

Behaviour of the maize transposable element *Ac* in *Arabidopsis thaliana*

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Summary

The somatic and germinal activity of the maize transposable element, *Ac*, has been analysed in progeny of 43 transformants of *A. thaliana* using a streptomycin resistance assay to monitor *Ac* excision. The ability to assay somatic activity enabled, for the first time, a detailed analysis of *Ac* activity in individual *A. thaliana* seedlings to be made. The effects of T-DNA copy number, generation, dosage at each locus, flanking sequences and orientation of the element were compared. The most striking observation was the variability in *Ac* activity in genotypically identical individuals and the poor penetrance of the variegated phenotype. In general, increasing *Ac* dosage increased both somatic and germinal excision frequencies. The majority of families from individuals selected as inheriting an excision event carried transposed *Ac* elements re-integrated in different positions in the genome.

Introduction

Transposon tagging is a powerful technique for isolating genes which encode unknown gene products, allowing genes, identified only by their mutant phenotype, to be cloned. In plants, a number of genes have been cloned from *Zea mays* (maize) and *Antirrhinum majus* (snapdragon) using endogenous transposable elements as molecular tags (e.g. Fedoroff *et al.*, 1984; Martin *et al.*, 1985).

For a species such as *Arabidopsis thaliana*, for which, until very recently (Peleman *et al.*, 1991) there was no active characterized transposon system, a potential approach is to introduce a known heterologous transposable element into its genome. This strategy has already been undertaken in several heterologous plant systems including tobacco, tomato, potato and *A. thaliana*

(Baker *et al.*, 1986; Jones *et al.*, 1989; Knapp *et al.*, 1988; Masson and Fedoroff, 1989; Schmidt and Willmitzer, 1989; Van Sluys *et al.*, 1987; Yoder *et al.*, 1988). The use of a heterologous element, present at single or low copy number, has the potential to facilitate the subsequent genetic analysis of the element in the host plant and the analysis of transposon-induced mutations. Several studies have employed the use of excision markers based on resistance to an antibiotic to select or monitor transposon excision. The transposon is cloned into the 5' untranslated leader of the fusion rendering it inactive until the element excises. Resistance to kanamycin was first used by Baker *et al.* (1987) to monitor *Ac* excision in tobacco. Schmidt and Willmitzer (1989) also used this assay in *A. thaliana* and demonstrated that the germinal excision frequency of *Ac* was considerably lower in *A. thaliana* than in tobacco or tomato. Studies using the frequency of germinal excision events as the basis for comparison suffer from the fact that timing of the excision event greatly influences the result. One early excision event can lead to a higher number of the progeny inheriting that excision product than many independent, late excision events. The ability to assay somatic activity allows the effects of modifications and copy number to be compared more accurately and also allows the activity in individuals to be monitored. Development of a chimeric streptomycin phosphotransferase (SPT) fusion (Jones *et al.*, 1989) allowed the monitoring of both somatic and germinal excision of *Ac* in tobacco. On streptomycin, sensitive cells bleach but do not die provided they are supplied with a carbon source (Maliga *et al.*, 1975). Streptomycin resistance appears to be cell-autonomous in cotyledons, thus, when seeds from the primary transformants are germinated on streptomycin-containing media, somatic excision of the transposon can be visualized as green sectors on bleached cotyledons. If the transposon excises in a cell that gives rise to gametes (germinal excision), then a progeny plant will inherit an active SPT fusion (that will be present in all cells) and will be fully streptomycin resistant.

Here, the streptomycin resistance assay has been used to carry out a detailed analysis of the somatic activity of *Ac* in *A. thaliana*. Somatic activity cannot easily be scored using an NPT::*Ac* construct as resistance to kanamycin is not a cell-autonomous phenotype in cotyledons. The ability to assay somatic activity as variegation on streptomycin-containing media has enabled us to test a number of parameters important in the establishment of an efficient transposon tagging system in *A. thaliana*. The

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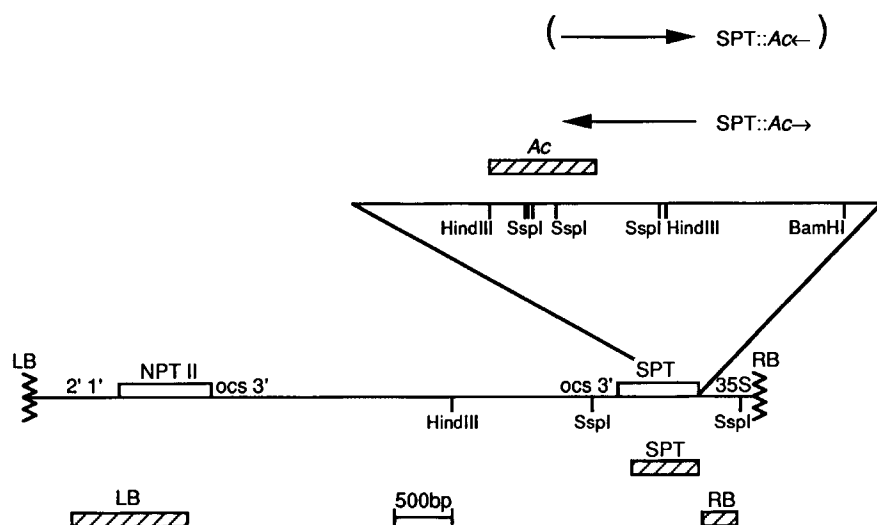


Figure 1. Schematic representation of the constructs introduced into *A.thaliana*. One orientation of the *Ac* element in the binary vector is shown.

NPT-II neomycin phosphotransferase II (Mazodier *et al.*, 1985); SPT, streptomycin phosphotransferase (Mazodier *et al.*, 1985); ocs, octopine synthase (De-Greve *et al.*, 1982). The fragments used as probes in the Southern blot analysis are indicated as hatched boxes. LB, left border probe; RB, right border probe; SPT, empty donor site probe; Ac, Ac probe.

effects of T-DNA copy number and dosage of *Ac* at each locus and the effect of various modifications of the *Ac* element on *Ac* activity are described here. We have also examined *Ac* activity in individuals within the same family and how this activity is inherited through several generations. Two other important parameters in a transposon tagging strategy, namely the frequency and independence of *Ac* re-insertions into the genome have also been analysed.

Results

Use of the streptomycin resistance assay to monitor Ac activity in A. thaliana

In order to establish that streptomycin would bleach seedlings of *A. thaliana* effectively, non-transformed seedlings of the Landsberg *erecta* ecotype were germinated on GM media with 1% glucose (Valvekens *et al.*, 1988) containing a range of concentrations of streptomycin sulphate. A concentration of 200 mg l⁻¹ was required for complete and consistent bleaching of the cotyledons. At this concentration of streptomycin, however, seedlings of *A. thaliana* carrying an SPT fusion driven by the T-DNA promoter 2' (pJJ2668; Jones *et al.*, 1989) showed poor resistance phenotypes (Dean *et al.*, 1991). In order to improve the resistant phenotype, constructs carrying a 35S-SPT fusion were made. This fusion was cloned into a binary vector carrying left border (LB) and right border (RB) T-DNA sequences as in van den Elzen *et al.* (1985) and a 1' NPT-II marker to be used for selecting transformed cells of *A. thaliana*. An *Ac* element was then cloned into the 5' untranslated leader of the 35S-SPT fusion in both orientations (and hence designated SPT::→*Ac*, SPT::←*Ac*). A plasmid carrying an *Ac* element with an introduced *Bgl*II

restriction site downstream of the transposase polyadenylation site (SPT::←*Ac*(Bg)) was also constructed (Jones *et al.*, 1990a). The addition of a unique site into the element was for future experiments in which *Ac* was to be marked by addition of an antibiotic resistance marker. A representative construct is illustrated in Figure 1.

