A versatile system for detecting transposition in *Arabidopsis*

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**Summary**

The maize transposable element *Activator (Ac)* has been shown to be active in a number of dicots, including *Arabidopsis thaliana*, whose small genome and short generation time have favored its wide adoption as a model organism for molecular genetic approaches to plant physiology and development. Using the *Ac* element and several bacterial and plant marker genes, we have devised a versatile system for identifying plants in which a transposon has excised and reinserted elsewhere in the genome. The transposons have been designed to facilitate the identification of insertions downstream of promoters and in the vicinity of enhancers by the inclusion of a β-glucuronidase (GUS) gene either lacking a promoter or having a minimal promoter sequence. The system permits the transposon and the source of transposase to be maintained either stably in separate plants or in the same plant. Plants in which transposition is occurring can be identified by the frequent somatic activation of the GUS gene. The herbicide chlorsulfuron is used as a selective agent to identify progeny plants in which the transposon has excised from its original insertion site within a chlorsulfuron-resistant acetolactate synthase gene. Additional selectable markers permit the identification of plants containing a transposed element, but lacking transposase. Here we describe our initial characterization of the system and demonstrate its reliability and efficiency in identifying plants with transposed elements.

**Introduction**

*Activator (Ac)* is a maize transposon that was first identified genetically by McClintock because of its ability to promote chromosome breakage at a specific chromosomal site designated the *Dissociation (Ds)* locus (reviewed in Fedoroff, 1983). The *Ac* element is the transpositionally active member of a family that includes a large and structurally heterogeneous group of elements, collectively designated *Ds* elements, which can transpose only if provided with a source of the *Ac*-encoded transposase (reviewed in Fedoroff, 1989). The element's transcription start sites and transposase-coding sequence have been identified, as have the terminal sequences required for transposition (Coupland *et al*., 1989; Kunze and Starlinger, 1989; Kunze *et al*., 1987). *Ac* has been shown to be active in a number of plants other than maize, including the increasingly popular dicotyledonous model plant *Arabidopsis thaliana* (Baker *et al*., 1986; Izawa *et al*., 1991; Knapp *et al*., 1988; Van Sluys *et al*., 1987; Yoder *et al*., 1988). Recent studies in *Arabidopsis* have addressed the transpositional behavior of *Ac*, as well as the development of marked *Ac*-derived transposons to facilitate transposon tagging (Altmann *et al*., 1992; Dean *et al*., 1992; Masterson *et al*., 1989; Schmidt and Willmitzer, 1989; Swinburne *et al*., 1992). The results of these studies, together with information on *Ac* and *Ds* behavior in other dicots (Dooner *et al*., 1991; Hehl and Baker, 1990; Jones *et al*., 1990; Yoder, 1990), focuses attention on several problems in the development of an efficient *Ac*-based transposon tagging system for *Arabidopsis*. These include the need for a reliable phenotypic assay to identify plants in which transposition is occurring, as well as genetic markers to identify transposed elements, map new insertion sites relative to donor sites, and eliminate plants which contain the transposase gene in order to stabilize newly transposed elements. In addition, it would be desirable to be able to identify insertions in and near genes by virtue of activation of a transposon-borne, promoterless reporter gene, as initially developed for *Drosophila* P elements using a bacterial β-galactosidase gene (Blair *et al*., 1989; Wilson *et al*., 1989).

To satisfy these requirements, we have assembled several transposons (termed *Ds-GUS* elements), each containing a promoterless bacterial β-glucuronidase (GUS) coding sequence as a reporter gene, as well as a second marker gene whose expression is independent of the integration site. The second marker gene, which confers resistance to hygromycin in plant cells, was included in the transposon to allow easy tracking of the element and facilitate the identification of insertion mutations by co-segregation of the mutant phenotype with the hygromycin-resistance trait. In order to permit the identification of plants in which

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an excision event has occurred, the transposons were cloned into the leader of a mutant A. thaliana acetolactate synthase (ALS) gene that confers resistance to the herbicide chlorsulfuron (Haughn et al., 1988). The transposon-disrupted ALS gene was introduced into A. thaliana by Agrobacterium-mediated root transformation on a T-DNA segment also containing a bacterial neomycin phosphotransferase (NPT-II) gene expressed from a plant promoter.

An Ac element was modified to provide a non-mobile transposase source by deleting one end and introducing a strong plant promoter upstream of the transposase coding sequence. This construct, termed the 35S-Ac transposase gene, was cloned into the T-DNA portion of an Agrobacterium transformation vector together with an A. tumefaciens Ti plasmid-derived tms2 gene, which encodes an indoleacetamide hydrolase (Thomashow et al., 1984). The capacity to express this gene is lethal to Arabidopsis in the presence of indole-3-acetamide or an analog (Karlin-Neumann et al., 1991). The purpose of this marker is to provide a negative selection for plants containing the 35S-Ac transposase gene, facilitating the recovery of newly transposed Ds-GUS elements in a genetic background lacking transposase. In the present communication we describe the system and provide molecular data demonstrating both the reliability and the efficiency of the selective agents in identifying plants with transposed elements.

Results

Ds-GUS and 35S-Ac transposase constructs

The tagging Ds-GUS transposons were assembled by cloning the GUS and aminocyclitol phosphotransferase (aph4) genes into transposition-defective (Ds) elements derived from an Ac element by deleting most of the internal sequence (see Experimental procedures for details). The three Ds elements selected retained either only the first and weakest of the Ac element's four transcription start sites (Ds325) or all of them, either with (Ds512) or without (Ds378) part of the element's untranslated leader sequence (Kunze et al., 1987). The complete GUS coding sequence was introduced into the element with (+) or without (−) a short core sequence from the CaMV 35S promoter (Figure 1a). A bacterial aph4 gene expressed from a CaMV 19S promoter was also cloned into each of the six Ds–GUS elements near the 3′ end. The Ds–GUS elements were inserted in each orientation into the leader sequence of a mutant chlorsulfuron-resistant ALS gene (Haughn et al., 1988). The Ds–GUS-disrupted ALS gene was transferred to an E. coli–Agrobacterium shuttle vector, giving an NPT-II-marked T-DNA region whose structure is shown in Figure 1a. Plasmids with the GUS gene in the same orientation as the 35S promoter were used to test the ability of the GUS gene to be expressed from a promoter and through the element's 5′ end. No expression of the GUS gene was expected when the GUS gene was in the opposite orientation from the 35S promoter unless a transposition or rearrangement had occurred to bring the gene into close proximity with either a promoter or enhancer.

Two different 35S-Ac transposase constructs were used, both derived from an intact Ac element (Figure 1b). In the Bam35S-Ac, the CaMV 35S promoter replaced the 5′ terminal 181 bp of the element, rendering it unable to transpose. In the Nae35S-Ac, the CaMV 35S promoter and the Ω leader sequence of the tobacco mosaic virus (Gallie et al., 1987) replaced the element's 5′ terminal 964 bp, eliminating most of the sequence corresponding to the untranslated leader sequence (this derivative was a kind gift of S. Scofield). Each of the 35S-Ac transposase constructs were then inserted between the T-DNA borders of a cloning vector that also contained the NPT-II gene as a plant transformation marker and a 35S-tms2 gene as a negative selectable marker (see Experimental procedures).
Introduction of transposons and transposase into A. thaliana

The Ds–GUS transposon and 35S-Ac transposase constructs depicted in Figure 1 were introduced into A. thaliana ecotype No-O by Agrobacterium–mediated transformation of roots, as described by Valkenek et al. (1988), using resistance to kanamycin for selection of transformed tissue. The plasmids were generally used singly with the objective of obtaining plants that contain either a Ds–GUS transposon or a 35S-Ac transposase T-DNA insert. In addition, roots were co-transformed with both Ac and Ds plasmids in an effort to assess the transposition activity of the system immediately. Plants regenerated from transformed calli containing a GUS gene were tested for its expression by histochemical staining (Jefferson, 1987). A low level of GUS expression was detected in some of the plants transformed with Ds–GUS plasmids shown in Figure 1a, particularly those that contained the core sequence of the 35S promoter. The staining was relatively pale and either uniform or confined to shoot meristems and some flower parts. None of the plants tested exhibited sectorial expression of the GUS gene. Therefore all plants obtained from independent transformants were allowed to set seed and their progeny analyzed, as discussed below. By contrast, calli and plants transformed with plasmids in which the GUS gene on the transposon was in the opposite orientation, putting it under the control of the 35S promoter of the ALS gene, expressed the GUS gene. Although the level of GUS gene expression in such plants was not quantified and showed considerable variation among transformants containing the same construct, GUS gene expression was moderate to strong in all cases, confirming that the element’s 5’ end did not substantially interfere with expression of the gene when it was adjacent to a strong promoter. GUS gene expression appeared to be most intense in transformants containing the Ds–GUS element with the shortest 5’ end, as well as the core sequence of the CaMV 35S promoter.

