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Promoter Fusions to the *Activator* Transposase Gene Cause Distinct Patterns of *Dissociation* Excision in Tobacco Cotyledons

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To explore the effects of altering the level of *Activator (Ac)* transposase (TPase) expression, a series of plasmids was constructed in which heterologous promoters were fused to the TPase gene. Promoters for the cauliflower mosaic virus (CaMV) 35S transcript and the octopine synthase *(ocs)* and nopaline synthase *(nos)* genes were tested. These fusions, and constructs expressing TPase from the wild-type *Ac* promoter, were introduced into tobacco, and their activity was monitored by crossing to a line carrying *Dissociation (Ds)* in a streptomycin phosphotransferase gene (*Ds*::SPT). The SPT marker provides a record of somatic excisions of *Ds* that occur during embryo development. The patterns of somatic variegation that resulted from transactivation by each fusion were distinct and strikingly different from the pattern triggered by the wild-type *Ac* constructs. Unlike wild-type *Ac*, which caused transposition throughout embryo development, each fusion gave rise to sectors of discrete size. Sectors triggered by the CaMV 35S fusion were largest, *ocs* sectors were intermediate, and *nos* were smallest. These patterns appear to indicate differential timing of the activation of these promoters during embryogeny. Measurement of transcript abundance for each transformant indicated that the CaMV 35S-transformed plants accumulated approximately 1000-fold more TPase mRNA than plants containing wild-type *Ac*, whereas *ocs*- and *nos*-transformed lines accumulated about 100-fold and 20-fold higher levels, respectively. Measurements of germinal excision frequencies driven by the chimeric TPase fusions, however, indicated that increasing transcription does not necessarily result in an increase in germinal excision. These measurements showed that the *ocs* and *nos* fusions have very low rates of germinal excision. Only the CaMV 35S fusion transformants were found to have higher rates than the *Ac* constructs, although significant pod-to-pod variation was observed. Gel blot analysis of DNA from progeny carrying germinal excision events resulting from the CaMV 35S fusion showed that excision is associated with reinsertion and that siblings sometimes carry the same transposition events. These findings suggest that in tobacco there is no direct proportionality between TPase expression and *Ac-Ds* transposition activity. This possibility has important implications for understanding the regulation of *Ac* transposition and for designing efficient gene tagging systems.

INTRODUCTION

The maize transposon *Activator (Ac)* has been shown to be able to transpose in tobacco (Baker et al., 1986), Arabidopsis and carrot (VanSluys et al., 1987), tomato (Yoder et al., 1988), potato (Knapp et al., 1988), and soybean (Zhou and Atherly, 1990). These findings suggest that by making transgenic plants carrying transposons of the *Activator* family, an insertional mutagenesis system could be established in plants that at present carry no known transposons.

The *Ac-Ds* system is well characterized. Studies in transgenic tobacco have shown that approximately 200 bp at each end of the element is required in *cis* (Coupland et al., 1989) and expression of the transposase gene is necessary in *trans* (Kunze and Starlinger, 1989) for *Ac-Ds* transposition. In tobacco and maize, there is a preference for transposition to linked sites (Greenblatt, 1984; Jones et al., 1990). Therefore, as in maize, the likelihood of tagging a specific target locus will be increased by starting the experiment with *Ac* or *Ds* at a position linked to the target (Döring, 1989). Typical germinal excision frequencies of *Ac* in maize are 2 to 5% (Dooner and Belachew, 1989). In transgenic tobacco, the germinal excision frequency of *Ac* is at least as high as, and probably higher than, in maize (Jones et al., 1991).

A puzzling aspect of *Ac* behavior is the relationship between *Ac* dosage and activity. In maize, as the number of copies of *Ac* is increased, the frequency of *Ac* or *Ds* excision is reduced and its timing delayed (McClintock, 1951). This has been termed the inverse dosage effect. In tobacco, however, increasing *Ac* dosage from one to two copies results in a positive effect on somatic and germinal excision frequency (Jones et al., 1989, 1991; Hehl and Baker, 1990). The reason for this interesting

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contrast in *Ac* behavior in the two hosts is not known. Conceivably, expression of high transposase levels (above a certain threshold) is inhibitory to transposition, and the thresholds differ in the two species. One way to investigate this idea is to determine the consequences for *Ds* excision of fusing the transposase gene to promoters of varying strengths.

