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Elevated Levels of *Activator* Transposase mRNA Are Associated with High Frequencies of *Dissociation* Excision in Arabidopsis

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The *Activator* (*Ac*) element of maize is active at a low frequency in Arabidopsis. To determine whether this is due to poor expression of the *Ac* transposase gene, we obtained and studied 19 Arabidopsis transformants containing fusions of the octopine synthase (*ocs*), nopaline synthase (*nos*), cauliflower mosaic virus (CaMV) 35S, or *Ac* promoters to the transposase open reading frame. These transformants were examined both for their ability to drive excision of a *Dissociation* (*Ds*) element from a streptomycin resistance gene and for the abundance of the transposase mRNA. Most transformants containing the CaMV 35S fusion have high levels of transposase transcript and drive high frequencies of somatic and germinal excision. These results demonstrated that Arabidopsis contains all of the host functions required for high frequency excision of *Ds*. Moreover, transposase mRNA abundance varied about 1000-fold among our transformants; this variation enabled us to demonstrate that for the *Ac*, *ocs*, and CaMV 35S fusions, raising the mRNA level is closely correlated with increasing excision frequency. We discuss our data in relation to the behavior of *Ac* in Arabidopsis, maize, and tobacco.

INTRODUCTION

The maize transposon *Activator* (*Ac*) is active in a wide range of plant species including several members of the Solanaceae, rice, *Triticum monococcum*, carrot, and Arabidopsis (Baker et al., 1986; Knapp et al., 1987; Van Sluys et al., 1987; Yoder et al., 1988; Houba-Herrin et al., 1990; Laufs et al., 1990; Izawa et al., 1991). Recently, it has become clear that *Ac* does not behave in the same way in all plant species. For example, in maize increasing the number of copies of *Ac* in the genome decreases the frequency of transposition (McClintock, 1948, 1951). This appears to be correlated with an increase in the abundance of the single mRNA encoded by *Ac* (Kunze et al., 1987). However, in tobacco an increase in the dosage of *Ac* results in an increase in excision frequency (Jones et al., 1989; Hehl and Baker, 1990). In addition, the frequency of transposition of *Ac* can vary between species. In tobacco the frequency of excision of *Ac* is very much higher than in Arabidopsis. For example, in one series of experiments tobacco plants homozygous for an *Ac* element inserted within a streptomycin resistance gene were crossed to wild-type plants, and 20% of the resulting progeny inherited an active resistance gene from which the element had excised. The minimum frequency reported was 7.5% (Jones et al., 1991). In contrast, after self-fertilization of Arabidopsis plants containing *Ac* inserted within a kanamycin resistance gene, there were no resistant individuals in most families and the average frequency with which they occurred was 0.5 to 1.0% (Schmidt and Willmitzer, 1989).

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The basis of the extreme difference in *Ac* activity in these two species is unclear. Conceivably, the low frequency of transposition in Arabidopsis could be the result of poor expression of the single gene encoded by the transposon or a consequence of reduced levels of or an absence of host proteins essential for high activity of *Ac*. The abundance of the *Ac* transcript has not previously been examined in detail in transgenic plants, but it is rare in maize, making up approximately 10^{-5} of all mRNA (Kunze et al., 1987; Finnegan et al., 1988; Fusswinkel et al., 1991). Moreover, upstream of the transcriptional start sites of the *Ac* mRNA there is no TATA box or other sequences characteristic of a normal plant promoter although the high G+C composition of this region has similarity to the promoters of housekeeping genes (Kunze et al., 1987).

To determine whether increasing the expression of the *Ac* transposase (TPase) in Arabidopsis increases transposition frequency, we have tested the effect of fusing the single *Ac* open reading frame (ORF) to four promoters. The efficiency of the different fusions is measured by testing the frequency with which they promote the excision of a *Dissociation* (*Ds*) element. This element is inserted within a streptomycin resistance gene so that the frequency of somatic and germinal excision can be measured (Jones et al., 1989; Dean et al., 1992). We demonstrate that the cauliflower mosaic virus (CaMV) 35S promoter fused to the *Ac* TPase ORF drives very high frequencies of somatic and germinal excision of *Ds* in Arabidopsis and that in general there is a positive correlation between the level of *Ac* TPase mRNA and excision frequency

in transformants containing fusions of *Ac* TPase to the *Ac*, octopine synthase (*ocs*), and CaMV 35S promoters.

RESULTS

Identification of Arabidopsis Transformants Homozygous for the T-DNA at a Single Locus

The fusions to the TPase gene of *Ac* were constructed by removing one end of the transposon and inserting a promoter in the DNA that encodes the long untranslated leader of the *Ac* mRNA (Scofield et al., 1992; see Methods). These *Ac* derivatives, which are shown in Figure 1, are referred to as stable *Ac*s and cannot themselves transpose (Coupland et al., 1989). A second *Ac* derivative, called *Ds*, which has both termini intact but contains a frameshift mutation that truncates the *Ac* ORF, was used to measure the effectiveness of the different fusions. In this construct, *Ds* was inserted within a streptomycin resistance gene such that excisions of the transposon from

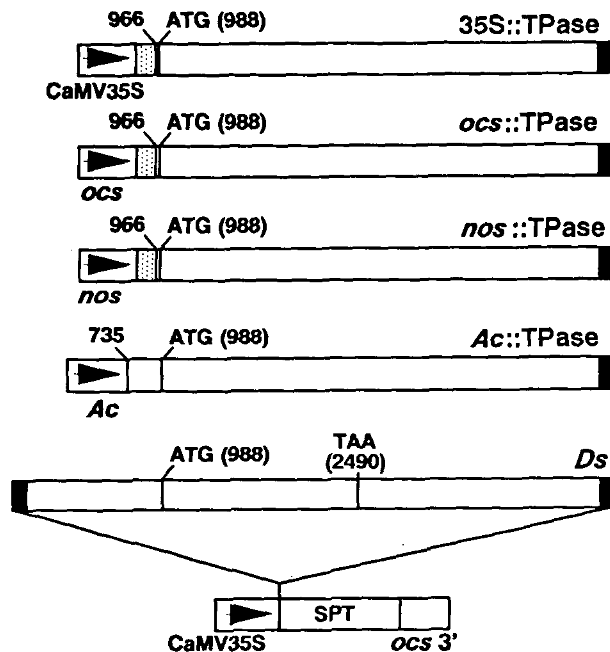


Figure 1. Diagrams of Transposase Fusions and *Ds*.

Arrows represent the promoters inserted in front of the TPase and streptomycin phosphotransferase (SPT) genes. The stippled boxes depict the Ω leader sequence of tobacco mosaic virus, and the solid boxes represent the termini of *Ac*. In the upper three TPase fusions, the Ω leader sequence is inserted at *Ac* coordinate 966, whereas in *Ac*::TPase, the *Ac* promoter is inserted at coordinate 735. The site at which translation of the TPase ORF is initiated is marked with ATG. The *Ds* contains a non-sense mutation within the TPase ORF, and this is marked by TAA. Further details of these constructions are given in the Methods section.

this gene would restore its activity, giving rise to cells resistant to the antibiotic.