To check its transposase activity, each 35S-Ac transposase plasmid was co-transformed with an ‘excision assay plasmid’ containing a 35S-GUS gene whose expression was disrupted by insertion of an unmodified Ds element (see Experimental procedures). Although the presence of the excision assay plasmid could not be selected for independently, the frequency with which a given kanamycin-resistant calli received both plasmids proved high. The percentage of kanamycin-resistant calli showing sectorial expression of the GUS gene ranged from less than 5 to 33, while calli from cells transformed with the disrupted GUS gene alone showed no GUS gene expression. Of the plants regenerated from co-transformed kanamycin-resistant calli in one experiment, 8% (Nae35S-Ac) and 13% (Bam35S-Ac) gave GUS-positive (GUS+) sectors (Table 1), while none were detected in plants regenerated from calli transformed with the Ds–GUS plasmid alone.

To determine whether the GUS gene on a Ds–GUS transposon could be activated somatically, roots were co-transformed with a Ds–GUS transposon and a 35S-Ac transposase plasmid. Among the kanamycin-resistant calli obtained after one such co-transformation, 11% showed GUS+ sectors (Table 1). Of these, seven yielded one or more plants that showed GUS+ sectors and set seed. The progeny of these plants were subsequently analyzed to obtain information about the utility of the selective agents in the identification of plants with transposed elements.

Genetic and molecular analysis of plants containing Ds–GUS and 35S-Ac T-DNAs

The phenotypes expected for plants containing the introduced Ds–GUS and 35S-Ac T-DNAs are listed in Table 2. Since the NPT-II gene served as the transformation marker for both the Ds–GUS and 35S-Ac T-DNAs, progeny containing either should be resistant to kanamycin. The phenotypes of kanamycin-sensitive (K') and kanamycin-resistant (K) seedlings are shown in Figure 2a. The 35S-Ac T-DNA carries the A. tumefaciens tms2 gene, which encodes an indoleacetamide hydrolase and confers sensitivity to naphthalene acetamide (NAM), an analog of the growth hormone derivative indole-3-acetamide. The growth rate of seedlings containing the tms2 gene is normal in the absence of NAM, but severely retarded in its presence. The phenotypes of NAM-resistant (N') and NAM-sensitive (N) seedlings are shown in Figure 2b. The Ds element

Table 1. Sectorial expression of the GUS gene in kanamycin-resistant calli

<table>
<thead>
<tr>
<th>Plasmid 1</th>
<th>Plasmid 2</th>
<th>Number of calli</th>
<th>% with GUS+ sectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excision assay</td>
<td>None</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Excision assay</td>
<td>Bam35S-Ac</td>
<td>63</td>
<td>13</td>
</tr>
<tr>
<td>Excision assay</td>
<td>Nae35S-Ac</td>
<td>56</td>
<td>9</td>
</tr>
<tr>
<td>Ds–GUS 6-1B2*</td>
<td>None</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>Ds–GUS 6-1B2*</td>
<td>Nae35S-Ac</td>
<td>123</td>
<td>11</td>
</tr>
</tbody>
</table>

*The Ds–GUS 6-1B2 transposon is based on Ds325 (Figure 1) and contains a CaMV 35S core promoter sequence upstream of the GUS gene.
Table 2. Phenotypes of plants differing in T-DNA and transposon constitution

<table>
<thead>
<tr>
<th>Genetic constitution</th>
<th>Kanamycin</th>
<th>Hygromycin</th>
<th>Chlorsulfuron</th>
<th>NAM</th>
<th>GUS* sectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ds T-DNA</td>
<td>r</td>
<td>r</td>
<td>lr</td>
<td>r</td>
<td>–</td>
</tr>
<tr>
<td>Ac T-DNA</td>
<td>r</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>–</td>
</tr>
<tr>
<td>Ac + Ds T-DNA</td>
<td>r</td>
<td>r</td>
<td>lr</td>
<td>s</td>
<td>+</td>
</tr>
<tr>
<td>EDS*</td>
<td>r</td>
<td>s</td>
<td>r</td>
<td>r</td>
<td>–</td>
</tr>
<tr>
<td>TrE*</td>
<td>s</td>
<td>r</td>
<td>s</td>
<td>r</td>
<td>–</td>
</tr>
<tr>
<td>EDS + TrE</td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>–</td>
</tr>
<tr>
<td>EDS + Ac T-DNA</td>
<td>r</td>
<td>s</td>
<td>r</td>
<td>s</td>
<td>–</td>
</tr>
<tr>
<td>TrE + Ac T-DNA</td>
<td>r</td>
<td>r</td>
<td>s</td>
<td>s</td>
<td>?b</td>
</tr>
<tr>
<td>EDS + TrE + Ac T-DNA</td>
<td>r</td>
<td>r</td>
<td>s</td>
<td>r</td>
<td>–</td>
</tr>
<tr>
<td>No T-DNA</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>r</td>
<td>–</td>
</tr>
</tbody>
</table>

*EDS, empty donor site; TrE, transposed *Ds–GUS* element.

†, some TrEs give an altered pattern of GUS* sectors, while others exhibit either a uniform or differential GUS staining pattern which can mask the GUS* sectors.

carries an *aph4* gene and plants containing the element are therefore also resistant to hygromycin B. The phenotypes of hygromycin-resistant (H*) and hygromycin-sensitive (H₀) plants are shown in Figure 2c.

We found that plants containing the *Ds–GUS* element inserted in the leader sequence of the chlorsulfuron-resistant ALS gene are much more resistant to chlorsulfuron than untransformed plants. At concentrations of the herbicide up to 100 p.p.m., the growth of seedlings containing the *Ds–GUS* T-DNA is only slightly retarded, while the same concentrations are lethal to untransformed seedlings (Figure 2d). Thus the integrity of the ALS gene can be tested in transplants containing *Ds–GUS* T-DNAs. This has proved to be a useful feature of the construct, since the NPT-II transformation marker is at the opposite end of the T-DNA segment from the ALS gene and incomplete T-DNA integration events have been reported to be relatively frequent (Deroles and Gardner, 1988; Spielmann and Simpson, 1986). The phenotypes of plants that contain a chlorsulfuron-resistant 35S-ALS transgene, one disrupted by a *Ds–GUS*, and no ALS transgene are referred to as chlorsulfuron-resistant (C'), low chlorsulfuron-resistant (C") and chlorsulfuron-sensitive (C₀), respectively.

The progeny of self-pollinated plants regenerated from K' calli transformed with either a 35S-Ac or a *Ds–GUS* plasmid were plated on kanamycin-containing medium to determine the percentage resistant to kanamycin. Of the 75 putative 35S-Ac and 79 putative *Ds–GUS* containing plants that have been tested to date, 69% and 68%, respectively, gave K' progeny (Table 3). Among these, 56% and 69%, respectively, showed the 3:1 segregation of K' and K₀ progeny expected for a single T-DNA insertion site. Many progenies showed either a lower or higher ratio of K'/K₀ seedlings than expected for a single T-DNA insertion site. While subsequent analysis revealed the presence of a variety of segregation-distorting embryo- and seedling-lethal mutations in the plants regenerated from transformed calli, it was initially assumed that plants giving more than 80% K' progeny contained more than one insertion site and these were not included in subsequent analyses. To determine whether a given insertion site carried one or more than one T-DNA copy, DNA was extracted from plants exhibiting a 3:1 segregation of the *Ds* T-DNA marker and analyzed by probing restriction endonuclease digests with labeled probes homologous to the genes on the respective T-DNAs, using different probes for the right and left borders (see Experimental procedures).

Of the 35S-Ac and *Ds–GUS* plants tested to date, 75% and 86%, respectively, contain a single T-DNA copy (Table 3). Finally, the intactness and expression of the *aph4* and the chlorsulfuron-resistant ALS genes were verified by plating the selected progenies with single *Ds–GUS* insertions on agar medium containing hygromycin, as well as on medium containing low (100 p.p.b.) and high (10 p.p.m.)

