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Transposable Elements Can Be Used To Study Cell Lineages in Transgenic Plants

E. Jean Finnegan,[1] Brian H. Taylor,[2] Stuart Craig, and Elizabeth S. Dennis

Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, Canberra A.C.T. 2601, Australia

The *β-glucuronidase* reporter gene has been used to develop a sensitive assay for the excision of transposable elements introduced into transgenic plants. The reporter gene, inactivated by the insertion of the maize transposable element *Activator* (*Ac*) into the 5′-untranslated leader, was introduced into the genome of tobacco by *Agrobacterium*-mediated transformation. Reactivation of the *β-glucuronidase* gene was detected in transgenic plants using a fluorometric or histochemical assay. Reactivation of the reporter gene was dependent on the presence of the transposase of *Ac*, and resulted from the excision of the *Ac* element. This assay, together with the improved methods for visualization, will provide a valuable and rapid method for studying the basic mechanism of transposition in plants and for developing modified transposable element systems suitable for gene tagging in transgenic plants.

INTRODUCTION

Transposable elements have no obvious phenotype unless they insert into and disrupt the expression of genes that have a visible phenotype. The movement of transposable elements in maize has been studied indirectly using insertion into genes, such as those expressed in the maize kernel, that can be inactivated with little or no deleterious effect on the plant and yet have a dramatic effect on the phenotype. These genes include those affecting biosynthesis of both the red and purple anthocyanin pigments of the aleurone layer of the kernel and the starch and storage proteins of the endosperm. Similarly, genes affecting flower pigmentation have been used to monitor the mobility of the *Tam* transposable elements in *Antirrhinum majus* (Bonas, Sommer, and Saedler, 1984; Martin et al., 1985; Sommer et al., 1985; Upadhyahya et al., 1985; Coen, Carpenter, and Martin, 1986). These naturally occurring transposable element-reporter gene combinations have enabled the activity of the element (that is, the frequency and timing of element excision) to be followed by recording the number and size of sectors in which gene activity has been restored.

Transposable elements, cloned from maize and *Antirrhinum*, have been introduced by *Agrobacterium*-mediated transformation into the genomes of other plants that do not have characterized transposable element systems (Baker et al., 1986; Van Sluys, Tempe, and Fedoroff, 1987; Knapp et al., 1988; Yoder et al., 1988). This advance offers great opportunities both for dissecting the function of transposons by reverse genetics and for extending the application of gene-tagging as a method for isolating genes, the products of which are unknown (Bingham, Levis, and Rubin, 1981; Fedoroff, Furtek, and Nelson, 1984; Martin et al., 1985; Moerman, Benian, and Waterston, 1986). Initially, there was no visual assay for excision of an introduced element in transgenic plants and so movement of foreign transposable elements was followed by DNA gel blot analysis of DNA from the transformed plants. These analyses not only require substantial amounts of plant tissue but also are time-consuming and provide only limited data. Excision of the element can be detected by the appearance of a new band corresponding to the empty donor site. The intensity of this band does not distinguish among a number of independent excision events each giving rise to a small sector of cells and a few events forming large sectors because each independent excision will contribute to the excision band.

A phenotypic assay based on excision of the maize transposable element *Activator* (*Ac*) from the untranslated leader of the neomycin phosphotransferase gene has been used to follow *Ac* excision in transgenic plants (Baker et al., 1987; Coupland et al., 1988; Knapp et al., 1988). *Ac* excision was assayed by selection for kanamycin-resistant calli either immediately following transformation or after a period of growth on nonselective medium.

A better understanding of the timing and frequency of excision events can be achieved with a visual assay for excision. Jones et al. (1989) described an assay based on

[1] To whom correspondence should be addressed.

[2] Current address: Department of Biology, Texas A & M University, College Station, TX 77843-3258.