The constructs were transformed into *A. thaliana* ecotype Landsberg *erecta* using *Agrobacterium* transformation. The primary transformants (T₁) were allowed to self-pollinate and progeny from 43 independent transformants (T₂) were germinated independently on either streptomycin- or kanamycin-containing medium. The phenotype of the seedlings plated on streptomycin-containing media ranged from highly variegated (HV ≥ 10 sectors per seedling) through a range of mid (MV = 2–10 sectors per seedling) to lightly variegated (LV = 1 sector per seedling), to completely bleached. A selection of the observed phenotypes are shown in Figure 2. The number of variegated and bleached seedlings for each T₂ family (progeny of the primary, T₁, transformant) are shown in Table 1. There was considerable between-transformant variability in *Ac* somatic excision frequencies (as monitored by variegation). Over half the transformants (27/43) showed no variegated seedlings in the progeny analysed. Variegated seedlings, when present, occurred at a very low frequency, and in only one case (SPT::→*Ac*-20) were there more variegated than bleached seedlings. The seedlings from this transformant displayed an array of variegated phenotypes, with both large and small green sectors. These will be derived from early and late *Ac* excision events, respectively, giving rise to green sectors containing different numbers of cells, depending on when the excision event took place. There does not appear to be any relationship between sector size and sector frequency. The boundaries of the green and white tissue

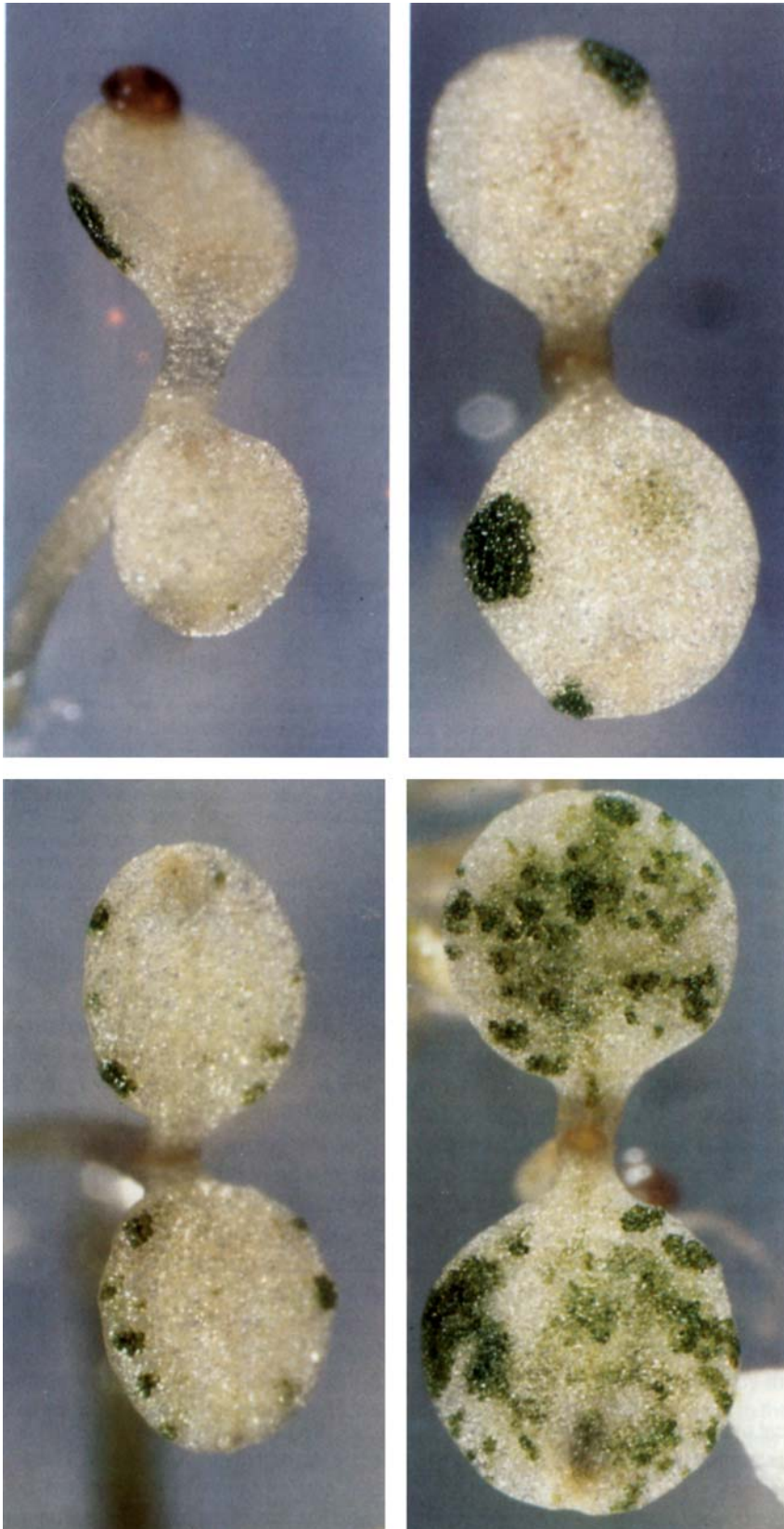


Figure 2. Representative examples of the various variegation phenotypes. The top row shows examples of LV and MV phenotypes and the bottom row shows two HV phenotypes. The variegation on streptomycin-containing media results from somatic Ac excision from the SPT::Ac locus of *A. thaliana* seedlings. Magnification $\times 14$.