Plants containing each of the four stable *Ac*s were cross-fertilized with individuals carrying *Ds* to measure the capacity of each TPase fusion to promote excision. To simplify the analyses in subsequent generations, only plants homozygous for the T-DNA at a single locus were used for these crosses. These transformed plants were constructed in the following way. The *Ac* derivatives were introduced into Arabidopsis by use of the root transformation method devised by Valvekens et al. (1988). The original transformed regenerant (T_1 generation) was allowed to self-fertilize, and its progeny were sown on kanamycin-containing medium. Only transformants that segregated 3:1 for kanamycin resistance in the T_2 generation were used for subsequent genetic analyses. Five to 10 kanamycin-resistant T_2 plants were self-fertilized, and the resulting seeds were sown on kanamycin-containing medium. By this method, T_2 individuals were identified that produced 100% kanamycin-resistant progeny when self-fertilized, and these were presumed to be homozygous for the T-DNA at a single locus. This was done for five independent transformants (A to E) carrying each of the following three constructs, nopaline synthase (*nos*)::TPase (i.e., stable *Ac* carrying the nopaline synthase promoter fused to the *Ac* ORF), 35S::TPase, and *Ac*::TPase, and for four transformants (A to D) carrying octopine synthase (*ocs*)::TPase. This protocol was also followed for seven transformants carrying the *Ds* construct. In the following experiments, only one *Ds* transformant was used to eliminate this parent as a source of variation in excision frequency. As demonstrated by gel blot analysis, the *Ds* line chosen contained a single copy of the T-DNA at a single locus (data not shown) so that the excision frequency of only one copy of *Ds* was measured in the following experiments.

Frequency of Somatic Excision of *Ds* Detected in F_1 Plants

T-DNA homozygotes derived from the 19 independent *Ac* stable transformants described in the previous section were cross-fertilized with the single *Ds* tester line. The resulting hybrid seeds were all heterozygous for both T-DNAs. These were sown on medium containing streptomycin, and the frequency with which clones of resistant cells appeared on the cotyledons was scored. Because neither parental line ever showed variegation, the appearance of these green clones is dependent upon the presence of both T-DNAs, which supports the conclusion that the sectors are due to *trans*-activation of *Ds* by stable *Ac*. This was confirmed by hybridization of DNA gel blots with probes derived from *Ds* and the streptomycin resistance gene.

The variegation phenotypes are illustrated in Figure 2, and the frequency with which each phenotype arose is displayed in Figures 3 and 4, and described in the following paragraphs. The gel blots are shown in Figure 5 and discussed in more detail later.

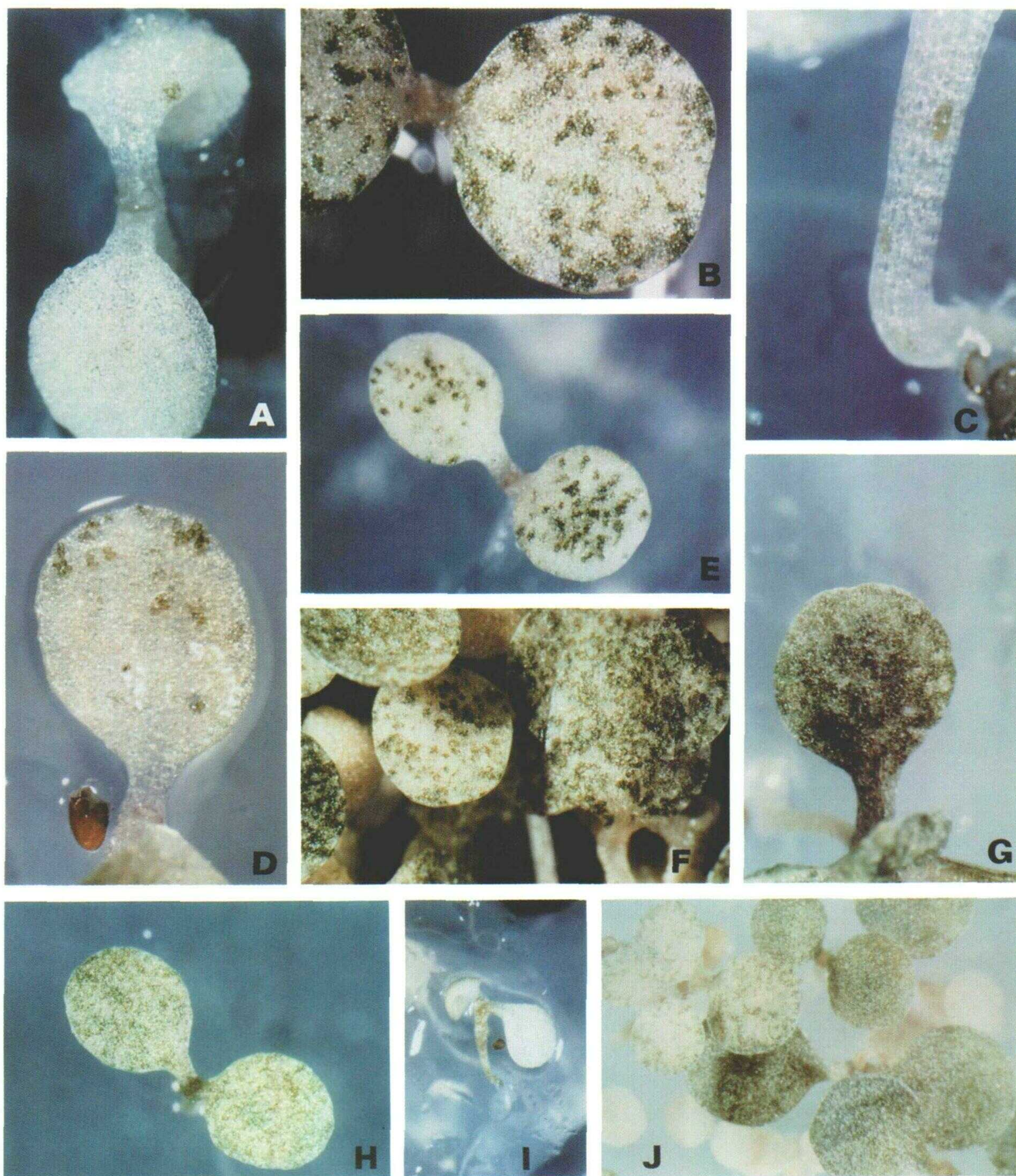


Figure 2. Phenotypes of Variegated and Full Green Seedlings Growing on Medium Containing Streptomycin.

(A) to **(C)** *Ac*::TPase F_1 plants are illustrated.

(A) Lightly variegated seedling.

(B) Highly variegated seedling.

(C) Seedling with variegated hypocotyl.

(D) to **(F)** *ocs*::TPase F_1 plants. All seedlings shown are highly variegated.

(G) to **(I)** 35S::TPase F_1 plants are illustrated.

(G) and **(H)** Highly variegated seedlings.

(I) Seedling with a green hypocotyl.

(J) 35S::TPase F_2 plants: the segregation of sensitive, full-green, and variegated seedlings.

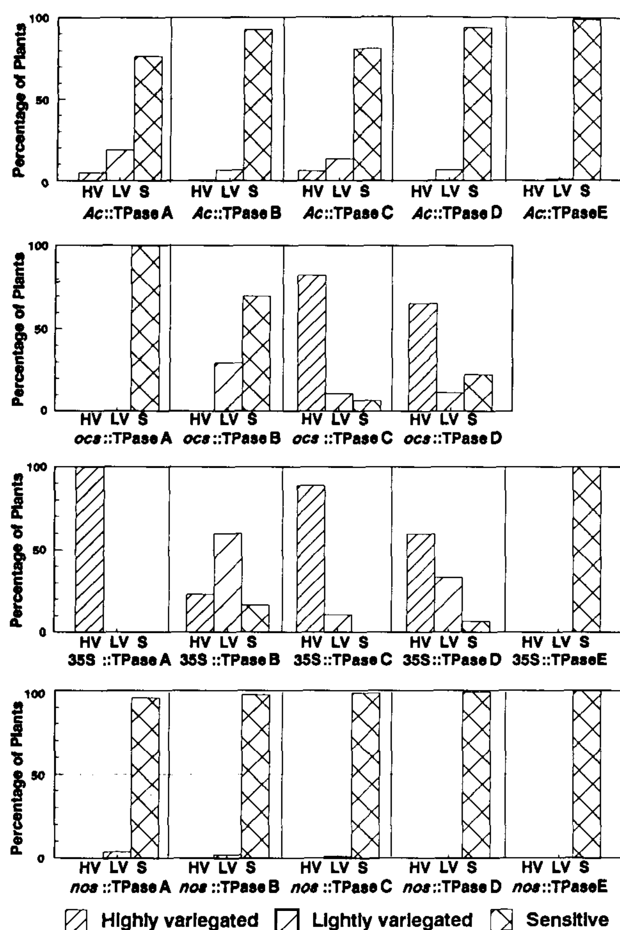


Figure 3. Frequency with which Variegated and Sensitive Seedlings Occur in Each F_1 Population.