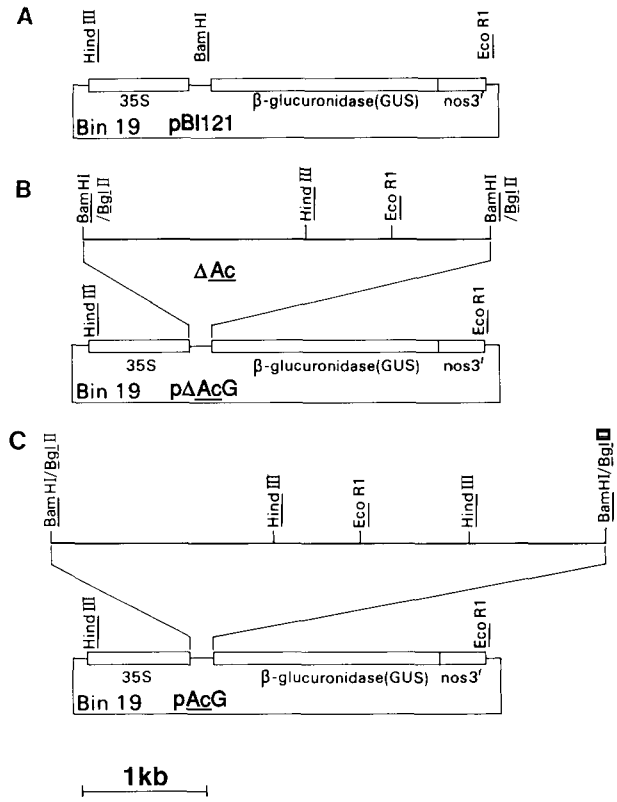

Figure 1. Binary Vectors Used for Transformation of Tobacco Leaf Discs.

(A) pBI121 contains the uninterrupted 35S-GUS-*nos*3′ chimeric gene.

(B) pΔ*Ac*G has an *Ac* element deleted for bases 2904 to 4106 cloned into the 5′-untranslated region at a BamHI site.

(C) p*Ac*G has the complete *Ac* element cloned into the same BamHI site.

Abbreviations are: 35S, cauliflower mosaic virus 35S promoter; GUS, coding region from *E. coli uidA* (*β-glucuronidase*) gene; *nos*3′, 3′ termination signals from nopaline synthase; Bin19, binary vector (Bevan, 1984).

excision of *Ac* from the untranslated leader of the bacterial streptomycin phosphotransferase (SPT) gene. Excision of *Ac* in seedlings germinated on medium containing streptomycin can be visualized as green (streptomycin-resistant) sectors on a bleached (streptomycin-sensitive) background.

The availability of the *β-glucuronidase* gene (GUS; Jefferson, Kavanagh, and Bevan, 1987), a sensitive reporter gene that can be assayed histochemically, has enabled us to develop a more general visual assay for excision of the *Ac* element in transgenic tobacco. We have cloned *Ac* into the untranslated leader of the 35S-GUS-*nos*3′ chimera (pBI121; Jefferson, Kavanagh, and Bevan, 1987). When this construct was introduced into tobacco, excision of *Ac* was detected by histochemical staining for GUS activity in leaf, stem, or root tissue.

While this manuscript was in preparation, a paper describing the independent development of a visual assay for *Spm* activity in transgenic tobacco based on the GUS reporter gene was published (Masson and Fedoroff, 1989).

RESULTS

We transformed tobacco leaf discs with plasmids pBI121, p*Ac*G, or pΔ*Ac*G, which are shown in Figure 1. GUS activity in leaves from independent transgenic plants was measured fluorometrically, and the results are presented in Table 1. The highest levels of GUS activity were obtained with extracts from plants containing pBI121 (Table 1), while plants containing the pΔ*Ac*G construction had little or no detectable activity. Plants transformed with p*Ac*G showed levels of activity up to 50% of that in plants containing pBI121. The level of GUS activity varied both between different leaves from the same plant and between individual transformants containing p*Ac*G. No GUS activity was detected in untransformed plants.