Table 1. Analysis of kanamycin and streptomycin resistance and T-DNA border fragments in T₂ progeny of *A. thaliana* transformants carrying SPT::Ac constructs

| Transformant | Resistant:sensitive (on kanamycin) | | Variegated:white (no. on streptomycin) | Border fragments | |
|----------------|------------------------------------|---------|---|------------------|----|
| | Number | Ratio | | LB | RB |
| SPT::←Ac-1 | 53:4 | 13.2:1* | 0:45 | 3 | 1 |
| -2 | 137:22 | 6.2:1* | 0:70 | 1 | 1 |
| -4 | 167:43 | 3.9:1 | 0:141 | 1 | 0 |
| -5 | 72:11 | 6.5:1* | 0:84 | 1 | 1 |
| -8 | 150:2 | 75:1* | 1:122 | | |
| -11 | 26:11 | 2.4:1 | 0:50 | | |
| -12 | 135:30 | 4.5:1* | 0:150 | | |
| -13 | 136:40 | 3.4:1 | 0:150 | | |
| -15 | nd | | 1:25 | | |
| -17 | 69:8 | 8.6:1* | 12:79 | nd | 3 |
| -19 | 195:57 | 3.4:1 | 0:200 | | |
| -20 | 77:32 | 2.4:1 | 11:110 | | |
| -22 | 107:37 | 2.9:1 | nd | | |
| -23 | 33:27 | 1.2:1* | 0:30 | | |
| -24 | 86:27 | 3.2:1 | 0:105 | | |
| -25 | 38:19 | 2:1 | 3:95 | | |
| -26 | 28:0 | | 1:0 | 0 | 0 |
| -28 | 23:0 | | 0:32 | | |
| -29 | 26:25 | 1:1* | 0:29 | | |
| -30 | 16:1 | 16:1 | 0:30 | | |
| -34 | 51:14 | 3.4:1 | 0:123 | 1 | 0 |
| -35 | 208:62 | 3.35:1 | 0:200 | | |
| -37 | 72:6 | 12:1* | 84:110 | 1 | 0 |
| SPT::→Ac-1 | 75:15 | 5:1 | 0:97 | 1 | 1 |
| -3 | 44:18 | 2.4:1 | 0:29 | | |
| -5 | 71:26 | 2.7:1 | 0:132 | | |
| -6 | 101:25 | 4:1 | 0:31 | | |
| -9 | 54:11 | 4.9:1 | 0:109 | | |
| -10 | 62:18 | 3.4:1 | 8:184 | 1 | 1 |
| -11 | 52:21 | 2.5:1 | 0:119 | | |
| -15 | 95:37 | 2.6:1 | 10:90 | nd | 1 |
| -18 | 87:21 | 4.1:1 | 2:194 | | |
| -20 | 75:1 | 75:1* | 90:44 | 1 | 2 |
| -22 | 46:23 | 2:1 | 0:82 | | |
| -25 | 61:5 | 12.2:1* | 32:48 | 2 | 3 |
| SPT::←Ac(Bg)-1 | 67:20 | 3.4:1 | 0:116 | | |
| -3 | 40:8 | 5:1 | 0:24 | | |
| -5 | 13:2 | 6.5:1 | nd | | |
| -6 | 13:6 | 2.2:1 | 0:15 | | |
| -7 | 62:13 | 4.8:1 | 16:48 | 1 | 2 |
| -8 | 42:6 | 7:1* | 2:80 | | |
| -10 | 47:0 | | 0:55 | | |
| -11 | 47:15 | 3.1:1 | 0:75 | | |

*Significantly different from a 3:1 ratio.
nd, not determined.

were always very sharp and microscopical examination of the sectors showed that streptomycin resistance is a fully cell-autonomous phenotype in the cotyledons.

No obvious differences were observed between families carrying the wild-type *Ac* element on the *Ac*(Bg) element. Neither the orientation of the element with respect to the

35S–SPT fusion, nor the introduction of the *Bgl*II site, appeared to influence *Ac* activity in *A. thaliana*. This is in contrast to the *Cl*aI site mutation in *Ac*, created by a Klenow treatment and ligation of the introduced *Bgl*II site, which reduced *Ac* excision frequency in tobacco 10-fold (Jones *et al.*, 1990a).

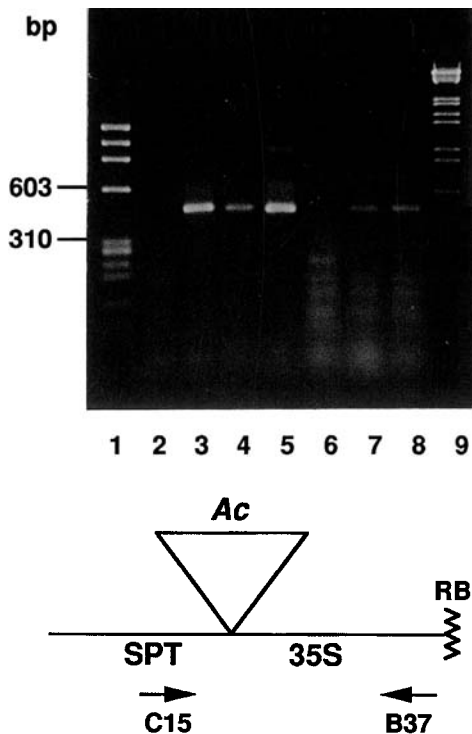


Figure 3. PCR analysis to detect somatic excision of *Ac*.

The oligonucleotides C15 and B37 amplify a 542 bp fragment once the *Ac* element has excised. Lanes 1 and 9 contain ϕ x174 *Hae*III and λ *Hind*III-*Eco*RI markers, respectively; Lanes 2–8 contain PCR amplification products from: 2, non-transformed Landsberg *erecta* DNA; 3, SPT::→*Ac*-1; 4, SPT::→*Ac*-15; 5, SPT::→*Ac*-20; 6, SPT::←*Ac*-2; 7, SPT::←*Ac*-4; 8, SPT::←*Ac*-5. The fraction of the amplified products loaded in each lane was not the same.

Also shown is a diagram of the position of the oligonucleotides C15 and B37.

As confirmation that the variegation on streptomycin was representing somatic excision of the *Ac* element, a *Ds* element was constructed, in which a frameshift mutation was introduced into the *Ac* open reading frame (by Klenow treatment of the unique *Eco*RI site within *Ac*). Progeny from six transformants carrying this *Ds* element in the SPT fusion showed no variegation on streptomycin-containing medium. As an additional confirmation that somatic *Ac* excision was occurring in the *Ac*-containing transformants, PCR experiments were performed on a sample of the T_2 families. Figure 3 shows this analysis on six families, three with each orientation of the *Ac* element, representing a range of variegated phenotypes. The PCR fragment from the 'empty donor' site is 542 bp. This is 84 bp larger than the region from the 35S–SPT fusion and is due to the 84 bp of *waxy* and linker sequence remaining in the 5' untranslated leader of the SPT fusion after *Ac* excision. A fragment of the correct size is present in all the samples except for lane 6. The PCR analysis was carried out on DNA isolated from relatively mature plants and the excision leading to the variegation in the cotyledons arises

in the developing seed. However, the samples in Figure 3 were not prepared in such a way to allow comparison to be made between the abundance of the 'empty donor' fragment and the variegation seen in the cotyledons.