Each row of histograms presents the results achieved with each transposase fusion, and each histogram contains the data obtained with one transformant. The percentage of plants with each phenotype in every F_1 population is shown. Seedlings with green or variegated hypocotyls have been included under lightly variegated (LV). These were generally a low percentage of seedlings (less than 5%) except in the case of 35S::TPase transformant B, where they made up 31% of the population. The number of plants in the F_1 populations varied from 50 to 384. HV, highly variegated seedlings; S, sensitive seedlings.

Ac::TPase

The majority of F_1 seedlings appeared entirely sensitive to streptomycin when germinated on medium containing the antibiotic. Up to 16% of the F_1 generation was lightly variegated, and four of the transformants carrying this construct gave rise to a small number of F_1 seedlings that were scored as highly variegated because there were many (arbitrarily defined as greater than approximately 20 per seedling) clones of streptomycin-resistant cells on their cotyledons (Figures 2 and 3). Although there is a dramatic phenotypic difference between the highly variegated and sensitive seedlings (Figure 2), both

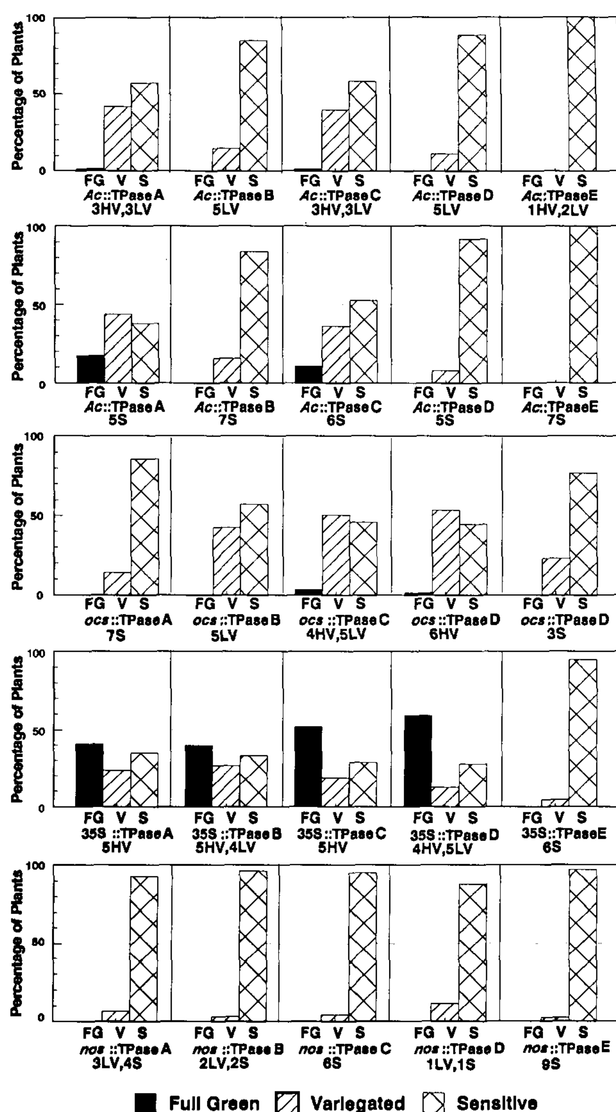


Figure 4. Frequency with which Full-Green, Variegated, and Sensitive Seedlings Occur in F_2 Populations.

The first two rows of histograms present the results obtained with *Ac*::TPase, whereas the other three rows show the data recorded with the other three fusions. Each box presents the combined F_2 data for several F_1 plants derived from the same transformant. The number and phenotype of the relevant F_1 seedlings are shown below each histogram (e.g., 5LV indicates that the parental plants were five lightly variegated F_1 seedlings). F_2 data collected from families derived from variegated and sensitive F_1 seedlings were combined for the *nos*::TPase transformants because the phenotype of F_1 seedlings had no apparent influence on the phenotypes detected in the F_2. The histograms show the percentage of plants exhibiting each phenotype. The number of individuals scored to compile each histogram varied from 225 to 1129. FG, full green seedlings; V, variegated seedlings; S, sensitive seedlings.

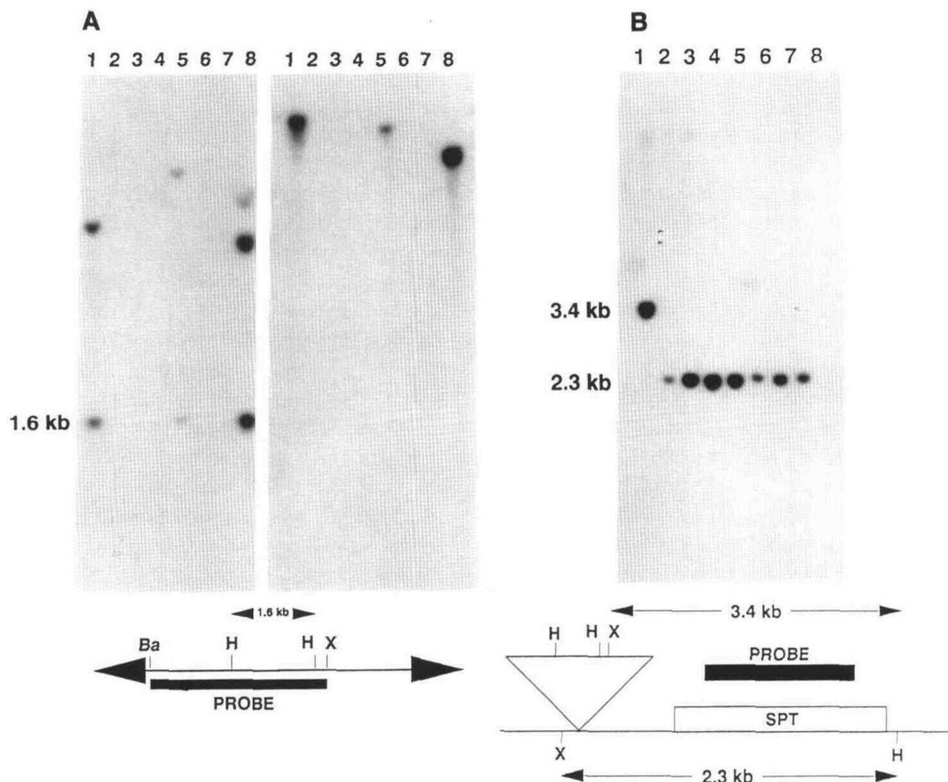


Figure 5. Analysis of the Structure of the SPT Gene and of the Location of *Ds* in Streptomycin-Resistant F_2 Plants.

All lanes contain DNA extracted from the *Ds* transformant (lane 1) or from seven streptomycin-resistant F_2 plants that have not inherited the 35S::TPase T-DNA (lanes 2 to 8).