Germinal excision frequencies of *Ac* are not uniformly high in all heterologous host plant species. A notable example is Arabidopsis, a plant whose suitability for genetic studies and small genome would, otherwise, make it well suited for transposon tagging strategies. Germinal excision frequencies have been measured to be as low as 0.2 to 0.5% in Arabidopsis (Schmidt and Willmitzer, 1989). Engineered transposase sources could achieve more favorable germinal excision frequencies in the system, and this provides an additional rationale for this experimental approach (Swinburne et al., 1992).

As a first step in evaluating the effects of altering *Ac* transposase expression, we have constructed a series of promoter fusions to the transposase (TPase) gene. Here, we report the relative effectiveness of the chimeric TPase gene fusions and the wild-type *Ac* constructs in the somatic and germinal transactivation of *Ds* inserted in a streptomycin phosphotransferase gene (*Ds*::SPT) (Jones et al., 1989). Our findings have interesting implications for understanding the regulation of the *Ac-Ds* system and the developmental regulation of the promoters used in these studies.

RESULTS

Construction of Promoter Fusions to the *Ac* TPase Gene and Their Introduction into Tobacco

To test the effects of altering the expression of *Ac* TPase, a series of chimeric TPase genes was constructed, as shown in Figure 1. Transcriptional fusions were made that bring the TPase gene under control of the cauliflower mosaic virus (CaMV) 35S promoter (Odell et al., 1984) or of promoters for the Agrobacterium genes octopine synthase (*ocs*) (DeGreve et al., 1983) and nopaline synthase (*nos*) (Depicker et al., 1982). The promoters were joined to the TPase gene at the NaeI site at nucleotide 964 of *Ac* that is 22 bp 5′ to the TPase translational initiation codon. Fusion at this site eliminates almost all of the 652-base 5′ untranslated leader of the *Ac* transcript. To ensure that an adequate leader sequence was present, an 82-bp fragment was synthesized (see Methods) carrying the tobacco mosaic virus (TMV) Ω leader sequence (Gallie et al., 1987) and inserted between the TPase and the promoter fragments. These constructs were designated 35S::TPase, *ocs*::TPase, and *nos*::TPase, respectively. Two plasmids were made to compare the activity of these fusions with that of *Ac*. The first is a derivative of *Ac*, which is unable to transpose due to a terminal deletion that does not interfere with the *Ac* TPase gene. This construct, designated *sAc*, for stabilized *Ac*, was generated by truncating the element at a BglII site introduced 177 bp from the 3′ end (Jones et al. 1989, 1991).

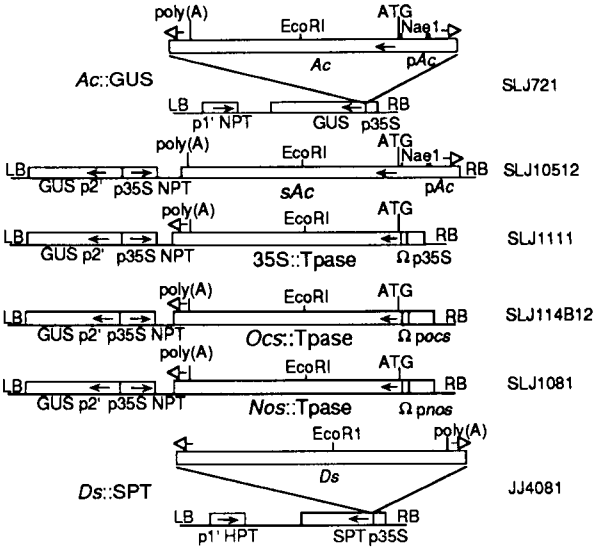

Figure 1. *Ac* and *Ds* T-DNA Constructs Used To Assess the Activity of TPase Fusions.

Five sources of TPase were used to transactivate *Ds* from construct JJ4081. Serving as a control is a construct containing an autonomous, wild-type *Ac* element inserted in a CaMV35S::GUS excision marker. *sAc* is a nonautonomous deletion derivative of *Ac* generated by cleaving at the BglII site introduced 177 bp from the 3′ end of *Ac* (Jones et al., 1989). Transcriptional fusions of the 35S, *ocs*, and *nos* promoters were made at the NaeI site 22 bp 5′ to the *Ac* translational initiation codon. In place of the long *Ac* leader, deleted almost entirely, a synthetic leader based on the TMV Ω sequence was substituted. LB and RB indicate the left and right border sequences of the T-DNA. NPT designates the neomycin phosphotransferase gene, which provides selection for transformation, on the vectors except JJ4081, which utilizes a hygromycin phosphotransferase (HPT) fusion as a selectable marker for plant transformation. The *sAc* and promoter fusion constructs contain T_R2'::GUS (β-glucuronidase) fusions permitting rapid screening for the presence of the TPase source T-DNA. The direction of transcription of each gene is indicated by a solid arrow. Open arrows mark the 11-bp inverted repeat sequences of *Ac*. The sequences of *Ac* associated with polyadenylation are indicated by poly(A). The EcoRI site is indicated because it was used in subsequent gel blot analysis (see Figure 4).