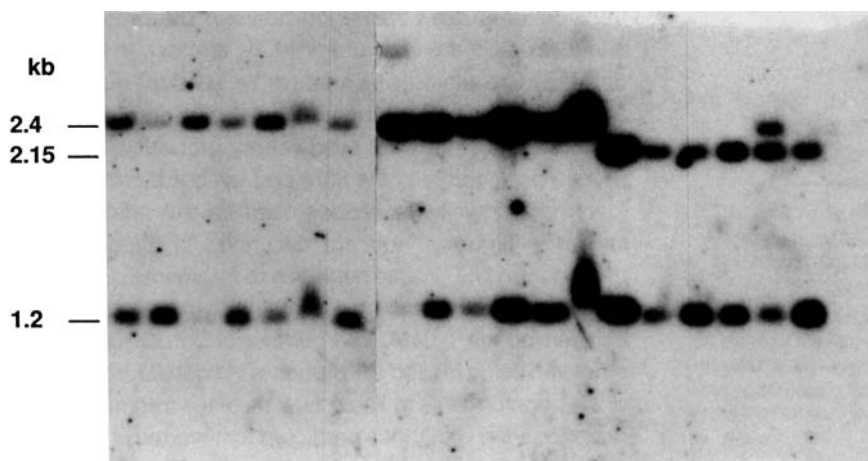
Relationship between Ac activity and T-DNA copy number

The score of kanamycin-resistant and sensitive individuals in the T_2 progeny is shown in Table 1. This gives an indication of the number of unlinked T-DNA loci in each transformant. In 60% of the transformants, the ratio of kanamycin-resistant to sensitive individuals is not significantly different from 3:1, indicating that the T-DNA segregates as a single locus. The majority of the other transformants show ratios indicative of the presence of either two linked or two unlinked T-DNA loci. In order to obtain a more accurate estimate of T-DNA copy number at each locus, DNA was prepared from a selection of the T_2 families, digested with *Hind*III and probed with DNA probes designed to show right and left border fragments (data not shown). The probes used for this analysis are shown in Figure 1 and are described in Experimental procedures. The probes hybridize to fragments of varying size depending on the next *Hind*III site in the plant DNA adjacent to the T-DNA. The number of border fragments observed using LB and RB probes is indicated for a subset of the transformants in Table 1. None of the fragment sizes corresponded with those predicted for inverted or tandem repeat integrations at one locus. However, the presence of repeat structures cannot be ruled out as incomplete T-DNA transfer would lead to fragments of unexpected sizes. In many of the individuals with a differing number of LB and RB fragments it is an RB fragment which is missing, e.g. SPT::→*Ac*-1 which has 3LB, 1RB and a kanamycin segregation ratio suggesting two unlinked loci. The absence of some RB hybridizing sequences can be explained in a number of ways. First, since the RB probe is relatively short (400 bp) and very close to the 25 bp RB repeat, this would frequently be lost if incomplete T-DNA transfer had occurred. From preliminary PCR and sequence analysis, we observed a lot of variability in which T-DNA sequences are found adjacent to the plant DNA. Lack of an RB fragment might also explain some of the apparent inactivity of *Ac*, since if the 35S promoter was not integrated the streptomycin assay would be ineffective. Secondly, lack of a border fragment could also be explained by the lack of a *Hind*III site in the plant DNA close enough to the T-DNA to yield a fragment which could be resolved in the gel systems.

The number of T-DNA loci and the T-DNA copy number estimate, where available, was then compared with the number of seedlings showing variegation. There is not a strict correlation between T-DNA copy number and *Ac* activity, as judged by variegation on streptomycin-

Table 2. Germinal excision frequencies in the T₃ generation

| Transformant | Streptomycin | | Percentage germinal excision |
|----------------|--------------|-----------|------------------------------|
| | Resistant | Sensitive | |
| SPT::→Ac-6 | 1 | 1356 | 0.07 |
| SPT::←Ac(Bg)-1 | 3 | 1484 | 0.2 |
| -7 | 53 | 3202 | 1.6 |
| SPT::←Ac-12 | 1 | 1280 | 0.08 |
| -17 | 92 | 1526 | 5.7 |
| -19 | 2 | 844 | 0.12 |
| -22 | 11 | 6350 | 0.17 |

**Figure 4.** Southern blot analysis to detect empty donor sites.

A Southern blot of plant DNA digested with *Ssp*I was probed with the SPT probe (shown in Figure 1). The 1.2 kb *Ssp*I restriction fragment results from excision of *Ac*. The 2.4 and 2.15 kb fragments represent the non-excised alleles. These bands hybridize more strongly in samples from transformants carrying multiple T-DNAs.

containing media. However, the families showing the highest levels of variegation, for example SPT::→Ac-20 tend to come from transformants containing multiple T-DNA inserts.

Germinal excision frequency of Ac

The low number of T₂ seed obtained from each transformant limited the number plated on streptomycin and kanamycin in the initial screens. Insufficient progeny were obtained to estimate the germinal excision frequency of *Ac* from these transformants. The germinal excision frequency was thus analysed in subsequent generations of eight of the transformants. In order to obtain enough seed to perform the analysis, 40 kanamycin-resistant T₂ seedlings from each transformant, including a mixture of heterozygotes and homozygotes for each T-DNA locus, were rescued from the kanamycin plates, transferred to the greenhouse into soil and allowed to self. The seeds were bulk-harvested from the 40 individuals. This T₃ seed was then sterilized and plated on streptomycin-containing medium. The frequency of fully streptomycin-resistant individuals in the T₃ families is shown in Table 2 and varied

from 0.07 to 5.7%. The actual number of independent germinal excision events cannot be elucidated from the number of streptomycin-resistant individuals, because the timing of the excision event can influence the number of streptomycin-resistant individuals inheriting the same excision event. The significantly higher number of individuals inheriting excision products in the progeny of SPT::←Ac-17 may have arisen from an early excision event encompassing a large sector of the plant. This question is analysed in more detail below.

In order to confirm that seedlings with the fully green streptomycin-resistant (FG) phenotype had indeed inherited excision products, DNA was isolated from the progeny of a selection of fully resistant seedlings. Southern blot analysis was performed using a probe from the SPT coding region (shown in Figure 1) to detect a characteristic 'empty donor' site. The analysis is shown in Figure 4 and illustrates that of the 19 families shown, all had the 1.2 kb *Ssp*I restriction fragment (the empty donor site) resulting from excision of *Ac*. Selfed progeny of the fully streptomycin-resistant individuals, when plated on streptomycin-containing media, gave the expected 3:1 ratio of resistant to sensitive seedlings, as shown in Table