Hand-cut sections of leaves or stems, pieces of leaf, or roots from plants transformed with p*Ac*G were stained with X-Gluc (5-bromo-4-chloro-3-indoyl-β-D-glucuronide; Jefferson, Kavanagh, and Bevan, 1987) to determine the location of cells expressing the GUS gene. Improved resolution was obtained by extraction of endogenous pigments followed by infiltration, under vacuum, with a medium of appropriate refractive index (for example, immersion oil). The histochemical staining of leaf sections cut from several independent transformants is presented in Figure 2. Plants transformed with pBI121 showed a uniform distribution of stain (Figure 2A), while in plants transformed with p*Ac*G, GUS activity was restricted to particular

Table 1. GUS Activity in Extracts Made from Individual Leaves Taken from Untransformed *N. tabacum* W38 or Plants Transformed with pBI121, pΔ*Ac*G, or p*Ac*G

Plant	Leaf 1	Leaf 2
	fluorometric units/ min/mg protein	
W38 (untransformed)	0.011	–
pBI121-2	31.8	32.1
pBI121-4	46.4	–
pΔ*Ac*G-1	0.18	–
pΔ*Ac*G-2	0.15	–
p*Ac*G-1	8.6	4.6
p*Ac*G-2	17.6	15.8
p*Ac*G-3	18.2	25.1
p*Ac*G-4	24.2	15.1
p*Ac*G-5	18.1	18.9