The second construct, designated *Ac*::GUS, consists of a wild-type, autonomous *Ac* element inserted in the 5′ leader of a CaMV 35S::β-glucuronidase (GUS) gene fusion (Jefferson et al., 1987).

The chimeric TPase genes and the 3′ deleted *Ac* element were each inserted into a binary vector, SLJ1006 (J. Jones, G. Bishop, and S. Scofield, unpublished data). This plasmid is based on the octopine Ti plasmid and carries a CaMV 35S promoter fusion to the neomycin phosphotransferase (NPTII) gene as a transformation marker and a fusion between the GUS gene and the T_R2' promoter (Velten et al., 1984) to permit rapid screening for presence of the T-DNA. The TPase genes were all inserted so that the promoters are adjacent to the right border of the T-DNA and transcribe the TPase gene

convergently relative to the NPTII gene. The autonomous *Ac* element was inserted into a related binary vector, SLJ 732, which carries a T_R1' ::NPT transformation marker (Velten et al., 1984) and a 35S::GUS fusion to monitor the excision of *Ac*, resulting in plasmid SLJ721 (J. Jones and S. Scofield, unpublished data).

Plasmids carrying the TPase promoter fusions were mobilized into Agrobacterium by triparental mating (Figurski and Helinski, 1979; Ditta et al., 1980), and the resulting strains were used to transform tobacco cultivar Petite Havana (Horsch et al., 1985). Six plants were regenerated for each TPase fusion construct. Four transformants carried active CaMV 35S fusions, and these were designated 35S::TPase C, D, E, and H. Four lines (A, G, H, and I) carried active *ocs*::TPase fusions, and two lines (C and H) carried active *nos*::TPase fusions. All of the fusion transformants showed 3:1 segregation for kanamycin resistance in selfed progeny, indicating a single T-DNA locus in each plant. Of the four active *sAc* primary transformants, two were single locus (B and G) and the other two (A′ and D) had five kanamycin resistance loci (data not shown).

Somatic Excision Phenotypes

The phenotypes conferred by the TPase fusions were monitored using a visual assay for the excision of *Ds* from a chimeric streptomycin phosphotransferase gene (*Ds*::SPT). The TPase source plants were crossed to another transgenic line, JJ4081D, homozygous for SPT::*Ds* (Jones et al., 1990). Excision of *Ds* from the 5′ untranslated leader of SPT restores expression of streptomycin resistance. In this assay, somatic excisions are observed as green sectors on white cotyledons of tobacco seedlings germinated on streptomycin-containing medium, and germinal excisions result in fully green seedlings. Cotyledons begin to form in dicotyledonous plants as the embryo makes the transition from globular to heart stage. Cell division in cotyledons is limited to the period of embryo development; the increase in size after germination results only from cell expansion. Therefore, sectors observed in this assay provide a record of *Ds* excisions that occurred during embryogenesis. Sector size reflects the timing of somatic excision; i.e., larger sectors result from earlier events, and smaller sectors reflect later excision. The shape of a sector is also influenced by the timing of excision because of changes in the orientation of cell division during cotyledon formation (Christianson, 1986).

Very obvious differences in the variegated phenotypes are evident when the different TPase sources transactivate the *Ds*::SPT tester line, as shown in Figure 2. Each fusion triggers excisions that give rise to sectors of distinct and consistent size. The phenotype associated with wild-type *Ac* or *sAc* is characterized by sectors of variable size, in that some sectors may comprise as much as one-half of a cotyledon (Figure 2E), whereas others consist of a few cells. Variegation resulting from the autonomous *Ac* construct, SLJ721, is only distinguishable from *sAc* by the occurrence of a more highly variegated phenotype in about 2% of progeny; this result is probably due to the positive effect of increased dosage brought about by acquisition of a transposed *Ac* (Jones et al., 1991). The multicopy *sAc* transformants A′ and D give rise to progeny with different degrees of variegation when crossed to *Ds*::SPT, presumably reflecting changes in dosage as the unlinked copies of *sAc* segregate.