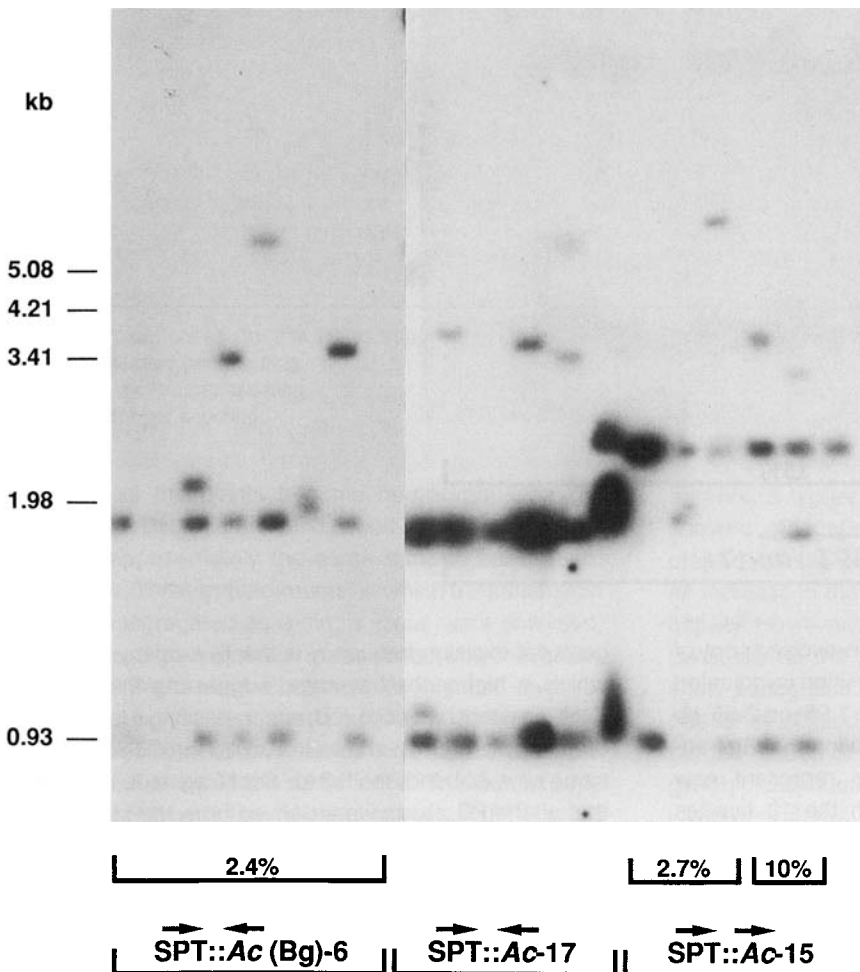
Table 3. Analysis of progeny from fully streptomycin-resistant seedlings

| Transformant | Classification | Streptomycin-resistant | Variegated | Streptomycin-sensitive | Ratio | Kanamycin |
|----------------|----------------|------------------------|------------|------------------------|------------------------|------------------|
| | | | | | r:var + s ^a | r:s ^b |
| SPT::←Ac(Bg)-7 | HV1-FG1 | 104 | 27 | 6 | 3.3:1 | 75:0 |
| | HV2-FG3 | 102 | 21 | 24 | 2.3:1 | 85:0 |
| | LV1-FG1 | 127 | 29 | 22 | 2.5:1 | 100:0 |
| | LV1-FG2 | 126 | 35 | 3 | 3.3:1 | 120:0 |
| | LV1-FG3 | 151 | 41 | 0 | 3.7:0 | 140:0 |

None of the ratios differed significantly from 3:1.

^ar:var + s = resistant:variegated + sensitive.

^br:s = resistant:sensitive.

**Figure 5.** Southern blot analysis of FG families to detect Ac re-insertions into the genome.

A Southern blot of plant DNA digested with *Ssp*I was probed with the Ac probe (Figure 1). Hybridizing fragments other than those at 2.55, 1.95, 0.91 kb represent new Ac insertions into the genome. The transformants from which the FG plants were selected are shown at the base of the figure along with the germinal excision frequency in that generation.

3. This result is important because it suggests that the variability of SPT::Ac variegation is not due to variability in penetrance of the 35S-SPT chimeric gene.

Frequency and independence of Ac re-insertions

The streptomycin resistance assay monitors excision of the element, but does not give any indication of the frequency of re-insertion of the element into the genome,

or if sibling FG plants carry independent transposition events. In order to address these questions, Southern blot analysis was carried out on progeny of 19 FG individuals and is shown in Figure 5. The families analysed are from three transformants, two of which contain a single T-DNA insertion and one of which contains three T-DNAs. Families of sibling FG plants, as well as FG plants generated by different parents and from different generations, are included. Those which are from sibling FG plants are

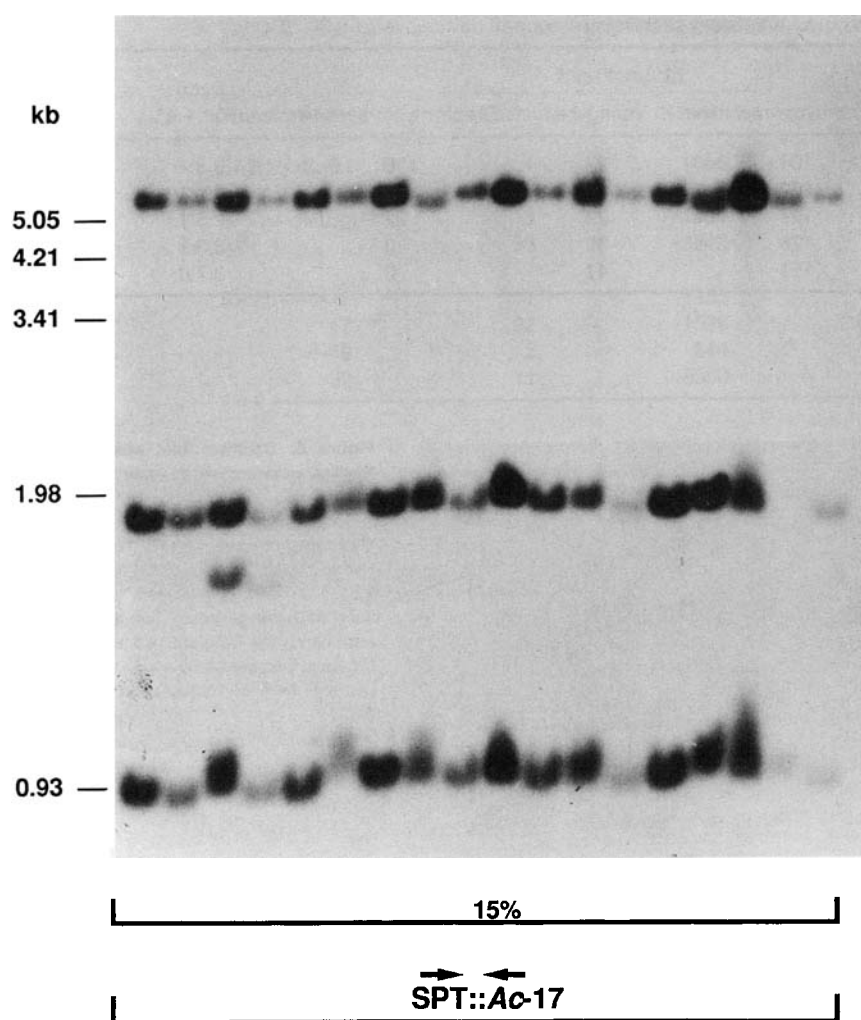


Figure 6. Southern blot analysis of 18 FG families picked from the T_3 progeny of the multiple T-DNA-containing plant SPT::←Ac-17 LV1 (Table 4).