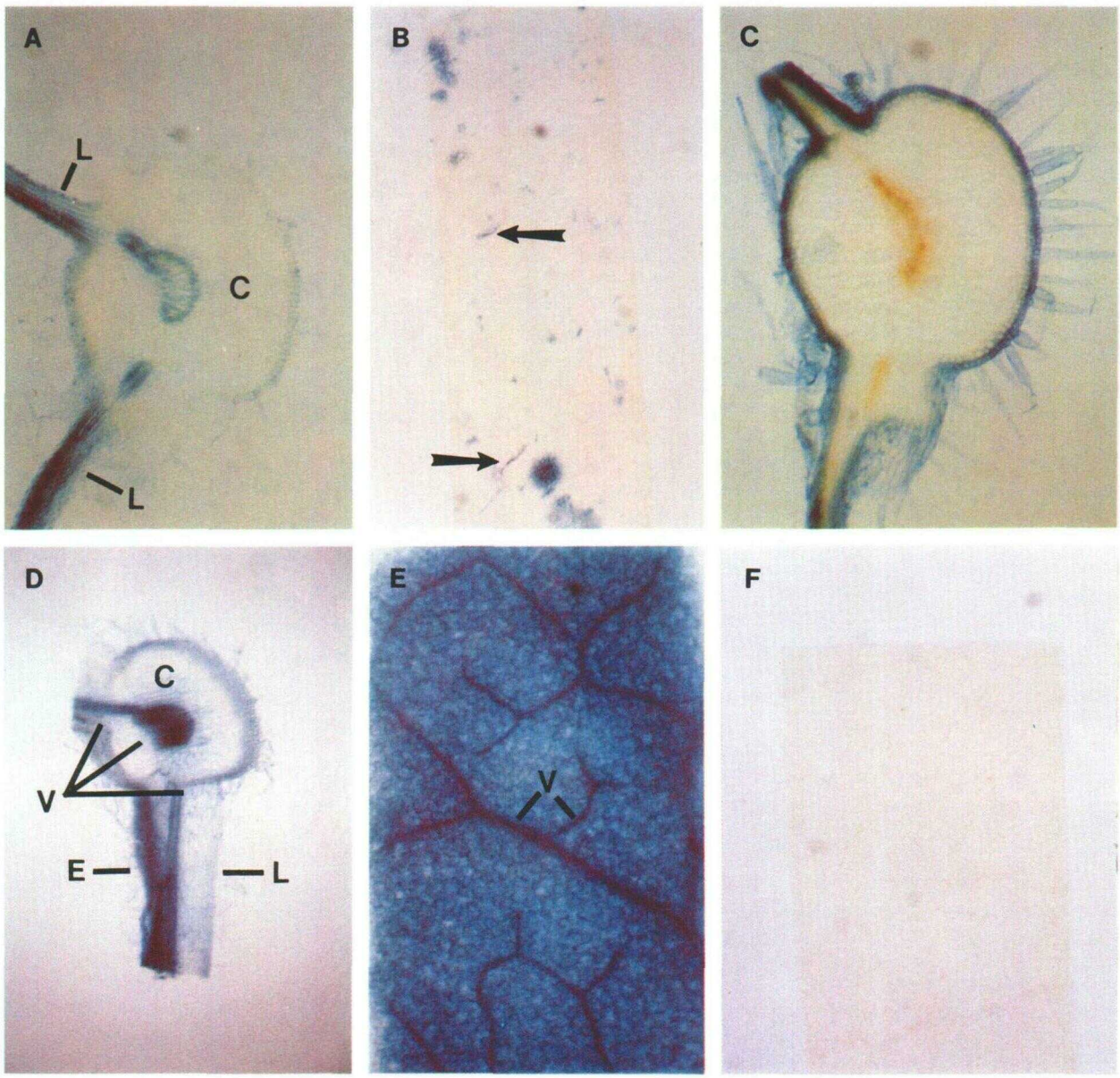

Figure 2. Histochemical Localization of GUS Activity in Transgenic Tobacco Leaf Tissue.

All tissues were excised from the base of 2 cm to 4 cm long leaves from 6 to 7 leaf plants grown in sterile culture.
(A), **(C)**, and **(D)** Cross-sections through the petiole-mid-rib region with short pieces of adjacent lamina (L).
(B), **(E)**, and **(F)** Surface views of lamina.
(A) Transformed with pBI121 showing GUS activity in all tissues except the petiole cortex (C).
(B) Transformed with p*Ac*G-1 showing localized GUS activity. Arrows indicate GUS-positive trichomes folded down onto the leaf surface.
(C) Transformed with p*Ac*G-3 showing GUS activity restricted to epidermal cells and trichomes.
(D) Transformed with p*Ac*G-4 showing GUS activity in vascular (V) and epidermal tissues. The lamina (L) is slightly twisted, resulting in overlapping staining of the upper epidermal (E) and vascular tissues.
(E) Surface view of leaf lamina from the plant described in **(D)**.
(F) Transformed with pΔ*Ac*G-1 showing absence of GUS activity.

cell lineages (Figures 2B, 2C, 2D), suggesting that GUS expression may have been due to localized excision of *Ac* during plant development. Transverse leaf sections from plant p*Ac*G-1 show that GUS activity has been restored in epidermal, mesophyll, and vascular tissue. All sectors observed in this plant were small; some sectors were restricted to the three cells of individual trichomes, suggesting that the *Ac* excision occurred in the cell giving rise to the trichome. We estimate that, in this plant, sector size ranged from as few as three cells to as many as several hundred cells. A surface view of a segment of leaf blade showing the pattern of sectoring in plant p*Ac*G-1 is shown in Figure 2B. The size and distribution of stained sectors were consistent for all leaves sampled from plant p*Ac*G-1 and from clones of this plant maintained in sterile culture. Roots from this plant also had blue staining sectors (not shown), indicating that *Ac* excises in different tissues. In other plants transformed with p*Ac*G, the stained sectors were larger and the distribution of the stain varied (Figures 2C, 2D). The transverse section of a leaf from plant p*Ac*G-3 shown in Figure 2C has blue staining only in epidermal cells and the attached trichomes. Neither vascular tissue nor mesophyll cells were stained in this leaf. In contrast, plant p*Ac*G-4 stained intensely in the epidermis and trichromes of the mid-vein and upper leaf surface and the vascular tissue. The lower epidermis, mesophyll of the lamina, and cortex of the mid-vein also stained, but at lower intensity (Figure 2D). A surface view of the same leaf seen in Figure 2E showed that staining was more intense in vascular tissue. No stained sectors were observed in plants transformed with pΔ*Ac*G (Figure 2F) or in untransformed tobacco (not shown). The sector size observed in this histochemical assay for GUS activity parallels the level of activity seen in fluorometric assays of leaf extracts from the same plants (Table 1).