Table 3. Analysis of putative kanamycin-resistant single transfectants

<table>
<thead>
<tr>
<th></th>
<th>Number of regenerants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35S-Ac T-DNA</td>
</tr>
<tr>
<td>Kanamycin-resistant</td>
<td>52 (69% of total)</td>
</tr>
<tr>
<td>Single T-DNA site</td>
<td>29 (56% of K')</td>
</tr>
<tr>
<td>Single T-DNA copy</td>
<td>12/16 (75%)</td>
</tr>
</tbody>
</table>

*Plants exhibiting 3:1 segregation of K'/K₀ progeny were assumed to have a single T-DNA insertion site.
concentrations of chlorsulfuron. Progeny of plants containing a single 35S-Ac were plated on 10 μM NAM to check the presence and expression of the tms2 gene.

Genetic characterization of co-transformed plants

Multiple plants were regenerated from each of seven K\(^+\) callus lines that exhibited GUS-positive sectors. All contained at least one 35S-Ac T-DNA and one Ds-GUS T-DNA insert, as judged by the presence of both the NAM-sensitivity and the hygromycin-resistant traits in the progeny. In four of the lines, markers characteristic of either the 35S-Ac or the Ds-GUS T-DNA (but not both) were detected in more than 75% of the progeny of the several regenerants tested, suggesting that there was more than one insertion site for one of the T-DNAs (data not shown). The three remaining lines exhibited the segregation ratios for hygromycin-resistance and NAM-sensitivity expected for a single insertion site for each T-DNA (Table 4). In two lines, designated 399-8 and 400-46, 75% of the progeny tested were kanamycin-resistant, suggesting close linkage of the insertion sites for the 35S-Ac and Ds-GUS T-DNAs. Upon further testing, roughly a quarter of the pooled progeny of the first two regenerants each of lines 399-8 and 400-46, respectively, proved sensitive to both hygromycin and kanamycin and sensitive to hygromycin but resistant to NAM (Table 4). In neither case were there any progeny plants that were resistant to both hygromycin and NAM, the expected phenotype for plants containing the Ds-GUS T-DNA. The three remaining lines exhibited the segregation ratios for hygromycin-resistance and NAM-sensitivity expected for a single insertion site for each T-DNA (Table 4).

In two lines, designated 399-8 and 400-46, 75% of the progeny tested were kanamycin-resistant, suggesting close linkage of the insertion sites for the 35S-Ac and Ds-GUS T-DNAs. Upon further testing, roughly a quarter of the pooled progeny of the first two regenerants each of lines 399-8 and 400-46, respectively, proved sensitive to both hygromycin and kanamycin and sensitive to hygromycin but resistant to NAM (Table 4). In neither case were there any progeny plants that were resistant to both hygromycin and NAM, the expected phenotype for plants containing the Ds-GUS T-DNA. Classification of the insertion sites for the 35S-Ac and Ds-GUS T-DNAs (Table 4). In the third line, designated 400-12, 84% of the progeny were kanamycin-resistant (Table 4). Less than 20% of the progeny were H\(^+\)K\(^+\) and H\(^+\)N\(^+\), and 3% of the progeny were H\(^+\)K\(^-\) and H\(^+\)N\(^-\) (Table 4). None of the progenies contained plants that were resistant to hygromycin, but sensitive to kanamycin, the expected phenotype of plants with a transposed element, but neither T-DNA (Table 2). Except for the presence of H\(^-\)K\(^+\) progeny plants in line 399-8, these observations are consistent with the interpretation that the 35S-Ac and Ds-GUS T-DNA insertion sites are extremely closely linked in lines 399-8 and 400-46, but loosely linked in line 400-12. As shown below, the H\(^+\)K\(^+\) progeny plants in line 399-8 were found to have arisen by excision of the Ds-GUS element, not by recombination between different T-DNA insertion sites. Lines in which T-DNAs are inserted at a single site, comprising a single genetic locus, proved useful in evaluating the correspondence between phenotype and genotype because empty donor sites and transposed elements could be readily identified by unique combinations of several markers (Table 2). Unfortunately, of the two lines with closely linked insertion sites, only 399-8 gave plants that were resistant to 100 p.p.b. chlorsulfuron, indicating that the ALS gene might have been truncated in the initial T-DNA insertion event in line 400-46 (Table 4).

All seven of the co-transformed lines analyzed exhibited GUS\(^+\) callus sectors and gave at least some progeny plants with GUS\(^+\) sectors. As noted earlier, none of the plants containing only a Ds-GUS T-DNA showed GUS\(^+\) sectors. To determine whether GUS\(^+\) sectors could be detected in all plants containing both a Ds-GUS and a 35S-Ac, cotyledons and leaves from R\(_1\) seedlings resistant to both hygromycin and kanamycin were tested in the three lines with a single copy of the transposon and transposase gene (Table 4). Except for the first two regenerants from line 399-8, all but one of the 398 H\(^+\)K\(^+\) plants tested exhibited GUS\(^+\) sectors of the type shown in Figure 3a. Among the pooled progeny of regenerants 1 and 2 in line 399-8, 15% of the H\(^+\)K\(^+\) seedlings showed no GUS-positive sectors. The H\(^+\)K\(^-\) progeny of such plants were more sensitive to chlorsulfuron than the parents and

<table>
<thead>
<tr>
<th>Transformant and regenerant</th>
<th>H(^+)/H(^-) (%)</th>
<th>K(^+)/K(^-) (%)</th>
<th>N(^+)/N(^-) (%)</th>
<th>C(^+)/C(^-) (%)</th>
<th>G(^+)/H(^+)/K(^+) (%)</th>
<th>H(^+)/K(^-) (%)</th>
<th>H(^-)/K(^+) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>399-8-1+2</td>
<td>78/116 (67)</td>
<td>90/117 (78)</td>
<td>113/149 (76)</td>
<td>73/97 (75)</td>
<td>156/183 (85)</td>
<td>33/116 (28)</td>
<td>7/116 (6)</td>
</tr>
<tr>
<td>399-8-4</td>
<td>69/119 (74)</td>
<td>65/114 (75)</td>
<td>122/161 (76)</td>
<td>76/180 (76)</td>
<td>154/154 (100)</td>
<td>21/115 (27)</td>
<td>1/115 (0)</td>
</tr>
<tr>
<td>400-12-1+2</td>
<td>69/117 (75)</td>
<td>97/115 (84)</td>
<td>101/123 (82)</td>
<td>67/88 (76)</td>
<td>100/101 (99)</td>
<td>15/177 (19)</td>
<td>3/89 (0)</td>
</tr>
<tr>
<td>400-46-1+2</td>
<td>90/120 (75)</td>
<td>69/120 (74)</td>
<td>121/170 (71)</td>
<td>0/100 (0)</td>
<td>143/143 (100)</td>
<td>24/117 (21)</td>
<td>0/117 (0)</td>
</tr>
</tbody>
</table>

\(1\) Total number of R\(_1\) seedlings tested.

\(2\) G\(^+\)/H\(^+\)/K\(^+\), number of H\(^+\)K\(^+\) seedlings with GUS\(^+\) sectors.

\(3\) The H\(^+\)G\(^+\) progeny were sensitive to a low concentration of chlorsulfuron and had a rearranged T-DNA.
Figure 2. Growth of transgenic Arabidopsis seedlings on selective media.
(a) K' (left) and K* (right) seedlings on agar medium containing 50 μg ml⁻¹ kanamycin. (b) N' (left) and N* (right) seedlings on medium containing 10 μM NAM.
(c) H' (left) and H* (right) seedlings on medium containing 20 μg ml⁻¹ hygromycin B. (d and e) C' (left, 35S ALS gene), C* (center, Ds-GUS), and C* (right, untransformed) seedlings on medium containing 100 p.p.b. and 20 p.p.m. chlorsulfuron, respectively.

Figure 3. Histochemical staining for GUS activity and growth on selective media.
(a) A cotyledon of a seedling containing both a 35S-Ac and a Ds-GUS T-DNA stained for GUS activity. (b) Progeny of a plant containing both a 35S-Ac and a Ds-GUS T-DNA plated at high density on medium containing 20 p.p.m. chlorsulfuron; C' seedlings have fully expanded cotyledons and long roots.
(c) C'H' (left, contains EDS and Tfl) and C'H* (right, contains EDS only) germinated on a medium containing both 20 p.p.m. chlorsulfuron and 20 μg ml⁻¹ hygromycin B. (d) Root segments from K' (left) and K* (right) seedlings grown on kanamycin-containing callus-inducing medium for 2 weeks.
showed a rearrangement affecting the sequence immediately adjacent to one end of the $\text{Ds-GUS}$ transposon. Since all of the $\text{GUS}^{\text{-}}$ siblings examined had the same phenotype and genotype, they probably represent the progeny produced on a mutant somatic sector of a plant (see below).