The germinal excision frequency was 15%. The 5.3 kb new Ac band is found in all 18 families.

shown on the figure in parentheses. A non-excised copy of Ac, probed with an Ac probe (whose position is indicated in Figure 1), gives *SspI* fragments of 1.95 or 2.55 kb (depending on orientation) and a 918 bp internal Ac fragment. *SspI* fragments of other sizes represent new integration sites of the Ac element. In the 19 families shown, 11 (58%) contain at least one new *SspI* fragment. Those lacking a new Ac position may have lost the element through lack of re-insertion, through insertion into a sister chromatid followed by mitotic segregation, or through re-insertion at a position unlinked to the T-DNA followed by meiotic segregation. As these were families derived from self-fertilization, an unlinked Ac would be lost through segregation 25% of the time if the sector carrying the excision covered the whole flower and 50% if it occurred after differentiation of the pollen and ova. The different families from sibling FG plants all contain different new Ac positions, showing that they were derived from different transposition events. However, we also analysed the progeny of 18 FG plants picked from the T_3 progeny of the multiple T-DNA-containing plant SPT::←Ac-17 LV1. The

germinal excision frequency in the T_3 progeny was 15%, which is higher than average, suggesting that an early excision event had occurred, encompassing a large sector of the plant. Figure 6 shows that all 18 families carried the same new Ac band, the 5.3 kb *SspI* fragment, confirming that all the FG plants were derived from the same transposition event. The frequency of co-segregation of this new Ac position with the streptomycin resistance in the T-DNA is significantly different ($P < 0.05$) from the expected value for an unlinked Ac element, suggesting that the transposition event was to a linked site. The 1.95 kb *SspI* fragment represents the non-excised allele. In one family this has been lost due to segregation of alleles during self-fertilization of the heterozygous parent. In two families a second new Ac band is also present.

Ac excision through three generations

Somatic and germinal Ac excision was monitored through several generations to investigate, first, whether the excision frequency increased when the dosage of Ac

Table 4. Frequency of somatic and germinal excision of Ac in T₂, T₃ and T₄ progeny from a single locus transformant

| Transformant | Generation | Classification | Percentage seedlings showing somatic activity | Percentage of variegated seedlings | | | Percentage germinal excisions | Kanamycin resistant:sensitive | | |
|----------------|----------------|----------------|---|------------------------------------|-----|------|-------------------------------|-------------------------------|--------|-------|
| | | | | HV | MV | LV | | | | |
| SPT::←Ac(Bg)-7 | T ₂ | | 25 | 50 | | | 0.4 | 62:13 | | |
| | | | 24 | 0 | | | (0/158) | 76:26 | | |
| | T ₃ | K2 | 33 | 11 | | | (0/229) | 59:14 | | |
| | | K3 | 90 | 5 | 50 | 45 | 3.0 | 109:0 | | |
| | | K6 | 32 | 0 | 50 | 50 | 1.0 | 75:0 | | |
| | | HV1 | 66 | 0 | | 100 | 2.4 | 70:0 | | |
| | | HV2 | 38 | 9 | 41 | 50 | 1.9 | 100:0 | | |
| | | HV3 | 17 | 0 | | | 4.2 | 100:0 | | |
| | | HV4 | 36 | 6.1 | | | 1.4 | 200:0 | | |
| | | HV5 | 79 | 15 | | | (0/92) | 76:0 | | |
| | | HV6 | 43 | 1.5 | | | 1.4 | 150:0 | | |
| | | HV7 | 64 | 6 | 33 | 61 | 5.7 | 70:0 | | |
| | | LV1 | 6.8 | 6 | | 94 | 0.2 | 115:47 | | |
| | | T ₄ | HV1 | K2 | 42 | 11.3 | | | 0.8 | 87:0 |
| | | | | K3 | 66 | 2.6 | | | 1.7 | 139:0 |
| | | | HV2 | K4 | 40 | 0 | | | (0/10) | 8:0 |
| | | | | K5 | 40 | 0 | | | 0.9 | 67:0 |
| | LV1 | | K1 | 47 | 0 | | | (0/127) | 111:0 | |
| | | | K2 | 39 | 8.5 | | | (0/122) | 104:0 | |
| | | | HV1 | 51 | 4.2 | | | 2.8 | 105:0 | |

The transformant chosen for this analysis showed the highest level of somatic activity of all the single locus transformants.

HV = >10 sectors per seedling.

MV = 2–10 sectors per seedling.

LV = 1 sector per seedling.

increased as the plants become homozygous for the introduced T-DNA, and second, whether the activity of Ac remained approximately the same through subsequent generations. Three transformants, in which the kanamycin resistance segregated as a single locus were analysed, but as the results were similar in each case, only the results from the transformant showing the highest level of Ac somatic activity are presented (Table 4). This transformant, SPT::←Ac(Bg)-7 carries one intact and one partial T-DNA copy at the same locus. The partial copy contains an RB, half of an Ac element but no NPT-II or LB sequences. Kanamycin-resistant, highly or lightly variegated or white individuals from each generation were selfed and their progeny analysed on streptomycin- or kanamycin-containing medium. As an example of these data, which are shown in Table 4, the kanamycin-resistant individuals K2 and K3 picked from the T₂ generation of SPT::←Ac(Bg)-7 were heterozygous for the T-DNA insert. Somatic excision frequencies in their progeny, 24 and 33% respectively, are very similar to those in the original T₂ family (25%). This would suggest that the Ac activity is maintained through at least one generation. This observation was also true for other transformants carrying single copy T-DNA integrations (data not shown). Other individuals picked, K6, HV1, HV2, HV3, HV4, HV5, HV6, HV7 and LV1 were homozygous for the T-DNA insert. Except

for HV4, a higher number of seedlings in these families showed variegation than in the K2 and K3 families. Increasing dosage of Ac is, thus, generally associated with an increase in the number of seedlings showing somatic activity. However, in no case, in either the T₃ or T₄ generations, did all the progeny from homozygous individuals show variegation. Also, there was a wide range in the frequency of seedlings which did show variegation in the progeny of homozygous sibs (between 17 and 90%). Thus genotypically identical individuals showed very different levels of Ac activity. All the individuals selected for analysis in the T₄ generation were homozygous. The number of seedlings showing variegation in the seven T₄ families analysed ranged from 42 to 66%. Thus Ac activity does not appear to be significantly different in the T₃ and T₄ families analysed.

The frequency of FG individuals in the progeny of the different transformants in the T₃ and T₄ generations varied considerably. The average value was higher for multi-locus transformants (data not shown) than for single locus transformants.

The average number of sectors per seedling was used by Jones *et al.* (1989) to investigate the effect of dosage on Ac activity in transgenic tobacco. It was not possible to do a similar analysis because the number of sectors observed in the progeny of homozygotes varied. For example, in the

T₃ generation the progeny of K6 shows 5% HV, 50% MV and 45% LV, whereas the progeny of its genotypically identical sib HV2 shows 100% LV.

There were also examples (not shown) of progeny of seedlings that had been scored as fully streptomycin sensitive (with no apparent variegation in the cotyledons) inheriting germinal excision events at varying frequencies. Thus, even though there was no *Ac* activity in the developing seed (where all the cell division involved in cotyledon formation takes place) somatic excisions must have occurred at some stage later in development in a cell lineage which eventually formed the gametes. This would then give FG plants in the progeny of individuals scored as fully streptomycin sensitive.