DNA gel blot hybridization was used to confirm that the restoration of GUS activity was due to the excision of the *Ac* element. Hybridization to DNA from the same plants transformed with p*Ac*G, pΔ*Ac*G, or pBI121 is presented in Figure 3. DNA digested with HindIII and EcoRI was first hybridized with the complete *Ac* element and then subsequently rehybridized using the GUS coding region. Reconstitution of the 35S-GUS gene by *Ac* excision will create a 3.0-kb restriction fragment that hybridizes only to the GUS probe; in the absence of *Ac* excision from p*Ac*G, a 3.3-kb fragment will hybridize to both *Ac* and GUS probes. The *Ac* probe will also hybridize to internal *Ac* fragments of 0.7 kb and 0.9 kb and to the 35S-*Ac* fragment (2.6 kb) (Figure 3A). A 3.0-kb fragment that hybridized only to the GUS probe was detected in p*Ac*G transformants 2, 4, 5 (Figure 3B) and, after a longer exposure, in p*Ac*G-3 (not shown). No 3.0-kb band was detected in p*Ac*G-1 even after prolonged exposure of the autoradiogram. This suggests that the proportion of cells carrying the excision band may have been too low to allow detection by this method, even

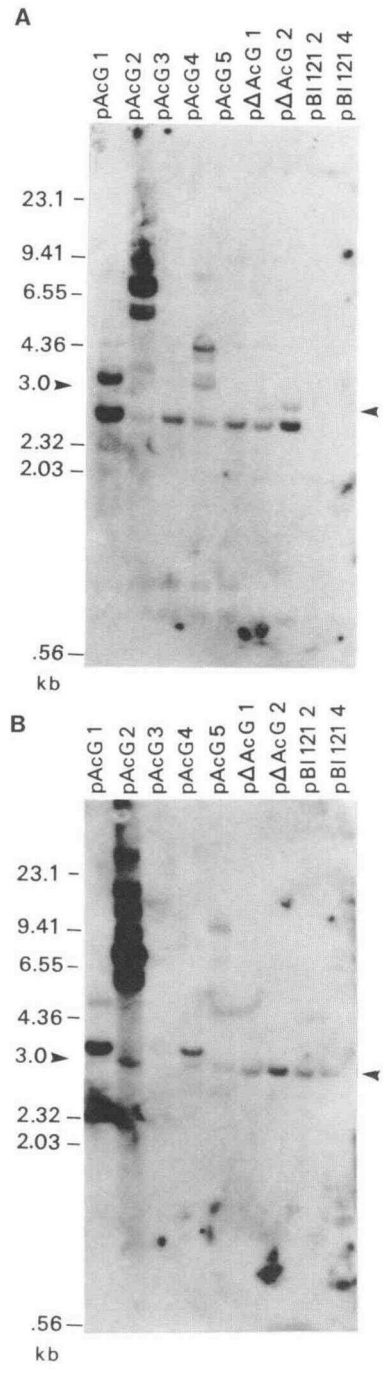

Figure 3. DNA Gel Blot Hybridization to DNA Isolated from Transgenic Tobacco Plants Digested with EcoRI Plus HindIII.