**Genetic properties of plants with excised and transposed elements**

The phenotypes of plants in which the $\text{Ds-GUS}$ transposon has excised and transposed to a new site should be distinguishable from the phenotypes of plants in which neither event has occurred by virtue of the ability of the chlorsulfuron-resistant ALS gene to be expressed at a high level (Table 2). Chlorsulfuron-resistant progeny are readily identifiable by their ability to germinate and grow normally on a medium containing up to 30 p.p.m. chlorsulfuron (Figure 2e). We have found that $C'$ seedlings can be identified even when seeds are plated at the very high densities of 600–700 per ml (Figure 3b). Plants that commenced development with an empty donor site (EDS) arising in the previous generation can be distinguished easily from plants with early somatic excision events by the rate of shoot and root growth. Moreover, plants containing only an EDS or only a transposed element (TrE) should be uniquely identifiable by their ability to grow on medium with the appropriate selective agents (Table 2). That is, a plant that has an EDS but no transposed element should be resistant to chlorsulfuron, but sensitive to hygromycin ($C'H^s$), while a plant that contains only a transposed element should be sensitive to chlorsulfuron, but resistant to hygromycin ($C'H'$, Figure 3c). Plants resistant to both selective agents should contain both an EDS and a TrE (or be heterozygous for an EDS and the original $\text{Ds-GUS}$ T-DNA). Because the transposase gene is immobile and its T-DNA contains a $\text{tms2}$ gene, plants that retain the 35S-Ac should be identifiable by their sensitivity to NAM.

The marker system was designed to facilitate the selection of plants containing a transposed element, but no transposase, in order to assure the stability of a newly arising insertion mutation. Thus among the progeny of plants containing a single transposon and a single transposase T-DNA at different chromosomal locations, plants that are resistant to chlorsulfuron, hygromycin and NAM should contain an EDS and a TrE, but no 35S-Ac transposase gene. Among the progeny of plants in which the transposon and transposase are inserted at the same chromosomal site, those that are resistant to hygromycin and NAM ($H'N'$) should contain only a TrE and lack both the EDS and the 35S-Ac transposase gene. Plants with the $\text{Ds-GUS}$ and 35S-Ac T-DNAs at a single location, obtained from cotransformed lines 399-8 and 400-46, were used to test the efficacy of the marker system for the rapid identification of excision and transposition events. In analyzing these plants, we have also made use of the fact that the T-DNAs contain an NPT-II gene, thereby providing an additional marker for the donor site. In the early stages of the present analysis, we did not appreciate the high mutation, excision and transposition frequencies in tissue culture and therefore initially pooled the seeds of more than one regenerant from a single transformant. That the frequency was quite high became apparent through the present analysis. For example, roughly a third of the $R_1$ plants grown from the pooled progenies of the first two regenerants from line 399-8 segregated an embryo lethal mutation, suggesting that the mutation was present in only one of the regenerants. Since the embryo lethal did not segregate with the T-DNA markers and appeared to be present in only one of the two regenerants, its origin could not be attributed to the T-DNA insertion event. And although some of the $R_1$ progeny exhibited chlorsulfuron resistance, none of the seven $C'$ plants grown to maturity segregated the embryo lethal, suggesting that the genetic event conferring chlorsulfuron resistance arose during development of the regenerant which did not carry the embryo lethal mutation and was not responsible for it. Such complications need to be borne in mind, but do not obscure the results.

Among the plants regenerated from line 399-8, the pooled progenies of plants 1 and 2 gave 6% chlorsulfuron-resistant $R_1$ seedlings, while plants 4 and 5 gave 2.1 and 70% $C'$ seedlings, respectively (Table 5). The $C'$ seedlings were tested for resistance to hygromycin and kanamycin either by transferring the complete plant to agar medium containing the drug or, if the seedlings were to be grown, root sections were put on a callus-inducing medium containing the drug (Figure 3d), and the remaining seedling was re-rooted on a medium lacking drugs (see Experimental procedures). Only roots carrying the corresponding drug resistance marker form callus on the callus-inducing medium containing the drug. We have recovered and analyzed 37 and 34 $C'$ plants from regenerants 1+2 and 4, respectively, in line 399-8 and found that all of them are sensitive to hygromycin, indicating that they contain an EDS, but no transposed element. Plants having only an EDS should also be resistant to kanamycin, but sensitive to hygromycin ($H'K'$). Such plants were recovered from the same progenies at the same frequencies as $C'H'$ plants (Tables 4 and 5). Although relatively small numbers of plants have been tested to date, no $H'N'$ or $H'K'$ progeny have been identified in these regenerants (Table 5). These latter phenotypes are those expected for a transposed element that has segregated away from an EDS (Table 2). By contrast, 70% of the $R_1$ progeny from regenerant 5 were resistant to chlorsulfuron and 76% of these were also resistant to hygromycin, suggesting that the element had transposed to a different chromosome from that bearing the EDS. If plant 5 contains a TrE
Table 5. Identification of plants in which excision and transposition has occurred

<table>
<thead>
<tr>
<th>Transformant</th>
<th>Regenerant</th>
<th>C'\text{total} (%)</th>
<th>C'H'/C' (%)</th>
<th>H'N\text{total} (%)</th>
<th>H'K'\text{total} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>399-8</td>
<td>1+2</td>
<td>37/620 (6.0)</td>
<td>0/37 (0)</td>
<td>0/165 (0)</td>
<td>0/96 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4/1656 (2.1)</td>
<td>0/34 (0)</td>
<td>0/60 (0)</td>
<td>0/110 (0)</td>
</tr>
<tr>
<td>4</td>
<td>3+4</td>
<td>154/220 (70)</td>
<td>66/87 (76)</td>
<td>18/79 (23)</td>
<td>9/28 (25)</td>
</tr>
<tr>
<td>5</td>
<td>1+2</td>
<td>-</td>
<td>-</td>
<td>0/99 (0)</td>
<td>0/99 (0)</td>
</tr>
<tr>
<td></td>
<td>3+4</td>
<td>-</td>
<td>-</td>
<td>40/191 (21)</td>
<td>60/251 (24)</td>
</tr>
</tbody>
</table>

Although the R₁ progeny of plants regenerated from line 400-46 could not be screened for chlorsulfuron resistance, we found that 21% and 24% of the pooled seeds from regenerants 3+4 gave H'N' and H'K' seedlings, respectively, while the pooled seeds from regenerants 1+2 gave neither class of seedlings. This suggested that there was a transposed, independently segregating element in the former, but not the latter regenerants.

Molecular characterization of R₁ plants with empty donor sites and transposed elements

To determine whether the phenotypes of plants, as assessed on selective media, accurately corresponded to their genotypes, we analyzed R₁ plants of regenerants from lines 399-8 and 400-46 expected to have the original T-DNA configuration, only an EDS, only a TrE or both. Although both lines contained a single copy each of the Ds-GUS and 35S-AcT-DNAs, only line 399-8 gave an Asp 718 fragment of the expected size (10.2 kb) for the complete ALS gene with a Ds-GUS element (see Figure 1a). Line 400-46, which gave no evidence of resistance to either low or high levels of chlorsulfuron (Table 4), contained no detectable ALS-homologous transgenic fragments, suggesting that the initial insertion event introduced an incomplete T-DNA with a truncated ALS gene (data not shown). DNA from progeny of line 399-8 that are resistant to a high concentration of chlorsulfuron contains a 4.9 kb Asp 718 fragment homologous to the ALS gene (Figure 4a). It is shorter by the length of the transposon (5.3 kb) than the 10.2 kb fragment detected in plants that exhibit the original marker configuration. The 10.2 kb fragment can also be detected with a sequence borne on the transposon (GUS or aph4) in plants having the original marker configuration (H'K'G'), but the shorter fragment shows no homology to the Ds-GUS-specific probes, supporting the interpretation of the latter as an EDS (Figure 4b). Plants that were selected for the presence of both an EDS and a TrE (C'H') have the short transgenic ALS fragment and a new, longer fragment homologous to the aph4 probe, showing that the donor site and the transposon are now on different Asp 718 fragments. Plants selected for the presence of the EDS, but no transposed element (C'H') contain the short, ALS-homologous fragment, but no aph4-homologous fragment, consistent with the absence of the hygromycin-resistance marker. It should be noted that a longer fragment homologous to the ALS probe can be detected in plants of all different constitutions; this is the endogenous ALS gene (Figure 4a).