(A) These two gel blots were hybridized to a probe made from the internal BamHI-XhoI fragment of *Ac*. The DNA samples run on the gel on the left were cleaved with HindIII, whereas those loaded on the gel on the right were cleaved with BglII.

(B) The gel blot was hybridized to a probe made from the SPT gene. The DNA samples were cleaved with a combination of XhoI and HindIII.

classes are genotypically heterozygous for both T-DNAs. Interestingly, the five *Ac*::TPase transformants varied in their capacity to promote *Ds* excision in the F_1 generation (Figure 3); less than 1% of the F_1 seedlings were variegated when *Ac*::TPase transformant E was used as a parent, whereas almost 17% were variegated when transformant A was used. Similar variation between transformants was seen with all constructs (Figure 3).

ocs::TPase

F_1 progeny derived from *ocs*::TPase transformants B, C, or D showed frequencies of variegation considerably higher than those detected with *Ac*::TPase (Figures 2 and 3). This increase was detected both in the numbers of variegated individuals and in the ratio of highly variegated seedlings to lightly variegated. Nevertheless, in two of these F_1 populations streptomycin-sensitive individuals with no phenotypic indication of *Ds* excision were detected along with variegated seedlings. *ocs*::TPase A only produced sensitive seedlings in the F_1 generation although some variegated individuals were detected in F_2 populations derived from sensitive F_1 seedlings (Figures 3 and 4).

nos::TPase

Surprisingly, none of the F_1 seedlings showed well-defined green sectors. Those scored as variegated exhibited a diffuse green color spread across the whole cotyledon. DNA gel blots of four *nos*::TPase transformants were hybridized to an internal fragment of *Ac*; this confirmed that the TPase gene was present in these transformants and had the expected structure. Subsequently, RNase protection experiments indicated that all five lines containing *nos*::TPase express the *Ac* mRNA in developing inflorescences (shown later).

35S::TPase

The frequency with which variegated seedlings arose in F_1 populations derived from 35S::TPase transformants A to D was in general very high. Indeed, 100 and 97% of the hybrids

created with 35S::TPase A and C, respectively, were highly variegated. Transformant B, apparently the least active of transformants A to D, still produced three times as many variegated seedlings in the F_1 generation as the most active *Ac*::TPase lines. In addition, the 35S::TPase construct often promotes such a high frequency of *Ds* excision that it is difficult to distinguish individual sectors. This phenotype is illustrated in Figure 2. Variegation to this degree was not recorded with the *Ac*::TPase construct but was seen for *ocs*::TPase transformants A and C. 35S::TPase transformant E produced no somatic sectors on the cotyledons of F_1 seedlings although this transformant is active because variegated individuals were detected in the F_2 generation (discussed later).

An intriguing aspect of the variegated phenotype detected in the F_1 generation of almost all crosses was the striking difference between genetically identical sibling plants. This is in contrast to the behavior of these constructs in tobacco where the phenotypes are extremely reproducible (Scofield et al., 1992). In a population of Arabidopsis plants that contain *Ac*::TPase and *Ds*, it is common to find a highly variegated individual adjacent to a sensitive one although both plants are genotypically identical. The 35S::TPase fusion increases the frequency with which highly variegated seedlings occur although most transformants containing this construct do not produce a population of only highly variegated individuals (Figure 3). This suggests that employing a stronger plant promoter to express the *Ac* TPase can reduce this variability but not abolish it.

Frequency of Germinal and Somatic Excision Detected in the F_2 Generation

If the promoter fused to the TPase gene is active in cell lineages that give rise to the gametes, then, as a result of *Ds* excision, a proportion of the hybrid F_1 plants will transmit a fully active streptomycin resistance gene to its progeny. These events, called germinal excisions, can be detected by self-fertilizing the F_1 plants and selecting F_2 individuals that are fully resistant to streptomycin (Figure 2). From every F_1 family, at least five plants exhibiting each phenotype were chosen; these were self-fertilized and each F_2 line was sown separately on medium containing streptomycin. The results obtained from F_2 lines derived from F_1 plants showing the same phenotype in the same F_1 population were combined. These are shown in Figure 4 and are discussed below.

Ac::TPase

Fifty-five F_1 plants carrying *Ac*::TPase and *Ds* were self-fertilized, and the resulting F_2 lines were sown separately on streptomycin-containing medium. Between 0 and 69% of seedlings in individual F_2 families were fully streptomycin resistant. In only two of the 55 lines was this proportion greater than 5%, and in most it was lower than 2%. In F_2 populations derived from *Ac*::TPase, therefore, the frequency with which fully streptomycin-resistant seedlings arise is usually low but is highly variable. This is emphasized by the streptomycin-resistant phenotypes of the 11 F_2 lines derived from *Ac*::TPase transformant A; a description of these is presented in Table 1. Moreover, these data indicate that with this TPase fusion there is no clear relationship between the appearance of sectors on the cotyledons of F_1 seedlings and the frequency of germinal excision; the range of germinal excision frequencies detected in the F_2 generation is similar whether the parental F_1 plant was variegated or sensitive. Nevertheless, transformants A and C, which produced the highest frequencies of variegation in the F_1 generation, also produced the highest frequencies of germinal excision (compare Figures 2 and 3). Therefore, whether an F_1 plant is variegated or sensitive is not an indication of whether that individual will produce germinal excisions, but a high frequency of variegated seedlings in a population increases the likelihood that seedlings from that population will produce germinal excisions. Another interesting feature of the F_2 generation is revealed by comparison of Figures 2 and 3. The F_2 families inheriting *Ac*::TPase contain a higher proportion of variegated seedlings than the corresponding F_1 generation. This increase in the proportion of variegated seedlings in the F_2 generation occurred with all constructs and possible explanations of this occurrence are given in the Discussion section.

ocs::TPase

The overall frequencies of germinal excision detected in the families containing *ocs*::TPase were not significantly higher than those detected with *Ac*::TPase. The higher frequency of somatic excision of *Ds*, which is driven by *ocs*::TPase in the F_1 generation, is not therefore extended to the frequency of germinal excision. Again, as described previously for the *Ac*::TPase transformants, the *ocs*::TPase transformants (A and C) that were the most active in the F_1 generation also produced the highest frequencies of germinal excision.

35S::TPase

35S::TPase transformants A to D drive very high frequencies of germinal excision of *Ds*. Forty-four F_2 lines derived from these transformants were tested on medium containing streptomycin, and the lowest proportion of fully resistant seedlings detected was 4%. Furthermore, in 36 of the families more than 30% of the seedlings showed this phenotype. The consistently high frequency of germinal excision detected with this fusion is emphasized in Table 2, which presents in detail the data for 14 F_2 lines derived from 35S::TPase transformant C. 35S::TPase transformant E showed no activity in the F_1 generation but did produce a low proportion of variegated seedlings in the F_2 generation (Figure 4).

Table 1. Frequency of *Ds* Excision Detected in F_2 Families Derived from a Cross between *Ac*::TPase Transformant A and the *Ds* Tester

	F_2 phenotypes			
F_1 phenotype	Full green (%)	Variegated (%)	Sensitive (%)	Total no. seedlings
Variegated	3.5	36	60.5	147
Variegated	0	49	51	145
Variegated	0	44.6	55.4	121
Variegated	3.9	38.3	57.8	128
Variegated	2.1	42.1	55.8	140
Variegated	0	40.1	59.9	137
Sensitive	5.5	55.2	39.3	145
Sensitive	72.9	0	27.1	133
Sensitive	3.8	49	47.2	157
Sensitive	1.9	61	37.1	105
Sensitive	3.5	55.5	41	144

Each row contains the results obtained from one F_2 family. The first column describes the phenotypes of the parental F_1 plants. The phenotypes of F_2 seedlings growing on medium containing streptomycin are shown in the second, third, and fourth columns. These are presented as the percentage of seedlings in the family that exhibit the phenotype. The fifth column lists the total number of individuals scored in each family.

nos::TPase

In contrast to the results obtained with the other three TPase fusions, no germinal excisions of *Ds* were observed in the F_2 populations derived from *nos*::TPase (Figure 4). However, F_2 seedlings exhibiting a diffuse green color on the cotyledons were detected, and these resembled those observed in the F_1 generation.