Probes used were purified 4.8-kb PstI fragment from *wx-m9* (McClintock, 1963) containing an intact *Ac* element **(A)** or pUC18 containing the GUS coding region **(B)**. Lanes 1 to 5, plants p*Ac*G-1 to -5, respectively; lanes 6 and 7, pΔ*Ac*G-1 and -2; lanes 8 and 9 plants, pBI121-2 and -4, respectively.

though both histochemical and fluorometric analyses showed that the GUS gene had been restored by *Ac* excision in some cells. In plants p*Ac*G-1, 4, and 3 (after longer exposure), a 3.3-kb band hybridizing to both the *Ac* and GUS probes was also detected, indicating that the *Ac* element remained linked to the GUS coding region in some cells. In plant p*Ac*G-2, the *Ac* and GUS probes hybridized to bands of higher molecular weight, suggesting that there may have been some rearrangement of the T-DNA during integration. The *Ac* element in this plant is still active, however, because a 3.0-kb excision band was observed. *Ac*-GUS is also present in high molecular weight DNA in plants p*Ac*G-3, 4, and 5. The 3.3-kb band could not be detected in p*Ac*G-5, suggesting that, in the majority of cells, *Ac* has excised. In plants transformed with pΔ*Ac*G, there was a 3.0-kb HindIII-EcoRI fragment that hybridized to both *Ac* and GUS probes, indicating that the deleted *Ac*, which lacks a portion of the coding sequence for the *Ac* transposase, remained linked to the GUS coding region.

DISCUSSION

Restoration of GUS activity has been used as a measure of *Ac* excision in transgenic tobacco using two different assays. In most primary transformed plants, excision of *Ac* was able to be confirmed by DNA gel blot analysis. The exception, plant p*Ac*G-1, showed a low level of GUS activity in a fluorometric assay and, after histochemical staining, showed many small sectors, indicating that there had been a number of independent *Ac* excision events late in development. No excision band could be detected by DNA gel blot analysis probably because the proportion of cells in which excision had occurred was too low (<10% total cell population) to yield a detectable band. A comparison of the three assays used in this study shows that restoration of GUS activity as measured by fluorometric or histochemical means is a more sensitive measure of *Ac* excision than DNA gel blot hybridization. This, together with the speed of the assays and the small amount of plant tissue sacrificed, makes the GUS reporter gene an attractive assay for *Ac* excision in transgenic plants.

Several phenotypic assays for *Ac* excision in transgenic plants have now been described. Baker et al. (1987) have used an *Ac*:*Npt*II reporter gene to select for cells in which excision has occurred. Selection was applied either immediately following transformation or after a period of growth on nonselective medium. While this assay does provide an estimate of the frequency of *Ac* excision at the callus stage, it is difficult to monitor excision in the whole plant, and, if kanamycin resistance has been selected in the first generation, cannot be used in subsequent generations. The visual assay using SPT: *Ac* also allows selection of plants in which *Ac* excision has occurred (Jones et al., 1989). Germination of seedlings on medium containing streptomycin has facilitated an elegant analysis of both somatic and germinal excision events in back-cross and self-progeny of primary transformants. An analysis of the effect of varying the dosage of SPT: *Ac* gene on the frequency of *Ac* excision has also been made in progeny plants. Because of the nature of the phenotype caused by streptomycin sensitivity, this assay is limited to an analysis of *Ac* excision in chlorophyll-producing tissue in plants that are susceptible to streptomycin. This assay is nondestructive, and the seedlings can be recovered for full analysis by removal to streptomycin-free medium. *Ac* excision later in development, in already green plants, may be difficult to assess because of the time needed for this tissue to bleach when cultured on streptomycin-containing medium.