The striking characteristic of the three promoter fusion phenotypes is the consistency of sector sizes. The 35S::TPase sectors are large and ovate with the long axis oriented longitudinally on the cotyledon. Their lengths range from 5 to 50% of the cotyledon, with most sectors being in the range of 10 to 20%. The sectors triggered by the *ocs*::TPase fusion are much smaller than those of the 35S::TPase fusion. The *ocs* sectors are slightly heterogeneous in size, with the larger sectors having an elongated shape, whereas the smallest are round. The *nos* fusion causes extremely small sectors, the largest consisting of about eight cells and the smallest being a single cell. The *nos* sectors are also distinguished by predominantly occurring on the lower side of the cotyledon (data not shown).

Independent transformants of a given fusion construction are quite similar in terms of the number of sectors per cotyledon. The 35S::TPase transformants crossed to the *Ds* tester have six to 10 sectors on average. The *ocs* and *nos* fusions result in so many sectors that the number is impossible to determine accurately because of the three-dimensional arrangement of those cells that have the potential to produce streptomycin-resistant sectors.

Crosses between heterozygous single locus TPase source plants and homozygous single locus *Ds*::SPT testers resulted in approximately 50% of the progeny exhibiting variegation. No loss of penetrance was observed in transactivating *Ds*::SPT through crosses with any of the TPase sources (data not shown).

Test cross progeny of variegating lines for each fusion have been examined for two further generations with no detectable change in the somatic excision patterns (data not shown).

Measurement of Germinal Excision Frequencies

Germinal excision frequencies were measured by growing plants that were doubly heterozygous for an *Ac* TPase source and *Ds*::SPT to maturity, and then crossing them to wild-type tobacco cultivar Petite Havana. Because one-half of the resulting progeny inherit the *Ds*::SPT T-DNA, 100% germinal excision would be indicated by 50% of the total progeny appearing as fully green on streptomycin plates. Reciprocal crosses were made in which the TPase source construct, *Ds*::SPT, double heterozygote was used as either the male or female parent so that differences in expression of the promoters in the male or female reproductive lineages might be observed. The results of this analysis are summarized in Figure 3.

The positive effect of multiple copies of *Ac* was evident when the germinal excision frequencies of the *sAc* constructs were examined. The double heterozygotes that were generated when *sAc* transformants A′ and D were crossed to the *Ds*::SPT line each contained three copies of *sAc* and resulted in germinal