Streptomycin-Resistant F_2 Plants Have Indeed Inherited Germinal Excision Events

Several experiments were performed to confirm that the F_2 plants scored as fully streptomycin resistant had inherited germinal excision events. First, the T-DNAs containing *Ds* and stable *Ac* will segregate in the F_2 generation, permitting the isolation of streptomycin-resistant plants that have not inherited stable *Ac*. Clearly, such plants must have inherited germinal excision events and cannot simply be highly variegated. As described in Methods, the T-DNA carrying stable *Ac* also contains a β-glucuronidase gene (*gus*), which allows its inheritance to be easily followed. Three hundred and thirty F_2 plants fully resistant to streptomycin were tested for β-glucuronidase (GUS) activity, and 100 did not contain the enzyme. This supports the hypothesis that the streptomycin-resistant individuals have inherited germinal excisions because 25% of them would not be expected to contain *gus*.

Second, 12 of the streptomycin-resistant, GUS-negative F_2 plants were self-fertilized, and the segregation of streptomycin resistance was tested in the F3 generation. In all 12 cases, streptomycin-resistant seedlings comprised 75 or 100% of the progeny, confirming that the F_2 plant contained a fully active streptomycin resistance gene.

Finally, DNA gel blots were performed on DNA extracted from the 12 plants. The DNA was cleaved with XbaI and HindIII, HindIII alone, or BglII alone. After cleavage with XbaI and HindIII and hybridization with a probe derived from the streptomycin resistance gene, a 3.4-kb fragment is expected if *Ds* is still within the gene and a 2.3-kb fragment is expected if *Ds* has excised. The 2.3-kb fragment was present in the DNA of all 12 plants, and the 3.4-kb fragment was absent (illustrated in Figure 5B for seven plants). When DNA from the 12 plants was cleaved with BglII and probed with *Ds* sequences, two of the plants contained *Ds* on a different-sized fragment than

Table 2. Frequency of *Ds* Excision Detected in F_2 Families Derived from a Cross between 35S::TPase Transformant C and the *Ds* Tester

	F_2 phenotypes				
F_1 phenotype	Full green (%)	Variegated (%)	Green hypocotyl (%)	Sensitive (%)	Total no. seedlings
Highly variegated	39	30	0	31	70
Highly variegated	38	29	0	34	77
Highly variegated	13	29	0	34	98
Highly variegated	58	4	0	38	154
Highly variegated	74	2	0	24	100
Highly variegated	34	32	13	21	76
Highly variegated	10	43	0	47	112
Highly variegated	56	21	2	21	112
Highly variegated	38	17	20	20	105
Highly variegated	72	0	7	20	123
Green hypocotyl	45	0	25	30	138
Green hypocotyl	60	3	10	27	115
Green hypocotyl	63	7	2	29	195
Green hypocotyl	24	7	28	41	90

The data in this table are presented in the same way as described for Table 1.

Ds at its original location. These two plants also contained the internal 1.6-kb HindIII fragment of *Ds* (Figure 5A).

Taken together, these data confirm that those plants scored as streptomycin resistant have inherited a germinal excision event and carry a streptomycin resistance gene with restored structure and that some of these plants contain the *Ds* inserted at a new location.

The Abundance of TPase mRNA Is Usually Correlated with the Activity of Stable *Ac*

The *Ac* transcript is rather rare in maize (Fusswinkel et al., 1991), and the experiments described above were performed with the expectation that a fusion of the CaMV 35S promoter to the TPase ORF would increase the abundance of the transcript. RNase protection experiments were performed to test this assumption and to examine whether the difference in activity between transformants could be correlated with the abundance of TPase mRNA.

A 278-bp fragment located within exon 3 of the *Ac* ORF was inserted in pGEM3, and an antisense riboprobe was synthesized. This probe was hybridized to total RNA extracted from developing inflorescences of the 19 *Ac* stable transformants. This mixture of RNA was then incubated with RNaseA and RNaseT1 to degrade the excess single-stranded riboprobe as well as the tails of the riboprobe that were not homologous to the *Ac* mRNA; the remaining double-stranded hybrids were denatured and run on a polyacrylamide gel that was subsequently autoradiographed. The intensity of the protected fragment obtained with each transformant was compared. Figure 6 presents some of the results obtained and the relative abundances of the *Ac* mRNA in the 19 transformants.

The four highly active 35S::TPase transformants (A to D) do indeed contain more *Ac* mRNA than any of the other transformants. There is between 12 and 31 times more TPase mRNA in 35S::TPase transformants A to D than in the most active *Ac*::TPase transformants. The low frequency of somatic and germinal excision found with 35S::TPase transformant E is a consequence of there being between sixfold and 15-fold less TPase mRNA in this transformant than in the other 35S::TPase transformants. However, as discussed later, this relationship is less straightforward when 35S::TPase transformant E is compared with transformants containing other constructs; for example, 35S::TPase transformant E promotes less transposition than *Ac*::TPase transformant C although it contains twice as much TPase mRNA.

All four *ocs*::TPase transformants contain less *Ac* mRNA than 35S::TPase transformants A to D. The variation in the abundance of TPase mRNA between *ocs*::TPase transformants is closely correlated with excision frequency. For example, transformants C and D contain between twofold and 4.5-fold as much mRNA as transformant B and show higher frequencies of both somatic and germinal excision.

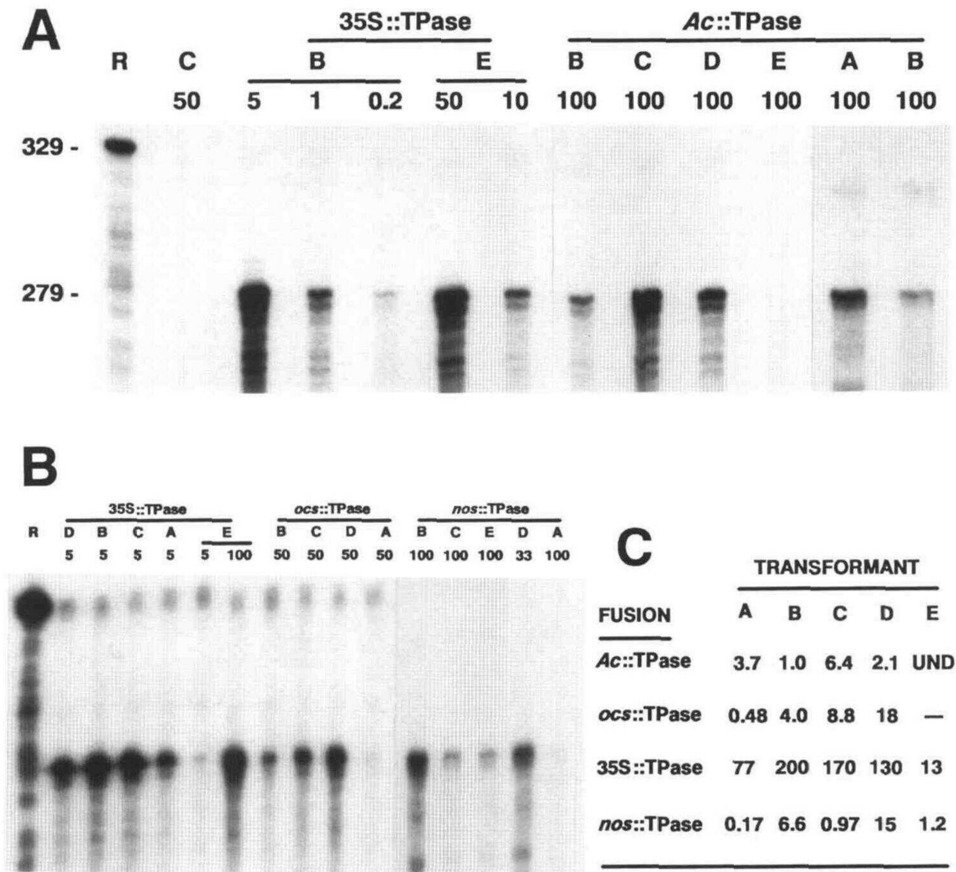
The variation in transposition frequency between *Ac*::TPase transformants correlates closely with mRNA abundance; transformants A and C show higher levels of expression and higher frequencies of excision than B and D. No TPase mRNA was detected in *Ac*::TPase transformant E although 0.8% of F_1 seedlings were scored as variegated. Currently, it is not known whether the abundance of TPase mRNA in this transformant is below the level of detection with our RNA protection assay or whether the five variegated F_1 seedlings were falsely scored. The observation that no variegated seedlings were detected in the F_2 population derived from this transformant would support the latter possibility.