Regenerant 5 of line 399-8 and regenerants 3+4 of line 400-46 gave a high proportion of progeny with marker combinations characteristic of transposed elements (Table 5). Progeny plants that were either H'N' or H'K' and should have a transposed element, but no EDS, were selected for analysis. DNA was extracted from leaves, digested with Ncol, an enzyme that cleaves once within the element and probed with a transposon-specific (aph4) probe to detect fragments that extend from the element into flanking sequences (Figure 4c). The fragments that were detected in the two progenies differed in size from those characteristic of the original T-DNA and from each other. It should be noted that Ncol cleaves the Ds-GUST-DNA at the initiation codon of the ALS gene and that the 400-46 parental DNA exhibits the same aph4-homologous Ncol fragments as line 399-8 (Figure 1). Sibling plants within each progeny contained element-homologous fragments of the same length, indicating that the elements are at the same site. This is consistent with the high fraction of R₁ plants bearing the marker combinations characteristic of a transposed element (Table 5), indicates that one transposition event is represented in each line and implies that it probably
Occurrence of transposition occurred during culture and prior to regeneration of the plants containing the transposed element.

Finally, plants selected from among the same progeny by alternative marker combinations, such as either H'K' or H'N' for a TrE and either H'K' or C'H' for an EDS, gave identical results. We conclude that the phenotypes of plants exposed to selective media accurately reflect their genotypes with respect to the structure of the T-DNA and the transposon.

**Initiating transposition by a genetic cross**

While the crosses to bring the transposon and transposase together are intended to be done with plants that are homozygous for their respective insertions, several crosses between Ds–GUS and 3SS–Ac plants were carried out before homozygous plants were available. This was done by using 3SS–Ac-containing plants as female parents and crossing them by Ds–GUS–containing male parents, growing and selfing the hygromycin-resistant F₁s, then identifying the plants that gave both NAM-sensitive and hygromycin-resistant progeny. Such test progeny were also assayed for the presence of GUS⁺ sectors. As in the previous experiments with co-transformants, H'N' progeny exhibited GUS⁺ sectors indicative of transpositional activation of the GUS gene and diagnostic for plants containing both a transposon and the transposase gene. Although only five plant progenies carrying both the transposon and transposase gene were obtained in these preliminary experiments, transposition events were detected in four of the five progenies by the criteria described above. In all four progenies, the C' plants had an EDS, as well as one to three transposed elements (data not shown).

**Sectorial GUS expression and transposition**

There is an excellent correlation between the presence of both transposon and transposase in a plant and the presence of sectors of cells expressing the GUS gene both in roots and shoots. In addition to the seedlings for which data are given in Table 4, we have tested 537 more R₁ and R₂ H'K' GUS⁺ plants that were analyzed all proved to have transposed elements or rearrangements in the immediate vicinity of the Ds–GUS T-DNA. For example, DNA from two H'K' GUS⁺ plants detected among the pooled progeny of the first two regenerants from line 399-8 exhibit Ds–GUS T-DNA fragments of altered mobility when cleaved with Asp 718.
Further analysis of the DNA from such plants revealed that there had been an inversion of the sequence between the transposon and the adjacent transposase gene leading to decreased expression of the ALS gene and immobilizing the transposon (Wright and Fedoroff, unpublished data).

Not unexpectedly, plants that are heterozygous for the 35S-Ac transposase gene show a lower frequency of GUS’ sectors than do plants that are homozygous for the gene. Thus GUS’ sectors are detectable in either all or virtually all plants containing both a Ds–GUS and a 35S-Ac T-DNA insert. Plants containing the 35S-Ac transposase gene and either of the two different transposed elements identified in co-transformed lines 399-8 and 400-46 show almost no GUS’ sectors, nor has uniform GUS expression in leaf tissues been detected in these two lines by the histochemical assay used here (Table 5, Figure 4c). The presence of both the transposase gene and Ds–GUS transposon is evidenced, however, by occasional small GUS’ sectors, as well as the markers associated with the transposon and transposase gene. Although we have not yet completed analysis of R2 progeny plants with transposed elements, those that we have examined so far fall into one of three different categories of staining pattern: (1) one that gives very infrequent small GUS’ sectors; (2) one that exhibits a differential pattern of GUS expression in development, but few or no GUS’ sectors; (3) one that gives a uniformly high level of GUS expression in all tissues.

Transpositional activation of the GUS gene

Among the first 33 R2 progenies of regenerant 4 from line 399-8 analyzed, we identified one in which 2% of progeny seedlings stained a uniform, intense blue color (Figure 5a). These seedlings were detected in the course of a large-scale screen of R2 progeny for GUS’ sectors. They were sensitive to chlorsulfuron and would not have been detected in a chlorsulfuron-based screen. This marker combination could arise by one of several genetic events, including a short-range intrachromatid transposition, an interchromatid transposition, or a rearrangement. Analysis of DNA from such seedlings revealed that while the original Asp 718 fragment was unaltered, the element had transposed within it and had reinserted, in inverted orientation, within 0.2 kb of its original insertion site (Figure 5b). This was inferred from the observation of novel NcoI (Figure 5c) and NcoI–Asp 718 fragments (data not shown) homologous to both the ALS and aphi4 genes. Supporting this inference was the further observation that progeny homozygous for the chromosome carrying the transposed element were more sensitive to chlorsulfuron than sibs that contained the original Ds–GUS T-DNA on the homolog. Thus strong activation of the GUS gene in all parts of the seedling resulted from reinsertion of the transposon just downstream from the 35S promoter of the ALS gene within whose leader it had originally been inserted and in the correct orientation for the GUS gene to be expressed. This observation provides further evidence that insertion of the transposon at sites within a gene will permit expression of the GUS gene.

Transposition frequency

The Ds–GUS element transposed to another chromosome during tissue culture in both of the co-transformed lines in which the 35S-Ac and Ds–GUS T-DNAs inserted at a
single site (Table 5). Only plants 1, 2 and 4 regenerated from line 399-8 commenced development with an untransposed element and could be tested for chlorsulfuron-resistant progeny. Two of the three R1 plants produced C' progeny, all of which contained an EDS, but no TrE. To determine the number of R1 plants that produce C' progeny, R1 plants that commenced development with an untransposed Ds-GUS element (HfK'G') were grown and the seeds harvested from individual plants were analyzed for excision and transposition events. We tested R1 progeny of 88 individual R1s derived from the pooled seed of R0 plants 399-8-1 + 2, as well as R2 progeny of 46 individual R1s from R2 plant 399-8-4 in sufficient detail to determine the proportion of C' plants, the heritability of the trait, and whether a TrE was present or only an EDS (Table 4), and we have obtained preliminary data on an additional 70 R1 individuals from plant 399-8-4. Between 1000 and 5000 seeds were plated on 10-20 p.p.m. chlorsulfuron for each progeny tested. Despite the fact that they represent sibling regenerants, the 399-8-1 + 2 R1 progeny differed markedly from the 399-8-4 R1 progeny in the frequency of plants yielding C' R2 progeny. Thus only three of 88 R1 plants (3.4%) from the 399-8-1 + 2 pool gave C' progeny, while eight of the first 46 R1s (17%) from R0 plant 399-8-4 gave C' progeny. Although the next 70 R1 plants from R0 plant 399-8-4 have not been analyzed as extensively as the group for which data are given in Table 4, the fraction of plants giving C' progeny is similar (23%).

Among the plants for which data are given in Table 4, the percentage of chlorsulfuron-resistant R2 progeny ranged from 0.3 to 26%, with most plants in the 1-10% range. About half of the plants that produced C' progeny were homozygous and half were heterozygous for the T-DNA. Since the population of plants examined contains twice as many heterozygotes as homozygotes, C' plants arise about twice as frequently in homozygotes as heterozygotes, an observation that is consistent with the higher frequency of GUS' somatic sectors in homozygotes as compared with heterozygotes. It is therefore likely that both the frequency of C' progeny and the somatic sectoring frequency reflect the transposase concentration.

Several conclusions emerged from the analysis of the C' R2 plants and their R2 progeny (Table 4). First, plants that grow rapidly under the selection conditions used almost invariably proved to have germinal excision events (see Figure 3b). Only one of 163 C'Hf R2 plants whose R1 progeny were analyzed proved to have been a misidentified C'Hf plant containing an untransposed Ds-GUS element (data not shown). An early somatic transposition event had occurred in the single misidentified plant and its R2 generation yielded 34% C' progeny. Second, most of the C' plants contained a transposed element, as judged by both genetic criteria and blot hybridization analysis of genomic DNA. Moreover, a single excision or transposition event dominated each progeny (Wright and Federoff, unpublished data). Thus, although two events have been detected in a single progeny, such as the EDS (1.0%) and the blue seedling progeny (2.6%) of 399-8-4 R1 plant 8, it appears likely that one event predominates numerically among most progenies by virtue of its developmental timing. Third, none of the EDS-containing progeny and almost none of the TrE-containing progeny of parent plants that contained the transposon and transposase T-DNAs on only one homolog (homozygotes in Table 4), gave R3 progeny that were homozygous for an EDS T-DNA and a T-DNA with an untransposed element. This implies that both male and female gametes giving rise to the C' progeny carried the EDS T-DNA and means that selection of the progeny of such homozygotes directly on both chlorsulfuron and hygromycin yields plants with transposed elements in a single step.