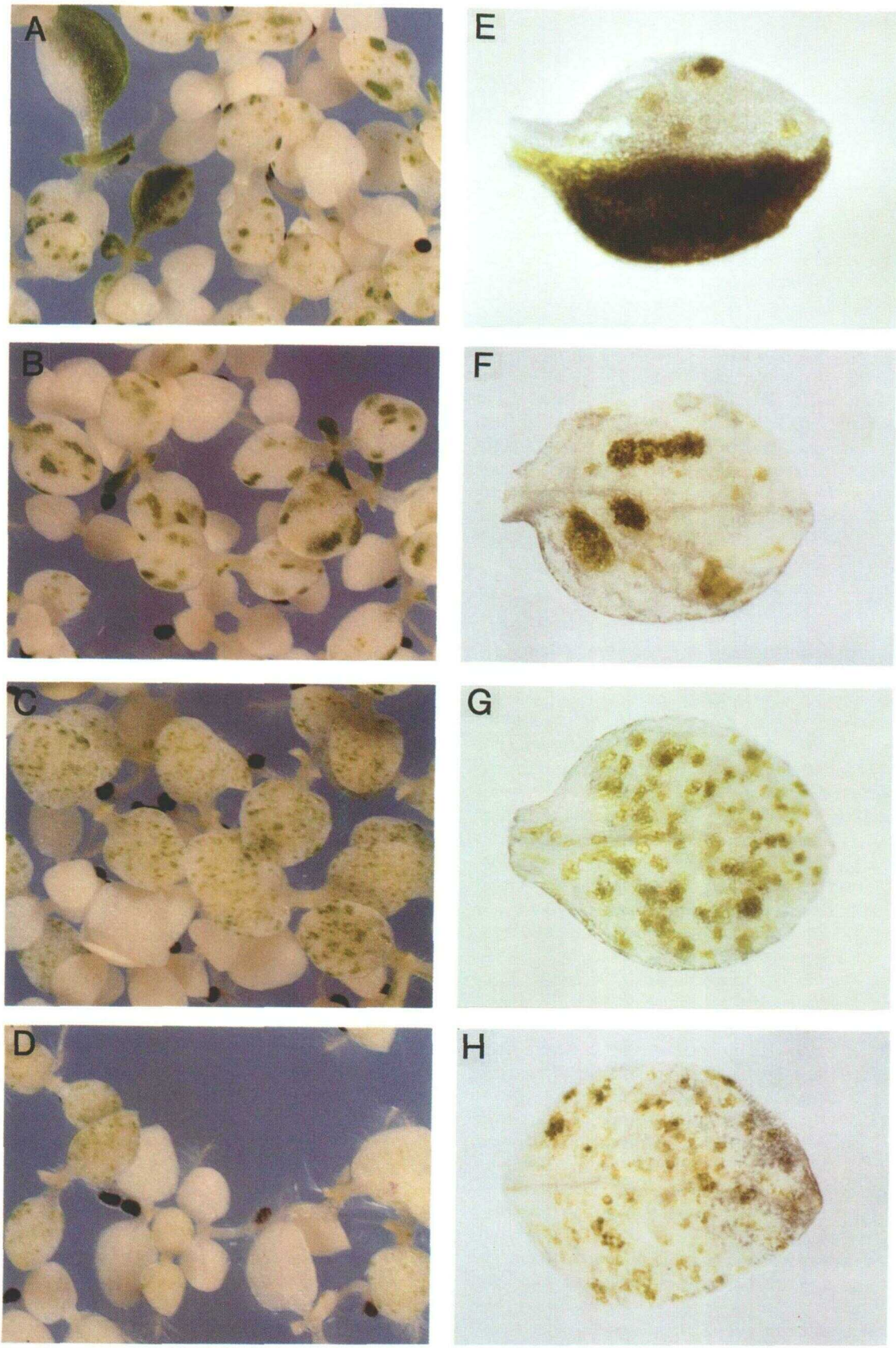

Figure 2. Somatic Variegation Resulting from Transactivation of *Ds*::SPT by Different *Ac* TPase Sources.

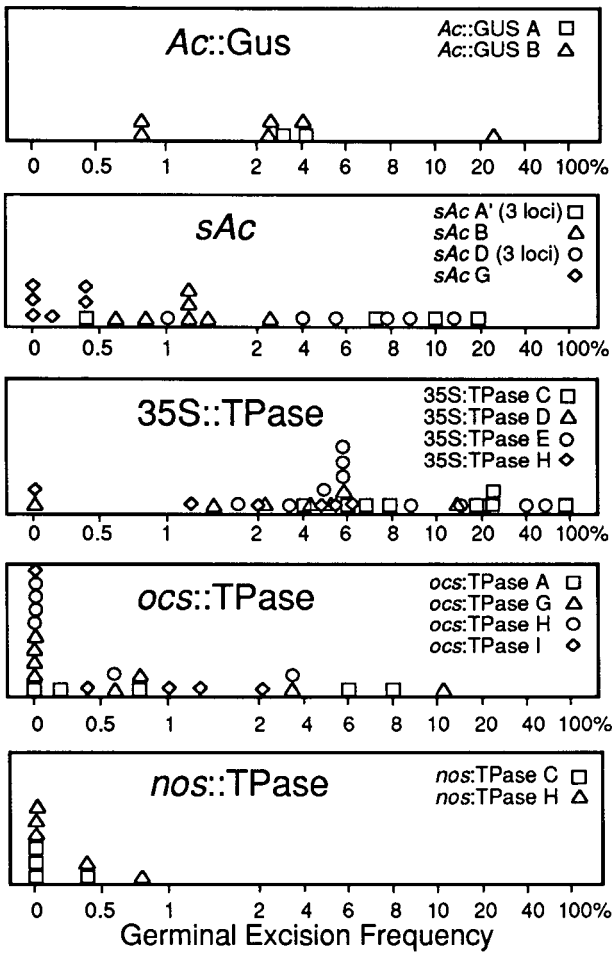

Figure 3. Germinal Transposition Frequencies Measured in Individual Pods from Crosses of TPase Source, *Ds*::SPT Double Heterozygotes to Untransformed Tobacco Cultivar Petite Havana.

These graphs present *Ds*::SPT germinal excision data for each transformant carrying each TPase source construct. The placement of each symbol along the nonarithmetic horizontal axis reflects the germinal excision frequencies of 1 to 14%, whereas the single copy *sAc* transformants ranged from 0 to 2.4%. The single copy *sAc* TPase double heterozygotes gave rise to germinal excision rates similar to or slightly lower than those measured for the single copy *Ac*::GUS transformants.

The *nos* fusions brought about a germinal excision rate of almost zero. The *ocs* fusion also showed very little activity, frequently giving rise to pods with no fully green seedlings. No obvious differences in the activities of any of the constructs emerged from the reciprocal cross analysis.