nos::TPase transformants A and D show levels of TPase gene expression comparable to *ocs*::TPase transformants C and D. However, the *ocs*::TPase transformants drive very high frequencies of somatic excision and promote some germinal excision of *Ds*, whereas the *nos*::TPase transformants do neither. It seems likely that the discrepancies between expression levels and transposition frequency found with the *nos*::TPase transformants are due to the promoter expressing the TPase gene in cell types in which *Ds* excision cannot be easily detected with the streptomycin resistance assay.

DISCUSSION

Ac::TPase drives a low frequency of somatic excision of *Ds* in the cotyledons and promotes a low frequency of germinal excision of *Ds*. This is consistent with the behavior of an autonomous *Ac* element in Arabidopsis (Schmidt and Willmitzer, 1989; Dean et al., 1992). The observation that among the *Ac*::TPase transformants those with higher mRNA levels also promote higher transposition frequencies suggests that the low excision frequency promoted by *Ac*::TPase is due to the low expression of the *Ac* TPase mRNA. The four *Ac*::TPase transformants in which *Ac* mRNA could be detected varied approximately sevenfold in the abundance of this mRNA, and across this range excision frequency and the level of mRNA are correlated. The increase in the frequency of excision detected in the F_2 generation of all populations derived from transformants in which the TPase gene was expressed at a low level is probably due in part to an increase in the dosage and, therefore, the expression level of the TPase gene in F_2 homozygotes. However, segregation of the T-DNAs in the F_2 generation will result in 56% of F_2 seedlings containing both T-DNAs, and only a third of these will be homozygous for *Ac*::TPase. Therefore, the increase in the number of variegated seedlings seen in most of these populations is too large to be explained entirely by this mechanism.

Exchanging the *Ac* promoter for the *nos*, *ocs*, and CaMV 35S promoters extended the range of TPase mRNA abundance to around 1000-fold. The four *ocs*::TPase transformants varied approximately 40-fold in the level of *Ac* TPase mRNA. *ocs*::TPase transformant B has a relatively low abundance of TPase mRNA (Figure 6) and produced no highly variegated seedlings in the F_1 generation of a cross to the *Ds* transformant; indeed, only 30% of the seedlings were lightly variegated and 70% were sensitive. Crosses using *ocs*::TPase transformant C produced 93% variegated seedlings, and this



FUSION	TRANSFORMANT A	B	C	D	E
Ac::TPase	3.7	1.0	6.4	2.1	UND
ocs::TPase	0.48	4.0	8.8	18	—
35S::TPase	77	200	170	130	13
nos::TPase	0.17	6.6	0.97	15	1.2

Figure 6. Determination of *Ac* TPase mRNA Abundance in Each Stable *Ac* Transformant.

(A) Lane R contains the riboprobe. The other tracks contain the products of the RNase protection experiments in which different RNA samples were used. Lane C is a control in which 50 μg of nontransformed Arabidopsis RNA was used. Above the other lanes the transformant from which the RNA was isolated and the amount of transformant RNA (in micrograms) are shown. A fivefold dilution series was performed with RNA from 35S::TPase transformants B and E, and this confirmed the relationship between band intensity and RNA abundance assumed in **(C)**. The full-length riboprobe was 329 bases and the protected fragment was 279 bases long. The extra, shorter protected fragments are a common feature of this type of experiment (Melton et al., 1984). All lanes are from one experiment, except the two on the extreme right (*Ac*::TPase A and B) that are from a second experiment.

(B) The remaining RNA samples presented in the same way as given in **(A)**. All 35S::TPase and *ocs*::TPase lanes are from the same experiment, whereas the lanes containing *nos*::TPase are from another experiment.

(C) The relative abundance of TPase mRNA in all transformants. *Ac*::TPase B was arbitrarily defined as 1. These figures are averages of two or three independent RNA protection experiments, except for that with *nos*::TPase A which was only performed once. UND, undetected; —, only four *ocs*::TPase transformants were analyzed. A detailed description of how the band intensities were measured is given in the Methods.

transformant contained twice as much *Ac* mRNA as transformant B. However, transformant D contained a further twofold increase in the amount of *Ac* mRNA and 78% of seedlings were highly variegated (Figure 3). These data suggest that for the *ocs*::TPase construct, excision frequency increases markedly with mRNA abundance until the level of mRNA detected in transformant C is reached. Beyond this point, increasing mRNA concentration further has little effect on excision frequency.

The five 35S::TPase transformants vary approximately 15-fold in the abundance of TPase mRNA. Transformants A, B, C, and D have high levels of mRNA and promote high frequencies of somatic and germinal excision of *Ds*. Between 84 and 100% of the F_1 seedlings generated by crossing these transformants to *Ds*-containing plants are variegated. We believe it likely that these four transformants approach the maximum level of excision frequency that can be achieved by increasing transcription of TPase mRNA.

The correlation between excision frequency and the abundance of TPase mRNA shown by different transformants containing the same construct seems convincing; however, comparisons of transformants harboring different constructs are more difficult. For example, 35S::TPase transformant E contains twice as much TPase mRNA as *Ac*::TPase transformant

C but drives much less transposition. This is probably due to the specificity of the *Ac* and CaMV 35S promoters. One possibility is that the constitutive CaMV 35S promoter is active at a low level in all cells of 35S::TPase transformant E (Benfey et al., 1989), whereas the activity of the *Ac* promoter might be limited to only some cells. If this is the case, then the concentration of TPase mRNA would reach a higher level in some cells of *Ac*::TPase transformants than in 35S::TPase transformant E. This could be studied by examining the activity of the *Ac* promoter in situ. However, this explanation seems less likely in comparisons between 35S::TPase transformant E and *ocs*::TPase transformant C. The latter shows high levels of somatic activity but has less TPase mRNA than the former. Because the RNA analyzed was isolated from inflorescences, it is possible that the level of *Ac* mRNA expressed in the cotyledons of *ocs*::TPase transformant C is higher than in those of 35S::TPase transformant E although this order could be reversed in the inflorescences. Similarly, *ocs*::TPase transformants C and D drive frequencies of somatic excision in the cotyledons that are comparable to those detected with the active 35S::TPase transformants although the frequency of germinal excision is much lower. This is probably due to the specificity of expression of the ocs promoter; perhaps it is more active in cell lineages that give rise to the cotyledons than in those that lead to gamete formation.