<table>
<thead>
<tr>
<th>R0</th>
<th>R1</th>
<th>Genetic constitution*</th>
<th>% C' in R2</th>
<th>TrE or EDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>399-8-1+2</td>
<td>82</td>
<td>Heterozygous</td>
<td>6.3</td>
<td>TrE</td>
</tr>
<tr>
<td>122</td>
<td></td>
<td>Heterozygous</td>
<td>2.4</td>
<td>TrE</td>
</tr>
<tr>
<td>126</td>
<td></td>
<td>Heterozygous</td>
<td>2.5</td>
<td>TrE</td>
</tr>
<tr>
<td>399-8-4</td>
<td>1</td>
<td>Heterozygous</td>
<td>3.6</td>
<td>TrE</td>
</tr>
<tr>
<td>46</td>
<td></td>
<td>Heterozygous</td>
<td>0.7</td>
<td>TrE</td>
</tr>
<tr>
<td>47</td>
<td></td>
<td>Heterozygous</td>
<td>1.6</td>
<td>TrE</td>
</tr>
</tbody>
</table>

*The genetic constitution of the R0 plant was either heterozygous or homozygous for the chromosome bearing the 3SS-Ac and De-GUS T-DNA insertion site.

Discussion

Since the initial report that the maize Ac element transposes in Arabidopsis (Van Sluys et al., 1987), investigators in several laboratories have devoted considerable effort not only to analyzing the element's behavior in this plant, but also to modifying the element in order to facilitate its use as a mutagenic agent (Altmann et al., 1992; Dean et al., 1992; Masterson et al., 1989; Schmidt and Willmitzer, 1989; Swinburne et al., 1992). The activity of the element has been monitored primarily by its own excision or that
of a transposition-defective \textit{Ds} derivative from the leader sequence of a streptomycin phosphotransferase (SPT) or NPT-II gene, assays initially developed to study \textit{Ac} in tobacco (Baker \textit{et al.}, 1987; Dean \textit{et al.}, 1992; Jones \textit{et al.}, 1989; Masterson \textit{et al.}, 1989; Schmidt and Willmitzer, 1989). Expressed from its own promoter, the \textit{Ac} element is substantially less active in \textit{Arabidopsis} than it is in either tobacco or tomato (Dean \textit{et al.}, 1992; Hehl and Baker, 1990; Schmidt and Willmitzer, 1989; Swinburne \textit{et al.}, 1992; Yoder, 1990). In recent studies it was found that replacement of the \textit{Ac} promoter by one of several stronger plant promoters substantially increases its ability to promote \textit{Ds} transposition early in plant development (Swinburne \textit{et al.}, 1992).

In \textit{Arabidopsis}, as in maize and other plants, the \textit{Ac} element can promote either its own excision or that of a \textit{Ds} element located elsewhere in the genome (Masterson \textit{et al.}, 1989; Swinburne \textit{et al.}, 1992). Two-element systems in which the transposon and transposase are genetically separable have the considerable advantage that new \textit{Ds} insertions can be stabilized by genetic segregation of the transposon from the transposase gene. \textit{Ds} derivatives marked with a drug-resistance gene have been constructed and shown to be mobile in \textit{Arabidopsis} only in the presence of the \textit{trans}-activating \textit{Ac} transposase (Masterson \textit{et al.}, 1989).

We have further modified the design of the \textit{Ac}-based transposon tagging system for \textit{Arabidopsis} to decrease the amount of labor involved in identifying plants with transposed elements, in general, and element insertions in and near genes, in particular. The results of studies in which the NPT-II or SPT genes have been used to monitor excision indicate that neither is optimally efficient and reliable as an indicator of excision activity or for the identification of germinal transposition events (Altman \textit{et al.}, 1992; Dean \textit{et al.}, 1992; Swinburne \textit{et al.}, 1992). Our experience with the \textit{present \textit{Ds}–GUS constructs in which a chlorsulfuron-resistant ALS gene serves as an excision assay marker shows that the rate of seedling growth on chlorsulfuron is an extremely accurate reflection of the seedling’s genetic constitution. Almost without exception, the seeds that germinate and grow the most rapidly on chlorsulfuron have received at least one copy of the intact chlorsulfuron-resistant ALS gene from a gamete. We find that under our growth conditions, seedlings with relatively large somatic transposition sectors almost always lag 3–5 days behind seedlings that received an intact 3SS-ALS gene from their parents. Whether the seedlings that exhibit large somatic GUS® sectors grow into plants that give a higher than average number of \textit{C}’ progeny is presently under investigation.

In addition to the high reliability of the chlorsulfuron selection for germinal events, the fact that chlorsulfuron inhibits seedling growth extensively permits to be plated at the very high densities of 4000–5000 in a single standard Petri dish. Finally, chlorsulfuron inhibition of growth is reversible and even untransformed seeds can be rescued from chlorsulfuron-containing medium, facilitating the genetic characterization of the progeny with respect to other markers. Thus, for example, after \textit{C}’ progeny are removed from the chlorsulfuron-containing medium, a number of seeds can be spread on a hygromycin-containing plate to determine how the hygromycin-resistance marker is segregating in the progeny.

In order to facilitate the identification of plants containing a transposed element, but no transposase source, we put immobilized, 3SS promoter-substituted \textit{Ac} elements adjacent to a negative selection marker, the \textit{Agrobacterium tumefaciens} \textit{tms2} gene, on a single T-DNA. The presence of this gene permits selection of plants that lack the mobilizing transposase gene by virtue of their ability to grow on NAM-containing medium. We have shown that plants that are resistant to both hygromycin and NAM contain the \textit{Ds}–GUS element, but no transposase gene. As noted in the text, we have observed that plants having the \textit{Ds}–GUS T-DNA on only one homolog (T-DNA heterozygotes) almost invariably give \textit{C}’ progeny that are either homozygous for the EDS T-DNA or heterozygous for the EDS T-DNA chromosome and the homolog lacking the T-DNA insert. This means that both male and female gametes arose from the cell lineage in which the excision (or transposition) event had already occurred. This, in turn, means that plants that carry the \textit{TrE}, but no \textit{Ac} element can be identified directly by plating seed on agar medium containing both hygromycin and NAM. If the transposition event occurs sufficiently early in the plant’s development and the element has transposed to a different chromosome or a distant site on the same chromosome, then such plants can be identified immediately among the progeny of the plant in which the transposition event has occurred. Indeed, \textit{H}’ \textit{N}’ \textit{R}2 seedlings were directly selected from 399–8–4 \textit{R}1 progeny plants 1 and 11 (Table 4). However, this selection can be carried out directly only if the fraction of progeny with a transposed element is relatively high, since only 25% will be homozygous for the homolog lacking the \textit{Ac} and \textit{tms2} T-DNA and, at most, 75% of these will carry the hygromycin-resistance marker of the \textit{Ds}–GUS element, unless it transposes to the homolog.

If the frequency of \textit{C}’ \textit{H}’ progeny of such heterozygotes is low, \textit{C}’ \textit{H}’ plants are grown and their seed plated on hygromycin and NAM to identify progeny containing the \textit{TrE}, but not the \textit{tms2}-linked \textit{Ac}. Given the relatively late timing of transposition characteristic of most of the present lines, we do not anticipate that secondary transpositions will be a major problem, although it is undoubtedly prudent to grow several \textit{H}’ \textit{N}’ siblings for seed collection. Transposed elements arising in T-DNA homozygotes contain an unarranged \textit{Ds}–GUS T-DNA on the homolog. Such plants
can either be outcrossed immediately and then analyzed as heterozygotes, or C′ EDS homozygotes that are also H′ can be identified first and then outcrossed. As detailed below, changes in the GUS sectoring pattern can be used to identify such seedlings.