Only the 35S::TPase fusions showed germinal excision frequencies above the *Ac* derivatives. Many pods were observed with rates of approximately 6% germinal excision, and this may represent the basal rate of germinal excision triggered by this construct; however, a great range of excision rates was observed. Pods were found covering the theoretical minimum and maximum rates, 0 to 100%. Such fluctuation in the rates of germinal excision measured for different pods is in stark contrast to the regularity of cotyledon variegation phenotype

frequency measured in a single pod. As only one-half of such progeny inherit *Ds*::SPT, 100% germinal excision would appear as 50% fully green seedlings. Therefore, the percentage of fully green seedlings arising in a pod has been doubled to give the germinal excision frequencies plotted here. Plates containing 150 to 1200 seedlings were scored for each data point. As no discernible difference in germinal excision frequency was observed when the double heterozygote was used as either the male or female parent, the graphs were generated from pooled data. Unless noted, all double heterozygote parents contain a single TPase source T-DNA locus. The mean germinal excision rate for the single copy *sAc* transgenic lines B and G was calculated to be 0.38%, the 35S::TPase lines had a mean rate of 6.83%, the *ocs*::TPase rate was 0.87%, and the *nos*::TPase rate was 0.09%. The Student's *t* test indicated that the 35S::TPase rate was significantly higher than the single copy *sAc* rate at the 1% probability level. The *ocs*::TPase and *nos*::TPase rates were significantly different from the *sAc* rate at the 5% probability level.

Figure 2. Somatic Variegation Resulting from Transactivation of *Ds*::SPT by Different *Ac* TPase Sources.

(A) to **(D)** Low magnification views of progeny from the test cross of TPase source, *Ds*::SPT double heterozygotes to untransformed Petite Havana (PH) plated on Murashige and Skoog medium supplemented with glucose and containing 300 μg/mL streptomycin.
(A) *sAc*, *Ds*::SPT × PH.
(B) 35S::TPase, *Ds*::SPT × PH.
(C) *ocs*::TPase, *Ds*::SPT × PH.
(D) *nos*::TPase, *Ds*::SPT × PH.
(E) to **(H)** High magnification views of single cotyledons of seedlings selected to illustrate characteristic somatic variegation of *Ds*::SPT by various TPase sources. Selected cotyledons from variegated seedlings germinated on streptomycin-containing media were fixed in 1% formaldehyde. Fixation causes the streptomycin-sensitive cells to collapse, permitting better visualization of the streptomycin-resistant excision sectors.
(E) *sAc*, *Ds*::SPT × PH.
(F) 35S::TPase, *Ds*::SPT × PH.
(G) *ocs*::TPase, *Ds*::SPT × PH.
(H) *nos*::TPase, *Ds*::SPT × PH.

and suggests that this fusion may create large sectors that may later contribute to reproductive lineages; therefore, very different rates may be observed from pod to pod, depending on the distribution and size of these premeiotic sectors. Further evidence for this hypothesis has come from another set of crosses (S. Scofield and J. D. G. Jones, unpublished data), which demonstrated that, on at least one occasion, an entire side shoot of a plant doubly heterozygous for 35S::TPase and *Ds*::SPT transmitted only the excised form of SPT.

Genomic Blot Analysis of Germinal Excisions Arising from CaMV 35S Fusions

To learn whether the excisions triggered by the fusions were bona fide transposition events, DNA was prepared from seedlings selected as carrying germinal excision events in crosses of double heterozygotes for the 35S::TPase and *Ds*::SPT with untransformed Petite Havana. The DNAs were digested with EcoRI, separated on 0.8% agarose gels, blotted, and probed sequentially with a series of fragments representing the 5′ and 3′ halves of *Ds*, the SPT coding sequence, or the octopine T-DNA right border sequence. The multiple probings were necessary because the JJ4081D transformant, although carrying a single hygromycin resistance locus, proved to be the result of a complex T-DNA insertion. This analysis, in combination with polymerase chain reactions specific for *Ds*-SPT and *Ds*-35S promoter junction sequences, indicated that JJ4081D contains one complete T-DNA flanked on each side by a truncated copy, each carrying only a 5′ half of *Ds* (data not shown).