Discussion

In this study we have analysed the transposition of the maize element *Ac* in transgenic *A. thaliana* plants. The use of the streptomycin resistance marker to monitor both somatic and germinal activity of the element has proved invaluable in this analysis. The activity of the element was compared in the progeny of a large number of transformants and also in subsequent generations for a smaller number of transformants. Use of the streptomycin resistance assay allowed the comparison of *Ac* somatic activity as well as germinal excision frequency between the different families. This is essential for comparative purposes, since it is not affected to the same degree by the timing of the excision event. However, it can only give information on *Ac* somatic activity at one point in development, namely, cotyledon formation in the developing seed.

The degree of between-transformant variability was very high in comparison to that found in tobacco (Jones *et al.*, 1989). This was only partly explained by the differing copy number of the introduced DNA. Some of the between-transformant variability might be caused by the factors that cause the variability in genotypically identical individuals. The site of integration of the T-DNA may also significantly influence *Ac* activity in *A. thaliana*. The analysis of T-DNA copy number revealed that, in several transformants, the number of left and right T-DNA border fragments were not always the same. This could have resulted from incomplete T-DNA transfer, especially at the RB, along with the presence of repeat structures at the site of integration. We are continuing this analysis using inverse PCR to clone out sequences which flank the T-DNA inserts. This should reveal whether incomplete T-DNA transfer is more common in *A. thaliana* than in other species where this problem has been or is being investigated (Gheysen *et al.*, 1987; Jones *et al.*, 1987; Jorgensen *et al.*, 1987).

The two orientations of the *Ac* element in the SPT fusion and the element carrying the introduced restriction site

(*Bgl*II) downstream of the transposase polyadenylation site all showed approximately the same levels of somatic activity. Thus there did not appear to be any influence of sequences within the T-DNA, for example, the 35S promoter driving the SPT fusion, on the expression of transposase.

Overall, the somatic activity of *Ac* was much lower than that reported for tobacco. Many seedlings judged to carry a T-DNA insert due to their kanamycin resistance showed no variegation on streptomycin. The non-Mendelian segregation of the activity of the SPT::*Ac* locus has not been observed with other introduced loci – the 1'-NPT-II and the 35S-SPT markers – in *A. thaliana*. The poor penetrance of the variegation phenotype thus seems to be associated with the presence of the *Ac* element.

The huge variability in *Ac* somatic activity in genotypically identical individuals is also in sharp contrast to that observed in tobacco. The variegation phenotype in tobacco was sufficiently predictive that homozygotes could be distinguished from heterozygotes based on the number of sectors per seedling. This enabled Jones *et al.* (1989) to show a positive effect of dosage on *Ac* excision frequency, an observation confirmed by Hehl and Baker (1990). This is in contrast to the situation in maize, where increasing copy number of *Ac* correlates with delay and reduction in frequency of *Ac* transposition (McClintock, 1948, 1951). In *A. thaliana*, the higher number of seedlings showing variegation in homozygous and multi-copy lines would indicate that, as in tobacco, there is a positive effect of *Ac* dosage on activity. The situation though, is far more complex than in tobacco, due to the poor penetrance of the SPT::*Ac* locus. In *A. thaliana*, a continuum of variegated phenotypes were found rather than the expected ratios of distinct classes. For example, in a self of a heterozygous individual, one-quarter of the progeny would be expected to be HV, one-half LV and one-quarter white. The data in Table 4 show that, where we have completed such an analysis, these categories were not observed. Thus the genotyping of individuals based on the variegation phenotype as achieved in tobacco (Jones *et al.*, 1989) is precluded. The basis for the variability in *Ac* activity in *A. thaliana* is unclear. In maize, inactivity of the element is associated with methylation of the CpG-rich region in the 5' untranslated transposase leader (Kunze *et al.*, 1988). Experiments are in progress to establish whether this might also be the case in *A. thaliana*. The relatively low level of activity may be associated with a poor expression of transposase in *A. thaliana*. Although this may be a factor, the presence of a few individuals in genotypically identical populations that show very high levels of variegation (>50 sectors per seedling) demonstrate that the transposase level need not be limiting. Interestingly, selection of the highly variegated individuals versus the lightly or non-variegated individuals did not

result in a heritable increase in *Ac* activity in the subsequent generation (data not shown). The lack of a sharp increase in the frequency of variegation in homozygous versus heterozygous individuals suggests that factors other than transposase expression are contributing to the low level and variability of the activity. Additional host factors required for transposition may be more variable in *A. thaliana* than in the other species where *Ac* activity has been studied. We plan to continue investigating this variability and also the reduced activity of *Ac* in *A. thaliana*.

The number of transposition events transmitted to the next generation is the critical factor in determining the feasibility of using the *Ac* transposon for insertional mutagenesis and gene-tagging experiments. In maize, germinal excision frequencies are usually 1–10% (Brink and Williams, 1973; McClintock, 1956). Furthermore, about 40–70% of maize plants picked as germinal revertants contain a transposed *Ac* (Dooner and Belachew, 1989; Greenblatt, 1984). In heterologous species, the germinal excision frequency in progeny of primary transformants has been estimated to be approximately 30% in tomato (Belzile *et al.*, 1989) and between 10 and 83% in tobacco (Hehl and Baker, 1990; Jones *et al.*, 1990). In *A. thaliana*, the germinal excision frequency of the wild-type *Ac* element was reported by Schmidt and Willmitzer (1989) to be significantly lower at 0.2–0.5%. They found varying frequencies and expressed these as minimal germinal excision frequencies to avoid the problem of an early excision event distorting the figure. We found a range of streptomycin-resistant individuals (0–62%) in the progeny of various transformants through different generations. We analysed the independence of a number of transposition events taken from the same progeny and the frequency of re-insertion of the element into the genome. In the families examined, where FG plants were picked from populations showing between 2.4 and 10% germinal excision, 58% had new *Ac* positions in the genome and these were all independent. In a population showing a relatively high number of streptomycin-resistant individuals (15%), all 18 families of FG plants analysed showed the same new *Ac* position (Figure 6). Thus selection of high germinal excision levels is not always advantageous for a transposon-tagging strategy as it may often result from an early excision event leading to a large number of progeny carrying a transposed *Ac* in the same new genomic location. A very active element may also result in a high level of somatic reversion of any mutation caused by insertion of a transposed *Ac* element which, if its gene product is non-cell-autonomous, may result in the masking of the mutant phenotype. The data presented show that individuals carrying transposed *Ac* elements integrated at new and independent genomic positions can be enriched for by pre-selecting germinal excision events from families showing a relatively low germinal excision

frequency. This is because of the relatively high degree of co-segregation of the excision marker with the new *Ac* element and the independence of the *Ac* re-integrations in families showing low germinal excision frequencies. Collection of a large number of these individuals and subsequent screening of their families for mutations caused by insertion of *Ac* is currently underway.

In the one family analysed in some detail, from the T₃ progeny of transformant SPT::←*Ac*-17LV1, the *Ac* insertion was to a site linked to the starting T-DNA. Transposition to linked sites has been observed in maize and tobacco (Greenblatt, 1984; Jones *et al.*, 1990b) and we are continuing to investigate this in *A. thaliana*. If transpositions are mainly to linked sites, this will have significant implications in a tagging strategy.