The histochemical assay we describe is complementary to the SPT assay in several features. Detection of *Ac* excision is not limited to green tissue; any tissue in which the 35S promoter is active can be assayed for excision by histochemical or fluorometric means. Staining of mature leaves, flowers, and roots makes it possible to follow excision of *Ac* in different tissues throughout plant development. The primary transformants studied in this survey show different timing and frequency of *Ac* excision. In plant p*Ac*G-1, *Ac* excised frequently late in development, resulting in many small sectors. However, *Ac* excision is not restricted to late in plant development because sectors in which the GUS gene is active are much larger in other transgenic plants carrying p*Ac*G—for example, p*Ac*G-3 and p*Ac*G-4. The position of insertion of an introduced gene influences the level of gene expression (Jones, Dunsmuir, and Bedbrook, 1985), which may in turn affect the timing and frequency of *Ac* excision. Histochemical staining for *Ac* excision not only provides information on the frequency and timing of excision, but also allows the localization of *Ac* excision to particular cell types. For example, in p*Ac*G-3, only epidermal cells derived from the tunica 1 cell layer of the somatic embryo stained, indicating that an excision event occurred early in this lineage. This assay, therefore, has considerable potential to facilitate an analysis of cell lineage during plant development, as in maize, where the visual phenotype, caused by insertion of a transposable element into a gene expressed in endosperm, has been used to determine the sequence of cell division (McClintock, 1978). Each sector arises from a single cell in which excision of the transposable element has restored gene activity. Histochemical staining of plants transformed with p*Ac*G will allow the sector size, shape, and cell type involved to be followed, which may in turn lead to an understanding of the development of the transgenic plant. The use of histolocalization is not without potential problems, as a variation in the tissue-specific pattern of expression of a 35S-GUS construct between individual transgenic tobacco and petunia has been reported (Benfey and Chua, 1989). This suggests that even

so-called constitutive promoters may be responsive to tissue-specific regulatory elements when inserted in different positions in the genome. Depending on the position of insertion, staining may occur in only some of the cell lineages in which the GUS gene has been restored, following *Ac* excision. We did not observe any variation in the localization of expression from pBI121 in leaves taken from five primary transgenic tobacco plants where staining occurred in epidermis, trichomes, mesophyll, and vascular cells.

Ac has potential as a tool for gene tagging in heterologous plant species. In tobacco, it transposes (i.e., excises and re-integrates) in both primary regenerants and their selfed progeny (Baker et al., 1988; Jones et al., 1989; Taylor et al., 1989). However, it may be necessary to change the timing or frequency of *Ac* transposition to increase the number of cells carrying the transposed *Ac* and thus facilitate identification and cloning of the mutation caused by insertion of the transposable element. Transactivation of the Δ*Ac* element in the pΔ*Ac*G construct by an unlinked *Ac* element will allow the effect of any modification of *Ac* sequence to be monitored by measuring the size and/or frequency of sectors in which GUS activity has been restored.

In maize, the characteristics of *Ac* transposition have been established—for example, the inverse correlation between the number of active *Ac* elements and the time during development and apparent frequency of *Ac*-mediated transposition events (McClintock, 1948). However, it is important that such characteristics are re-established when *Ac* is introduced into a new background because there may be consequences for gene tagging. Jones et al. (1989) have already shown that the excision frequency of *Ac* does not show an inverse correlation to dosage in transgenic tobacco. Other environmental factors may affect the frequency of transposition of a wild-type *Ac* element. For example, *Tam*3, a transposable element from *Antirrhinum*, transposes 1000-fold more frequently when grown at 15°C than at 25°C (Harrison and Fincham, 1964; Carpenter, Martin, and Coen, 1987). We have investigated the effect of temperature on *Ac* excision in transgenic tobacco and found that the excision frequency, as measured by GUS activity in a fluorometric assay, was not affected by growth temperature (E.J. Finnegan, unpublished results).

The transposable element-reporter gene combination described here, in concert with the improved resolution obtained by tissue processing following the histochemical detection of GUS activity, provides a sensitive and convenient means to assay excision in transgenic plants. It can be adapted for other elements [for example, *Spm* (Masson and Fedoroff, 1989)] and can be used in any plant susceptible to *Agrobacterium*-mediated transformation. It is anticipated that the availability of this assay will facilitate the construction of improved vectors for gene tagging in a large number of plant species.