The *nos*::TPase transformants show little correlation between excision frequency and *Ac* mRNA abundance. The somatic activity detected with *nos*::TPase was qualitatively different from that seen with the other TPase fusions. Well-defined sectors were not observed; rather, there was a diffuse greening of the cotyledons. Currently, we can only explain the difference in appearance of the sectors by proposing that the *nos* promoter is expressed either in different cell lineages than the other promoters or perhaps at a different stage during embryo development. For example, the data of Scofield et al. (1992) suggest that the *nos* promoter is expressed very late during cotyledon development in tobacco. We are examining the pattern of expression of the *nos* promoter in Arabidopsis seedlings to try to explain the lack of clear variegation detected with *nos*::TPase in Arabidopsis, but, currently, we believe that the lack of correlation between TPase mRNA level and excision frequency with the *nos*::TPase transformants is due to the streptomycin assay not efficiently detecting the *Ds* excisions promoted by this fusion in Arabidopsis.

The *Ac*::TPase fusions behave differently in tobacco and Arabidopsis. In Arabidopsis many seedlings that contain both *Ac*::TPase and *Ds* show no phenotypic evidence of excision, and those that are variegated show a highly variable number of sectors. The *Ac*::TPase fusion, therefore, shows low levels of penetrance and expressivity in Arabidopsis. This variation can in part be overcome by increasing the expression of the TPase gene because *Ac*::TPase transformants that show an increased abundance of TPase mRNA show an improved penetrance, and the highly expressed *ocs*::TPase and 35S::TPase transformants show almost 100% penetrance. How increased penetrance is related to increased rates of transcription is unclear. It seems possible that the *Ac* promoter might be active at different rates in different seedlings of the same population with the result that they appear highly variegated, lightly variegated, or sensitive. If the activity of the promoter is different in individuals of the same family, then our mRNA measurements would represent an average figure for the entire population. In this case, perhaps high mRNA measurements in the RNase protection experiments simply indicate a relatively large proportion of plants that are expressing the TPase gene at a high level, and, therefore, the penetrance and expressivity of the fusion would be higher in this population. If increasing the expression of the TPase gene in Arabidopsis increases the penetrance of the fusion, then perhaps the more constant phenotypes observed in tobacco are the result of the *Ac* promoter being more active in tobacco than in Arabidopsis. This does not appear to be the case; our preliminary comparisons of the level of *Ac* mRNA in tobacco leaves and Arabidopsis inflorescences indicate that if there is a difference then the TPase mRNA is more abundant in Arabidopsis. The difference in the tobacco and Arabidopsis phenotypes might be a consequence of poor processing of the *Ac* mRNA in Arabidopsis or less efficient translation of the mRNA resulting in a lower concentration of the TPase protein. An alternative explanation is that the *Ac* TPase must interact with host factors to bring about transposition and that this occurs more efficiently in tobacco than Arabidopsis.

The different patterns of variegation detected with 35S::TPase in Arabidopsis and tobacco are equally difficult to explain. This construct produces many more sectors on the cotyledons of Arabidopsis than on those of tobacco. Again, this difference is unlikely to be caused at the level of transcription because the TPase mRNA appears to be equally abundant in tobacco and Arabidopsis plants containing this construct. One of the models of Scofield et al. (1992) proposes that the pattern of infrequent, large sectors on tobacco cotyledons might be due to the TPase protein reaching very high levels; these high TPase protein levels might then inhibit excision, preventing the formation of small sectors. This inhibitory mechanism is postulated to act in a way similar to the inverse dosage effect in maize but to require a higher concentration of TPase. If this model is correct, there are two ways in which this inhibitory mechanism might not come into play with this fusion in Arabidopsis. Either the level of TPase protein at which inhibition is induced is higher than in tobacco or maize so that the level of TPase produced by 35S::TPase does not inhibit excision in Arabidopsis, or less TPase protein is formed with the same level of transcription in Arabidopsis so that the TPase protein does not reach the inhibitory concentration.

Much of the interest in *Ac* behavior in transgenic plants is derived from its potential utility as an insertional mutagen. Genes inactivated by insertion of the transposon can be subsequently cloned by using the element as a probe, a process called transposon tagging. An active transposon system has not yet been reported in Arabidopsis, and there is, therefore, great interest in employing *Ac* in this model species. However, the low and variable activity of *Ac* in Arabidopsis has so far

made it difficult to use (reviewed by Balcells et al., 1991; Haring et al., 1991). The high frequency of somatic and germinal excision promoted by 35S::TPase clearly indicates that Arabidopsis cells provide all the host functions required for frequent *Ac* excision.

Is efficient transposon tagging, therefore, now a possibility in Arabidopsis? In general, we believe that it is, although there are considerations apart from the frequency of excision (Coupland, 1992). The first of these is the frequency of reinsertion of *Ds* after excision. Experiments that exploited an *Ac* insertion in the *Bronze* gene of maize demonstrated that 58% of F_1 plants that inherited a copy of the *Bronze* gene from which *Ac* had excised did not also contain a transposed *Ac* (Dooner and Belachew, 1989). Preliminary data obtained from our populations suggest that approximately 20% of Arabidopsis F_2 plants that inherited an active streptomycin resistance gene after activation by 35S::TPase also inherited a transposed *Ds* (D. Long, J. Swinburne, and G. Coupland, unpublished data); however, this result needs to be examined in more detail.

The second requirement for transposon tagging is that a large number of independent transposition events should be generated. Currently, we have studied one F_2 family which had several individuals that contained a transposed *Ds*, and, in this case, the position of *Ds* was the same in all individuals examined. This result suggests that the 35S::TPase construct drives excision early during the development of the F_1 plant, giving rise to a large somatic sector that is subsequently involved in gamete formation. To use 35S::TPase in a transposon tagging strategy, it is necessary, therefore, to generate a large number of F_1 plants to produce the independent events required.

METHODS

Plasmid Constructions

Ac::TPase was constructed by assuming that the *Ac* promoter was contained within *Ac* coordinates 32-355. This region was isolated as an RsaI-BglII fragment from an *Ac* derivative containing a BglII linker inserted within the untranslated leader sequence of *Ac* (Coupland et al., 1988). Because the main transcriptional start site of *Ac* is around coordinate 330 (Kunze et al., 1987), it was assumed that the RsaI-BglII fragment would contain the promoter region of *Ac*. This 324-bp fragment was ligated to a second *Ac* derivative that contains a BglII linker inserted within the untranslated leader at coordinate 735. The result is a stabilized *Ac* because it does not contain the first 31 bp that are essential for transposition. A 380-bp segment of the untranslated leader sequence between coordinates 355 and 735 has also been removed in this fusion. *Ac*::TPase was then inserted into a binary vector based on pCL0111 (Dean et al., 1992). The TPase fusion was inserted as a SacI-SmaI fragment into the SacI-HpaI sites of the binary vector. In this construct the 3′ end of the *Ac* fusion is located 283 bp from the right border of the T-DNA. This T-DNA also contains a fusion of the 1′-promoter to the neomycin phosphotransferase II gene coding sequence that acts as a selectable marker during transformation.

Plasmids SLJ1111, SLJ114B12, and SLJ1081 were used to provide the 35S::TPase, *ocs*::TPase, and *nos*::TPase fusions, respectively. In addition to the TPase fusions, these plasmids carry a neomycin phosphotransferase II gene transcribed by the CaMV 35S promoter and a *gus* gene transcribed by the 2′ promoter. The construction of these plasmids is described by Scofield et al. (1992).