DNA gel blot analysis is presented in Figure 4 of all the individuals carrying germinal excision events arising in a single pod from the test cross of a 35S::TPase E, *Ds*::SPT double heterozygote. The filter was hybridized with a probe from the 3′ half of *Ds*. Because only the intact *Ds* in JJ4081D hybridizes to the 3′ *Ds* probe, a single 7.8-kb band is seen in the JJ4081D control sample (Figure 4, lane 3). The disappearance of this band resulting from germinal excision of *Ds* is seen in all the test cross progeny (Figure 4) as are new bands indicating reinsertion of *Ds*. An EcoRI band of 13 kb is present in DNA of the 35S::TPase E parent. This indicates that the single kanamycin resistance locus present in the 35S::TPase E transformant carries just one copy of T-DNA. The 13-kb band segregates among the test cross progeny. Absence of the 13-kb TPase band in conjunction with an excised *Ds* is direct proof that these progeny contain germinally transposed *Ds*'s.

A common 3.7-kb transposed *Ds* band is present in the germinal excision progeny shown in lanes 4, 6, 7, 8, 10, 11, 12, and 14 of Figure 4. This provides more evidence for the suspected occurrence of large clonal, premeiotic excision sectors. Also, it is interesting to note that the germinal excision progeny in lanes 7, 10, 13, and 15 all lack the 13-kb TPase source band and contain more than one transposed *Ds* band. These individuals indicate that more than one transposition of *Ds* may occur within a single generation.

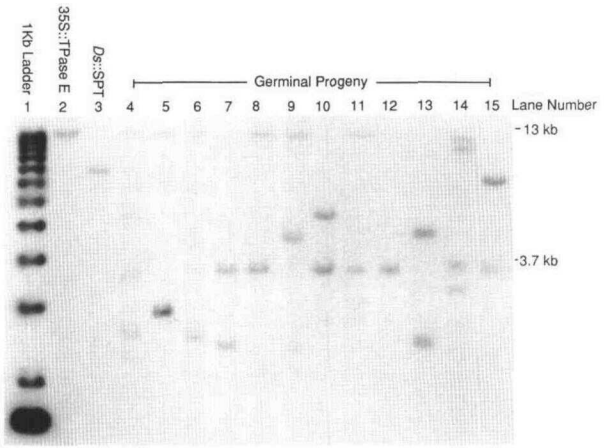

Figure 4. Genomic Blot Analysis of DNA from Siblings Selected as Having Inherited a Germinally Excised Form of the SPT Marker from the Test Cross of a 35S::TPase Fusion E, *Ds*::SPT Double Heterozygote by Untransformed Petite Havana.

All the genomic DNA samples were digested with EcoRI and probed with a 2.0-kb EcoRI fragment from the 3′ end of *Ds*. Lane 2 contains DNA from the 35S::TPase fusion E parent, and lane 3 contains DNA from the 4081D *Ds*::SPT parent. Lanes 4 to 15 contain DNA from the fully green test cross progeny.

Quantitation of TPase mRNA Levels by RNase Protection Analysis

RNase protection experiments were carried out to compare the relative levels of TPase mRNA accumulation among the various transformants. Total RNA was prepared from leaves of different TPase source, *Ds*::SPT double heterozygotes. Aliquots of the leaf RNA were hybridized to labeled RNA transcribed from the plasmid pSP649. This plasmid contains the 0.9-kb EcoRI-HindIII fragment of *Ac* oriented so that SP6 RNA polymerase synthesizes RNA complementary to the *Ac* message (Kunze et al., 1987). After digestion of the hybrids with RNases A and T_1, the length of the protected RNA should be 753 bases.

Figure 5A shows an autoradiogram from an RNase protection experiment in which RNA samples from all the active CaMV 35S, *ocs*, and *nos* transformants, and two *sAc*-transformed plants, A′ and G, were examined. This array shows not only the great difference in transcript abundance between different promoter fusions, but also reveals differences, if any, between transformants carrying the same construct. The CaMV 35S transformants showed very little between-transformant variation, whereas the *ocs*-, *nos*-, and *sAc*-transformed plants showed more variability. The fact that the *sAc*-transformed plant A′ accumulates more than *sAc*-transformed plant G is consistent with *sAc* A′ having three T-DNA loci, whereas *sAc* G contains just a single locus, assuming a simple structure for each introduced locus. The variation in RNA levels within the *ocs* and *nos* transformants cannot be explained on the basis

of locus number because all are single locus transformants, again assuming simple T-DNA structures. It is interesting that the differences in mRNA accumulation between transformants carrying the same construct are not reflected by any discernible effects on somatic or germinal excision. This is in sharp contrast to the behavior of *sAc*-transformed lines that showed a very clear positive dosage effect in both somatic and germinal excision assays.