Experimental procedures

Construction of the SPT::*Ac* plasmids

All three constructs, SPT::→*Ac*, SPT::←*Ac* and SPT::←*Ac*(Bg) were made by cloning different *Ac* elements into the same binary vector plasmid, pCL0111. This binary vector was constructed by first combining fragments carrying the T-DNA LB and RB sequences described in van den Elzen *et al.* (1985), an NPT-II fusion driven by the 1' promoter (Velten *et al.*, 1984) and octopine synthase (*ocs*) 3' sequences (DeGreve *et al.*, 1982), all carried on the broad host range plasmid pRK290 (Ditta *et al.*, 1980). It also carried a 35S–SPT fusion containing *ocs* 3' sequences (Jones *et al.*, 1991), which carried overlapping *Sst*I and *Xho*I sites in the 5' untranslated leader. This plasmid was termed pCL0101. Plasmid pCL0111 was made by cloning a 2 kb *Sal*I–*Xho*I fragment carrying the NPT-II gene from pCEN14 into the *Xho*I site of pCL0101 and the orientation was selected to leave an intact *Xho*I site immediately downstream of the transcription start site. This acted as a stuffer fragment which was removed in the subsequent cloning step, enabling the *Sst*I and *Xho*I sites in the 35S–SPT fusion to be used as cloning sites for unidirectional insertion of the different *Ac* elements. The three *Ac* elements, one in each orientation and one with a *Bgl*II site inserted by oligonucleotide mutagenesis at position 4390 (Jones *et al.*, 1990a), were cloned into the *Sst*I and *Xho*I sites as *Sst*I and *Sal*I fragments isolated from plasmids pJJ4361, pJJ4368 and pJJ3553. This will be described elsewhere. A typical construct is shown diagrammatically in Figure 1.

Plant material and transformation

The binary plasmids were mobilized by triparental mating (Figurski and Helinski, 1979) to the rifampicin-resistant *Agrobacterium tumefaciens* strain C58C1 harbouring the disarmed Ti-plasmid pGV2260 (Deblaere *et al.*, 1985). This strain proved more effective than the LBA4404 strain as was also found by Valvekens *et al.* (1988). The integrity of the constructs in *Agrobacterium* was checked by rescuing the plasmids back into *E. coli* and comparing the restriction patterns before and after mobilization into *Agrobacterium*. Root transformation of *A. thaliana* ecotype Landsberg *erecta* was performed as previously described (Valvekens *et al.*, 1988). Transformants were selected on 50 mg l⁻¹ kanamycin monosulphate (Sigma) in the shoot-inducing medium. The explants were maintained at 20°C with either a 16 or 24 h

photoperiod. The Landsberg *erecta* transformants did not root efficiently so the plants were allowed to flower and set seed *in vitro* (in Magenta pots). Efficient seed set was achieved by ensuring that the humidity remained low in the Magenta vessels. The original transformants was defined as the T₁ generation and its progeny were the T₂ generation, etc. The ploidy of a random sample of 13 of the transformants was established using root tip squashes. Only one of these, SPT::←Ac-28 was tetraploid.

Estimation of locus number and phenotypic selection for transposition

Seeds were surface-sterilized by a 2-min treatment in 70% ethanol followed by 10 min in 10% bleach solution. After five washes in water, the seeds were dried. To monitor the number of segregating T-DNA loci in the transformants, sterile seeds were plated on GM medium (Valvekens *et al.*, 1988) including 1% (w/v) glucose and 50 mg l⁻¹ kanamycin monosulphate. Resistant and sensitive seedlings were scored about 10 days after germination. Ac excision events were monitored in the progeny of the transformants by germinating sterile seed on GM medium containing 1% (w/v) glucose and 200 mg l⁻¹ streptomycin sulphate (Sigma). The seedlings were scored 14–18 days after germination for white (sensitive), variegated or fully green (resistant) phenotypes.

Isolation of genomic plant DNA

A modified miniprep CTAB method, based on a method from Janice Keller (DNAP, Oakland, Ca, USA) was used to prepare plant DNA for Southern analysis. Two to five grams of frozen leaves were ground in 25 ml 140 mM sorbitol, 220 mM Tris-HCl (pH 8), 22 mM EDTA, 800 mM NaCl, 1% Sarkosyl, 0.8% CTAB and incubated for 20 min at 65°C, with occasional vigorous shaking. After addition of 10 ml chloroform and inverting for 20 min, the samples were centrifuged at 2800 g for 15 min in an H6000 rotor in a Sorvall RC3C centrifuge. The nucleic acid was then precipitated from the aqueous phase by addition of 17 ml isopropanol. The pellet was resuspended in 4 ml TE pH 8 (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and an equal volume of 4 M lithium acetate added to precipitate high molecular weight RNA. After centrifugation the DNA was precipitated from the supernatant by addition of 2 volumes of ethanol. The pellet was dissolved in 0.9 ml TE, and after addition of 100 µl 3 M sodium acetate was extracted, first with phenol, then phenol/chloroform and finally chloroform. The DNA was precipitated from the aqueous phase by addition of 2 volumes of ethanol and dissolved in 100–250 µl TE. The yield was usually >10 µg g⁻¹ tissue.

Southern blot analysis

Between 0.5 and 2.5 µg of genomic plant DNA were digested with either *SspI* or *HindIII*. Following separation on a 0.8% agarose TBE gel, the DNA was transferred to Hybond N membranes (Amersham) by capillary blotting. The membranes were pre-hybridized and hybridized according to the Hybond N protocol. The washing conditions were 2 × (2 × SSC, 1% SDS) for 20 min at 65°C and 2 × (0.1 × SSC, 1% SDS) for 20 min at 65°C. The membranes were exposed to X-ray film (Kodak), in cassettes with intensifying screens, at -70°C. The 904 bp 3' *HindIII*-*EcoRI* internal Ac fragment was used as a probe to detect new Ac positions. For empty donor sites a 700 bp *Clal*-*NcoI* fragment from the SPT coding region was used. The LB probe was a 1 kb

Clal-*SphI* fragment carrying the 1' and 2' promoters and the NPT-II coding region. The RB probe was a 400 bp *SspI*-*XhoI* fragment carrying the T-DNA RB sequences to the *BamHI* site and the 35S promoter. The positions of the probes are indicated on Figure 1. The probes were labelled using a random priming procedure.

PCR analysis

The PCR reactions were carried out using oligonucleotides C15 and B37. The sequences of the primers used were; C15: 5'TATCCAGCTCGAGTGGGTGGTGGAG3'; B37: 5'CCATCGT-AGGTGAAGGTGGAAATTAAT3'. The reactions were performed in 0.6 ml Eppendorf tubes in a reaction buffer of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatin, 0.5% Tween 20 with 1 mM of each primer, 0.2 mM each nucleotide, 50 ng *A. thaliana* genomic DNA and 2.5 U Amplitaq Taq I Polymerase. The samples were overlaid with 100 µl paraffin oil to prevent evaporation. An MJ Research Inc. PCR machine was used with 35 cycles of denaturing at 94°C for 60 sec, annealing at 55°C for 60 sec and extension at 72°C for 120 sec.

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