Transposons containing a reporter gene were first developed in *Drosophila* to detect developmentally regulated promoters and enhancers (Bier et al., 1989; Wilson et al., 1989). T-DNA-based tagging vectors incorporating either a drug-resistance marker or the GUS gene have been developed and used successfully in plants (Kerbendt et al., 1991; Konz et al., 1989). Reporter genes with no promoter or a minimal promoter have the potential of facilitating the identification of insertions in and near genes, but which do not disrupt gene function. In designing the present transposons, we included both a drug-resistance marker with a plant promoter, as well as the bacterial GUS gene. The various *Ds* elements we used differed in the amount of 5′ terminal Ac sequence that precedes the promoterless GUS gene, retaining either only the first and weakest transcription start site or all of them (with or without some additional downstream sequence). We also made variants of each that included the ‘core’ sequence of the CaMV 35S promoter. To date, our tests suggest that the GUS gene is capable of being expressed through the 5′ end of all of the different *Ds* elements and that all are capable of transposing. The co-transformants analyzed in greatest detail here contained a *Ds* element with a 35S core promoter. The GUS gene is capable of being expressed from the adjacent 35S promoter either at the original insertion site just upstream of the GUS gene’s translational start site or after transposition and reinsertion within the gene sequence at some distance from its 5′ end (Figure 5), thus demonstrating the feasibility of using the transposon to detect developmentally regulated promoters. We have already obtained lines in which the GUS gene is differentially expressed during development and are currently engaged in analyzing them. We believe that transposon-borne reporter genes have several advantages over those carried on a T-DNA. These include the ability to avoid either seed or tissue-culture transformation, both of which may be mutagenic, in producing insertions. In addition, transposon insertions are reversible and provide the ability to verify the association of a given mutation with the insertion by selecting revertants after reinsertion of a transposase gene by a genetic cross. Finally, there is evidence that many Ac transpositions are to nearby sites in maize, tobacco and tomato, which offers the possibility of efficient mutagenesis of nearby sequences (Dooner et al., 1991; Moreno et al., 1992; Osborne et al., 1991).

An unexpected property of plants containing both a 35S-Ac and a *Ds*-GUS T-DNA is the presence of many small GUS + sectors in all parts of the seedling, particularly in the cotyledons (Figure 3a). In the co-transformed lines analyzed here (399-8 and 400-46), T-DNA homozygotes exhibit a markedly higher frequency of GUS + sectors than T-DNA heterozygotes, indicating that the sector frequency is related to the T-DNA dosage. Although we have not separated the effect of Ac dosage from that of *Ds*-GUS dosage, we suspect that the effect is attributable to the increased dosage of the transposase gene. A positive correlation between Ac dosage and somatic excision frequency has been reported in tobacco, and increased somatic excision frequencies have been observed in *Arabidopsis* when the Ac transposase gene is expressed from stronger promoters than its own (Hehl and Baker, 1990; Jones et al., 1989; Swinbourne et al., 1992). Plants that either do not give GUS + positive sectors or show an altered pattern of sectors have all, to date, proved to have either a transposed element or some other rearrangement.

Especially useful is the observation that plants with transposed elements and a 35S-Ac element exhibit far fewer somatic sectors than the parent plants. This has been observed in all T-DNA plants analyzed so far, except those in which the GUS gene has been activated following transposition and its expression masks somatic sectoring wherever the GUS gene is expressed at a high level (Fedoroff, unpublished data). *Ac* elements undergo short-range transpositions, even within genes and are known to transpose from one sister chromatid to the other in maize (Athma et al., 1992; Chen et al., 1992; Moreno et al., 1992). It is possible that the high frequency of GUS + sectors in seedlings containing the original *Ds*-GUS T-DNA is attributable to the frequent somatic insertion of the element into a site near one of the strong promoters within the T-DNA. In particular, there are five strong promoters in the immediate vicinity of the transposon in the co-transformed lines containing the two T-DNAs at a single insertion site and two such promoters in plants having unlinked transposon and transposase T-DNAs. Elements that have transposed away from the original T-DNA lack these nearby promoters and the frequency with which short-range transpositions of the *Ds*-GUS element from a new site would strongly activate the GUS gene is not only likely to be lower, but will probably differ from site to site, depending on the proximity of other strong promoters. Our interpretation of the somatic GUS sectoring pattern is supported by the identification of a germinal event which resulted in the strong expression of the GUS gene throughout the plant following its excision and reinsertion, in inverted orientation, within the ALS gene just downstream of its original insertion site. Thus we believe that the high frequency of GUS + sectors in plants containing a transposase source and the *Ds*-GUS transposon in its original position within the T-DNA is probably attributable to the predominance of short-range transpositions in somatic tissue. We have not yet analyzed enough different germinal transposition events to know
the relative frequency of short-range transpositions, but we have detected only one germinal event of the type described in Figure 5 and most of the germinal transpositions for which we have segregation data are to unlinked sites (Fedoroff, unpublished data). It should be noted, however, that transpositions into the ALS gene would not be detected in the chlorsulfuron screen because they disrupt ALS gene expression.

Sectorial GUS expression provides a reliable marker to identify plants with the desired genetic constitutions. The sectoring frequency is sufficiently different between T-DNA homozygotes and heterozygotes so that these can generally be distinguished simply by removing and staining a cotyledon. The difference in sectoring frequency between plants that have the \textit{Ds\textendash}GUS transposon in its original position and plants in which it has transposed to a new site is quite marked and has proved useful in identifying plants with transposed elements that arise in T-DNA homozygotes. As noted for T-DNA heterozygotes, most \textit{TrE}\textendash}containing progeny of a self-pollinated parent plant arise from the fusion of male and female gametes produced by tissue of the same genetic constitution. This means that they receive either two EDS chromosomes or one EDS chromosome and one chromosome containing an un-rearranged \textit{Ds\textendash}GUS-T-DNA. Both EDS homozygotes with a transposed element and EDS/original \textit{Ds\textendash}GUS T-DNA heterozygotes (with or without a \textit{TrE}) are C'H'. However, the former have few or no somatic GUS\textsuperscript{*} sectors, while the latter have many (Fedoroff, unpublished data).

We have characterized two cases in which only an EDS is present in a substantial fraction of progeny plants. Molecular analysis shows that these plants are devoid of \textit{Ds\textendash}GUS DNA sequences, implying that they did not arise by a transposition event that inactivated the transposition marker genes. The frequency of C'H\textsuperscript{*} seedlings was fairly high in both progenies (6% and 2%), but not high enough to suggest that the excision event occurred in tissue culture prior to regeneration of either plant. Thus in both cases, the progeny with an EDS appear to have arisen from somatic excision events occurring fairly early in plant development. This inference was supported by the observation that the eight EDS plants analyzed were either homozygous for the EDS or heterozygous for the EDS and the homologous chromosome lacking the T-DNA, the expected outcome if both male and female gametes were derived from tissue in which the excision event had occurred (Fedoroff, unpublished data). Enough progeny plants were tested for the H\textsuperscript{*} marker on the \textit{Ds\textendash}GUS to have confidence that its loss was not simply attributable to meiotic segregation. Such EDS sectors could have arisen either by excision of the element without reinsertion or by its excision from one sister chromatid and its reinsertion into the other near the original insertion site, a type of transposition event that appears to be common in maize (Chen \textit{et al.}, 1992; Greenblatt, 1984).

Plants that contain an EDS, but no reinserted element, are easily identified among the progeny of both T-DNA heterozygotes and T-DNA homozygotes of the type analyzed here. As noted above, EDS progeny arising in a T-DNA heterozygote exhibit no hygromycin-resistance. EDS progeny of a T-DNA homozygote can be identified by selecting first for chlorsulfuron resistance and then determining the fraction of C' seedlings that are H\textsuperscript{*} and show an altered pattern of GUS staining. One-third of the C' EDS plants should be homozygous and therefore H\textsuperscript{*}, while 2/3 should be heterozygous for the EDS and the original T-DNA and therefore H'. All C'H' progeny should show GUS\textsuperscript{*} sectors and, upon progeny testing, be C'C' heterozygotes. These predictions have been tested and verified for the C' progeny of \textit{R}, plant 28 derived from regenerant 399-8-4 (Table 4). It has been reported that only about half of the germinal excision events promoted by \textit{Ac} in tobacco and \textit{Arabidopsis} give rise to lines containing an EDS, but no \textit{TrE} (Altman \textit{et al.}, 1992; Jones \textit{et al.}, 1990). Among the 11 \textit{R} plants that yielded germinal excision events analyzed here, 10 had a transposed element (one of which was not detected by C', but by virtue of uniform GUS staining) and two had an EDS sector. Moreover, preliminary genetic analyses indicate that most of the transposed elements listed in Table 4 segregate independently of the EDS markers, implying that the \textit{Ds\textendash}GUS transposed to a different chromosome (Fedoroff, unpublished data).