The *Ds* construction contains an *Ac* derivative in which the EcoRI site was filled in using the Klenow fragment of DNA polymerase I; this gave rise to a frameshift mutation (Dean et al., 1992). This element was then inserted within the streptomycin phosphotransferase gene in vector pCL0111 (Dean et al., 1992).

All of the binary vectors used are based on pRK290 (tetracycline resistant) and were mobilized into *Agrobacterium tumefaciens* C58C1 by use of pRK2013 that contains the IncP conjugation system (Ditta et al., 1980). The nononcogenic Ti plasmid pGV2260 was present in the Agrobacterium strain and supplied the *virulence* gene functions required for transfer of the T-DNA (Deblaere et al., 1985). The internal BclI-XbaI fragment of *Ac*, which is located in exon 3, was inserted in the polylinker of pGEM3, and this was used as a template to synthesize the riboprobe.

Transformation of Arabidopsis

Arabidopsis thaliana Landsberg *erecta* was transformed by infection of root explants with Agrobacterium according to the method of Valvekens et al. (1988). To kill the Agrobacterium, augmentin (200 mg/L) was used as well as vancomycin (750 mg/L). Transformed shoots were selected with 50 mg/L kanamycin as described by Valvekens et al. (1988). In our hands, regenerated shoots never produced roots. Bolting plants were transferred to magenta pots where they set seed. These seeds were collected directly and tested for resistance to kanamycin. Approximately 3 months elapsed from the cocultivation of root explants until the collection of seeds.

Antibiotic Selection of Germinating Seedlings

Because large numbers of populations of seeds were selected on agar plates with kanamycin and streptomycin, a quick method of seed sterilization was developed. One population of up to 300 seeds was incorporated into a "packet" by folding a sheet of 7 × 7 cm Miracloth (Calbiochem) and closing this with a plastic-coated paper clip. The packets (up to 40) were soaked in a Sterilin 250-mL specimen container in 70% ethanol for 2 min and 0.5% SDS, 5% sodium hypochlorite for 15 min. Until this stage, the method can be performed on a laboratory bench. The packets are then transferred to the flow hood where they are washed at least three times in sterile water for approximately 10 min. Finally, packets were opened with forceps on Petri plates and allowed to dry before sowing.

Seeds were sown on GM medium (Valvekens et al., 1988) containing 50 mg/L kanamycin or 200 mg/L streptomycin. The plates were placed in the cold room for 4 to 7 days to promote germination and then transferred to the growth room. Phenotypes were scored after 2 to 3 weeks. In some experiments, those seedlings that were variegated on streptomycin-containing medium were classified as either highly variegated (HV) or lightly variegated (LV). Seedlings were defined as HV if they exhibited a total of at least approximately 20 green sectors on the cotyledons.

Genomic DNA Isolation and DNA Gel Blot Analysis

Genomic DNA was isolated from rosette leaves just before the plants started to bolt. The method of Chang et al. (1988) was used. For each sample, 5 μg of DNA was digested with the appropriate enzymes, and the fragments were separated on a 1% Tris-borate-EDTA (TBE)–buffered agarose gel that was subsequently transferred to a Hybond N membrane. DNA was immobilized on the damp filter by UV stratalinking using a reading of 2400 on a Stratagene stratalinker and subsequently baking the filter under vacuum at 80°C. Filters were prehybridized, hybridized, and washed according to Sambrook et al. (1989). DNA fragments to make probes were isolated from agarose gels using a Gene Clean kit (Bio-101, La Jolla, CA) and were labeled using a random priming method (Feinberg and Vogelstein, 1983).

Assay for GUS Activity

GUS activity was assayed essentially as described by Jefferson (1987). Two to three rosette leaves were harvested and crushed in an Eppendorf tube with approximately 0.1 g of Polyclar AT/polyvinylpolypyrrolidone and 100 μL of extraction buffer. The mixture was centrifuged for 5 min in a microcentrifuge, and 100-μL aliquots of the supernatant were assayed in microtiter dishes using 4-methylumbelliferyl β-D-glucuronide as substrate assay buffer. Each sample was tested in triplicate by incubation at 37°C for 0, 2, and 16 hr. The reaction was then stopped by the addition of sodium carbonate and the microtiter dish was exposed to UV light. Positive samples could be easily identified as those showing an increase in fluorescence after 2 and 16 hr of incubation (Jefferson et al., 1987).

RNA Extractions

Inflorescences containing approximately four to eight developing siliques were harvested, wrapped in aluminium foil, weighed, frozen in liquid nitrogen, and stored at −70°C until use. Total RNA was prepared according to Logemann et al. (1987). Tissue (0.2 to 1.0 g) was ground in liquid nitrogen using a mortar with pestle. The powder was transferred to either an Eppendorf tube or a 50-mL polypropylene tube containing 2 volumes of guanidine hydrochloride buffer (Logemann et al., 1987) for each gram of tissue. With some batches of tissue, it was necessary to increase the amount of buffer to 4.5 volumes per gram of tissue to reduce the viscosity of the final solution. The mixture was immediately homogenized with a vortex, and three phenol/chloroform extractions were performed. The RNA was then precipitated by the addition of 0.7 volumes of ethanol and 0.2 volumes of 1 M acetic acid and was finally dissolved in water. The RNA concentration was determined by the orcinol colorimetric reaction in duplicate or triplicate samples (Schneider, 1957). A_{260} was also measured, and, assuming an OD_{260} of 1 indicates an RNA concentration of 40 mg/L, the orcinol reaction values were between 25 and 70% higher. RNA integrity and lack of DNA contamination were determined by electrophoresis through a 2% agarose gel in TBE buffer.

RNase Protection

Single-stranded riboprobes were synthesized as described by Sambrook et al. (1989) using SP6 RNA polymerase and approximately 125 μCi of α-^{32}P-UTP (3000 Ci/mmol). Probes were used within 24 hr. A riboprobe of 2 to 5 $\times$ 10^5 cpm contained in approximately 1 μL of Tris-EDTA (TE) buffer was mixed with 50 or 100 μg of RNA (if necessary wild-type RNA was added up to 50 μg) in 30 μL of hybridization buffer. The RNA was denatured at 90°C for 10 min and was hybridized for 13 to 16 hr at 47°C. RNaseA and T_1 treatment, proteinase K digestion, and phenol/chloroform extractions were performed as described by Sambrook et al. (1989). Yeast tRNA (20 μg) was added to the double-stranded RNA fragments, and the mixture was precipitated with ethanol at −20°C for 2 to 3 hr. After centrifugation, pellets were washed once with 75% ethanol, briefly dried at 42°C, and dissolved in 2.5 or 6 μL (depending on the well size of the gel) of loading buffer. RNA was denatured at 90°C for 10 min, immediately chilled on ice, and loaded on a denaturing 6% acrylamide, 8M urea gel. Electrophoresis was performed on a Raven tank system using 1× TBE buffer. The gel was fixed with 10% acetic acid and 10% methanol for 30 min, transferred to filter paper, and dried under vacuum at 80°C. The gel was exposed to x-ray film at −70°C for 2 to 20 days, and the signals were quantified using a Loeb-Joyce model Chromatoscan 3 densitometer. 35S::TPase transformant E or *ocs*::TPase transformant A were included in all experiments, and these were used as points of comparison between experiments. This protocol assumes that the amount of RNA detected in different RNase protection experiments with these two transformants was identical. When all values had been obtained, an average reading for each transformant was calculated and the figure for *Ac*::TPase B was converted to 1. All other values are given relative to this transformant.

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