METHODS

Gene Constructs

An *Ac* element was cloned from the *P-VV* locus of maize on an 8-kb SalI fragment (T.A. Peterson, unpublished; Chen, Greenbatt, and Dellaporta, 1987). *Ac* was located on an AccII-XhoI fragment that retained only 21 bp and 26 bp of flanking P-DNA. BglII linkers were added and the element was cloned into the unique BamHI site in plasmid pBI121 [the cauliflower mosaic virus 35S promoter driving the GUS gene linked to *nos*3′ termination signals (Jefferson, Kavanagh, and Bevan, 1987)] to create p*Ac*G. The BamHI site is located between the 35S promoter transcription start and the translation start of the β-glucuronidase gene. Maps of these constructs are shown in Figure 1.

A defective *Ac* (Δ*Ac*) was created by deletion of three internal SphI fragments spanning bases 2904 to 4106 of the *Ac* element. This deletion encompasses part of the sequence encoding the *Ac* transposase (Coupland et al., 1988) and so autonomous transposition cannot occur. This Δ*Ac* element was cloned, on a BglII fragment, into the BamHI site in pBI121 to create pΔ*Ac*G (Figure 1).

Transformation

The plasmids pBI121, p*Ac*G, and pΔ*Ac*G were each transferred into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983) by the triparental mating procedure of Ditta et al. (1980). *Nicotiana tabacum* cv W38 leaf pieces were transformed as described by Ellis et al. (1987).

Histochemical Staining

Leaves 2 cm to 3 cm long were removed from plants in sterile culture and dissected into ice-cold 1% glutaraldehyde in 25 mM sodium phosphate buffer, pH 7.0. Following evacuation, tissues were fixed on ice for a total of 30 min, washed 4 × 5 min in the same ice-cold buffer, and then incubated in GUS assay buffer (0.1 M NaH_2PO_4, pH 7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 1 mM *X*-glucuronide) for approximately 16 hr at 37°C in the dark. After a brief rinse in buffer, tissues were dehydrated through a graded series of ethanol to extract the chlorophyll, with repeated changes in absolute ethanol until all pigment was lost. The ethanol was replaced with acetone (5 × 10 min) followed by a 1:1 mixture of acetone/immersion oil for 1 hr, and then undiluted oil for at least 1 hr. Traces of acetone were removed under vacuum. Leaf pieces have been stored for several weeks in oil with no visible changes, but they should not be kept in acetone, as a low level of GUS reaction product extraction may occur. Tissues were mounted under coverslips and photographed with a Wild M5A stereo microscope using Extachrome professional 50 ASA or 160 ASA film.

Fluorometric Assay

Individual leaves from tobacco plants grown in vitro were ground with a pestle and mortar in 500 μL of lysis buffer (50 mM NaH_2PO_4, pH 7.0, 10 mM EDTA, 10 mM β-mercaptoethanol), spun to remove

debris, and then assayed. One hundred microliters of assay buffer (lysis buffer with 1 mM MUG) was added to 100 μL of extract (or diluted extract) and incubated at 37°C for 30 min. The reaction was stopped by the addition of 1.2 mL of 0.2 M Na_2CO_3, and the fluorescence was measured in a Hoefer Scientific DNA fluorometer with excitation at 365 nm and emission at 460 ± 10 nm. The protein content of each extract was determined (Spector, 1978), and GUS activity was calculated as fluorometric units per minute per milligram of protein.

DNA Gel Blot Analysis

DNA was isolated as described by Taylor and Powell (1982) from in vitro grown *N. tabacum*. DNA was digested with EcoRI plus HindIII at 3 units/μg of DNA for 16 hr in 33 mM Tris acetate, pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol. The digested DNA was fractionated by electrophoresis on 0.7% agarose gel, and then transferred to nitrocellulose by blotting as described by Taylor et al. (1985). Hybridizations were according to Taylor et al. (1985) except that probe DNA was labeled using a random primer labeling kit (Bresatec, SA). Probes used were a 4.8-kb PstI fragment consisting of an *Ac* element flanked by *wx* DNA or a pUC18 plasmid carrying a BamHI fragment containing the GUS coding region.

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