In summary, the \textit{Arabidopsis Ac} transposon-tagging system described here incorporates several novel and labor-saving modifications. This includes the ability to screen large numbers of progeny in a small volume, identify germinal transposition events reliably, select for progeny containing the transposed element, but not the transposase gene, and detect insertions downstream of promoters by virtue of the activation of a transposon-borne reporter GUS gene. We have demonstrated the reliability of the marker genes in identifying progeny of the desired genetic constitution. To date, we have not encountered difficulties attributable to the phenomenon of 'co-suppression', in which the expression of a transgenic marker, either one that is homologous to an endogenous gene or one that is present in more than one copy, is lost (Jorgensen, 1990, 1991). We have not seen discrepancies between the molecular and the genetic phenotypes, as determined by the ability to grow on selective media, except for the gradual disappearance of the NAM-sensitive phenotype in late regenerants after protracted maintenance in tissue culture of one line containing multiple copies of a 35S-Ac T-DNA. This has permitted us to rely on the genetic screens, which are efficient and rapid, to create lines with different transposition events.

We have observed considerable variation from plant to
Experimental procedures

Construction of the Ds-GUS transposons and 35S-Ac transposase genes

The Ds512 element was derived from an Ac element (Fedoroff et al., 1983; Pohlman et al., 1984) by replacing the sequence between the DraI and XhoI sites with an XhoI linker. The Ds325 and Ds378 elements were obtained by partial Bal31 digestion of the XhoI-cut Ds512 element, followed by repair synthesis with the Klenow fragment of DNA polymerase I (Boehringer Mannheim) and ligation to an XhoI linker. The 35S promoter of the GUS gene in the pBI221 plasmid (Clontech) was replaced by a fragment containing the sequence extending from nucleotide -46 to +6 of the CaMV 35S promoter (Odell et al., 1985). The GUS gene of this plasmid, with or without the minimal 35S promoter, was transferred to the XhoI site of each of the three internally deleted Ds elements by a blunt-end cloning. A 19s-apb4 gene (Kaster et al., 1983) extracted from the pMONT54 plasmid (kindly provided Dr. S. Rogers of Monsanto Co.) was cloned into the StyI site nearest the Ac element's 3' end. The entire Ds-GUS transposon was cloned into the XhoI site 64 bp upstream of the ALS coding region from pH5S2 (kindly provided by J. Mauvais of DuPont Co.). The chlorsulfuron-resistant ALS gene (Pohlman et al., 1984) and its double 3SS promoter region were subcloned from pH5S2 into Bluescript (Stratagene) in such a way that each end was flanked by an Asp718 site. Derivatives of the 35S-ALS gene containing the various Ds-GUS transposons in both orientations were transferred to the Asp718 site of pCGN1549 (kindly provided by K. McBride of Calgene), an E.coli-AgrOshuttle vector that carries an E. coli NPT-II gene expressed from a mannopine synthase promoter within the T-DNA borders (McBride and Summerfelt, 1990). The excision assay plasmid was derived from the pMONT530 plasmid (kindly provided by S. Rogers of Monsanto Co.) by cloning the GUS gene from pBI221 downstream of the 35S promoter and introducing a Ds element, derived by deleting the internal HindIII fragment of Ac, just upstream of the GUS gene, disrupting its ability to be expressed.

The Bam35S-Ac was obtained by replacing the 5' terminal BamHI fragment of the Ac element (Pohlman et al., 1984) with a CaMV 35S promoter fragment (Odell et al., 1985). The Nael35S-Ac element in plS71101, which was the kind gift of S. Scofield, contains 208 bp of the CaMV 35S promoter and the 82 bp ORF sequence from the 5' leader of the tobacco mosaic virus major transcript inserted at the NaeI site 22 bp upstream of the first ATG of the Ac's ORF (Kunze et al., 1987). Fragments containing either of the two 35S-Ac elements were cloned into the Asp718 site of two different shuttle vectors, pCGN1548 and pCGN1578, which differ from each other in that the NPT-II gene of the former is expressed from a mannopine synthase promoter, while that of the latter is expressed from a 35S promoter (McBride and Summerfelt, 1990). Prior to the introduction of the 35S-Ac, both shuttle vectors were modified by the addition of the A. tumefaciens tms2 gene, which encodes an indoleaceticamide hydrolase. A DsGUS fragment containing the entire tms2 coding sequence (Solisky and Thomashow, 1984) was transferred to pMT40 (Thomashow et al., 1984), cloned downstream of a 35S promoter, and transferred as a DsGUS-Asp718 fragment from an intermediate Bluescript vector into both pCGN1548 and pCGN1578.

Introduction of Ds-GUS and 35S-Ac into A. thaliana

pCGN vectors carrying the 35S-Ac transposase and Ds-GUS transposon constructs were transferred to A. tumefaciens strain LBA4404 by tri-parental mating (Hodelka et al., 1983). A. thaliana ecotype Nio-O roots were transformed as described by Valvekens et al. (1988), with minor modifications. Control roots were transformed with a pMON530 derivative containing a GUS coding sequence expressed from a 35S promoter. To assess transposase function, roots were co-transformed with the excision assay plasmid described above and each 35S-Ac-containing plasmid. Each 35S-Ac transposase and each Ds-GUS transposon plasmid was transformed individually into roots. In addition, some roots were co-transformed with both types of plasmid and selected for kanamycin resistance. Co-transformed calli were identified by histochemical detection of sectorial GUS gene activity. Regenerating shoots commonly gave rise to clumps of shoots from which multiple plants representing the same transformation event were regenerated. Rooted plants were transferred to soil and allowed to set seed.

Detection of markers in progeny seedlings

To assess expression of the various markers introduced on the 35S-Ac and Ds-GUS T-DNAs, R1 seeds produced by the regenerated plants (R0) were surface sterilized in 0.5% Na hypochlorite containing one drop of Tween 20 per 50 ml and germinated on Murashige and Skoog medium (Gibco) containing 1% sucrose and one or more of the following: kanamycin (Sigma), 50 μg ml⁻¹; hygromycin B (Calbiochem), 20 μg ml⁻¹; chlorsulfuron (DuPort Co.; kindly provided by J. Mauvais; commercially available from Chem Services, West Chester, PA), 100 p.p.b. or 10–30 p.p.m.; naphthalene acetamide (Sigma), 10 μM. For selecting for GUS activity, seeds were suspended in 0.7–0.8% agar-containing medium with chlorsulfuron and plated directly in small or large Petri dishes. Screening could be done at seed densities up to 600–700 per ml medium. However, such high densities required the higher chlorsulfuron concentrations (20–30
We thank Pat Gary of Arabidopsis. This work was supported by D. Meinke of Oklahoma State University for advice on growth as well as a fragment of the Ac element to determine the copy number of plant DNA.

Analysis of plant DNA

DNA was extracted from Arabidopsis leaves as described by Dellaporta et al. (1983). Restriction endonuclease digestion was done under conditions specified by the supplier of the enzyme, generally Boehringer-Mannheim or New England Biolabs, except that spermidine HCl was added to a final concentration of 5 mM. DNA digests (300 ng) were fractionated on a 0.7% agarose gel, transferred to Genescreen (DuPont) and crosslinked by UV irradiation (Stratalinker). The DNA fragments used for probes were a 2.4 kb XhoI fragment containing the NPT-II gene from pCGN1547 (McBride et al., 1987) and a 1.1 kb XhoI-Asp 718 fragment containing only the ALS gene from the same plasmid, a 1.9 kb XhoI-HindIII fragment containing a 19s allele from the random primer labeling kit (Use). DNA from plants identified by Southern blot analysis containing the 35s promoter and ALS gene from pMH52 and the XhoI-Asp 718 fragment containing only the ALS gene from the same plasmid, a 1.9 kb XhoI-HindIII fragment containing a 19s allele from the random primer labeling kit (Use). DNA from plants identified by segregation analysis to contain a single 35s-Ac insertion site was digested with SpII and probed with the NPT-II gene sequence, as well as a fragment of the Ac element to determine the copy number of the right and left T-DNA borders. DNA from plants identified genetically to contain a single Ds-GUS insertion site was digested with SpII and probed with an NPT-II (left border) probe, followed by a 3SS-ALS probe (right border). Southern blot hybridization was carried out as described by Church and Gilbert (1984).

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References


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Note added in proof

Since submission of this manuscript, there have been two additional publications on Ac-based transposon-tagging describing the transposition of marked Ds elements from either the NPT-II or SPT genes activated by immobilized, promoter-substituted Ac elements (Greweling et al. 1992) Proc. Natl Acad. Sci. USA, 89, 6085–6089 and Bancroft et al. (1992) Mol. Gen. Genet. 233, 449–461.