion rates for *Tag1* most resembles that described for *Ac* in dicots but not in maize. In maize, the timing of *Ac* excision in kernel development can be delayed with increasing dosage of *Ac* (McClintock 1950; McClintock 1951), but this effect is dependent on the level of transposase and the dosage and composition of the transactivated element (Heinlein and Starlinger 1991; Heinlein 1996). In dicots, there is a consistent increase in germinal transposition with increasing *Ac*

copy number (Jones *et al.* 1989; Hehl and Baker 1990; Keller *et al.* 1993). Very high levels of transposase expression, however, have been found to inhibit *Ac* transposition, perhaps because of the aggregation of the transposase (Scofield *et al.* 1993; Heinlein *et al.* 1994).

The inheritance of *Tag1* revertant alleles from the male and the female parents appears to be the same; therefore, approximately half of the germinal revertants in a silique arose from excision events that occurred in the female parent and half in the male. In maize, *Mutator* activity is inherited more efficiently through the female than the male (Walbot 1986; Bennetzen 1987; Lisch *et al.* 1995). For *Ac* at the *R-nj* locus, the frequency of reversion was 2–3 times higher when the male (as compared with the female) contributed the *Ac* element (Brink and Williams 1973).

These and many other studies (reviewed in Fedoroff 1989; Fedoroff and Botstein 1992; Saedler and Gierl 1996) have shown that transposition can be regulated in plants. *Tag1* excision activity also displays developmental regulation in Arabidopsis. This regulation is most pronounced in the shoot and generates independent germinal revertants.

Transposable elements have been very useful for isolating genes based solely on mutant phenotypes and for enhancer and gene trapping (reviewed in Walbot 1992; Coupland 1994; Osborne and Baker 1995; Sundaresan *et al.* 1995). They offer an alternative with both advantages and disadvantages to the use of T-DNA from Agrobacterium as an insertional mutagen. Gene tagging systems have been established in Arabidopsis using the maize elements *Ac*, *Spm*, and their derivatives *Ds* and *dSpm*. One may ask if *Tag1* may also serve as an useful insertional mutagen for tagging genes. Our results indicate that transgenic plants carrying a single copy of *Tag1* display low-germinal excision rates, generally lower than 1%. However, for plants that have multiple copies of *Tag1*, the rate is much higher. The highest rate we have observed is 26% for homozygous progeny of TG-3, which have seven copies of *Tag1* in the hemizygous state. This rate is encouraging for mutagenesis. We have also found that new insertion bands were observed in approximately three-fourths of the plants that had undergone a *Tag1* excision event. In 47 germinal revertants from a single plant, we found no reinsertion events that gave the identical Southern blot pattern, indicating that *Tag1* excision is occurring late enough to produce independent revertants. These results suggest that *Tag1* will be useful for insertional mutagenesis. At the very least, *Tag1* should be used as a probe to check new unstable mutations in transgenic lines that contain *Tag1*. The *Ac*-containing lines of Landsberg *erecta* have so far produced three tagged mutants: the original *chl1::Tag1* mutant (Tsay *et al.* 1993), a *cup-shaped cotyledon* mutant (Aida *et al.* 1997) and a *pinhead* meristem-defective mutant (Lynn *et al.* 1997), all having *Tag1* insertions that allowed cloning of the corresponding gene.

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