With the result in Figure 5A as a guide, an RNase protection experiment using serially diluted RNAs was performed to more accurately establish the relative *Ac* transcript levels for each type of construct. The results are shown in Figure 5B. From this analysis, we have determined that the CaMV 35S transformants accumulate approximately 2000-fold as much TPase mRNA as the single locus *sAc* G transformant. The strongest *ocs* transformant, H (Figure 5B, lane 19), expresses about 100-fold more than the *sAc* G transformant (Figure 5B, lane 2), whereas the weakest *nos* transformant, C (Figure 5B, lanes 11 to 17), expresses about 16-fold more TPase mRNA than *sAc* G.

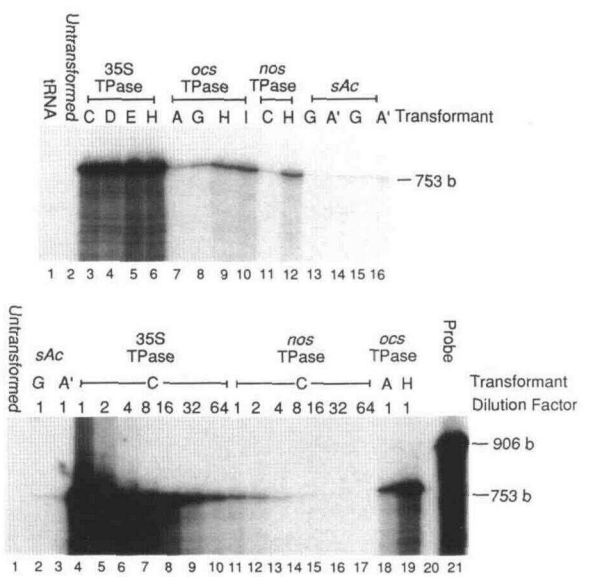

Figure 5. RNase Protection Analysis of the Expression of *Ac* TPase mRNA in Leaves of Transgenic Tobacco.

(A) A comparison of expression of all TPase fusion transformants to *sAc*-transformed lines A′ and G. A labeled 906-base riboprobe (see **[B]**, lane 21) complementary to the *Ac* transcript was hybridized to total leaf RNA prepared from TPase source, *Ds*::SPT double heterozygotes and digested with RNases A and T_1. The molecules protected from digestion were then separated on a 3.5% polyacrylamide/urea denaturing gel. The expected length of the RNA protected by the processed *Ac* transcript is 753 bases. Control digestions of the probe plus 50 μg of tRNA and the probe plus 50 μg of Petite Havana RNA are shown in lanes 1 and 2, respectively. Lanes 3 to 14 indicate probe protection by 50 μg of total RNA from each TPase fusion transformant and *sAc* sources G and A′. Hybridizations to 150 μg of *sAc* G and A′ RNA were performed in lanes 15 and 16, respectively, in an effort to enhance the signal from the weak *Ac* promoter.

(B) RNase protection by serially diluted TPase source RNAs. Representative samples were chosen on the basis of expression levels shown in **(A)** for dilution analysis. The RNA samples were loaded as follows: lane 1, 50 μg of untransformed Petite Havana; lane 2, 50 μg of *sAc* G; lane 3, 50 μg of *sAc* A′; lane 4, 50 μg of 35S::TPase C; lane 5, 25 μg of 35S::TPase C; lane 6, 12.5 μg of 35S::TPase C; lane 7, 6.25 μg of 35S::TPase C; lane 8, 3.13 μg of 35S::TPase C; lane 9, 1.56 μg of 35S::TPase C; lane 10, 0.78 μg of 35S::TPase C; lane 11, 50 μg of *nos*::TPase C; lane 12, 25 μg of *nos*::TPase C; lane 13, 12.5 μg of *nos*::TPase C; lane 14, 6.25 μg of *nos*::TPase C; lane 15, 3.13 μg of *nos*::TPase C; lane 16, 1.56 μg of *nos*::TPase C; lane 17, 0.78 μg of *nos*::TPase C; lane 18, 50 μg of *ocs*::TPase A; lane 19, 50 μg of *ocs*::TPase H; lane 20, no sample; lane 21, undigested probe. From the dilution series, the following relationships were determined: *nos*::TPase C is 16× > *sAc* G; 35S::TPase C is 128× > *nos*::TPase C; 35S::TPase C is 64× > *ocs*::TPase A; 35S::TPase C is 16× > *ocs*::TPase H.

DISCUSSION

Comparison of the Patterns of Somatic Excision of *Ds*::SPT Caused by TPase Fusions and *Ac*

We have constructed a series of promoter fusions to the *Ac* TPase gene and compared the ability of these TPase sources to transactivate a *Ds* element with that of wild-type *Ac*. This analysis shows very marked differences among the TPase gene fusions in patterns of somatic excision.

The cotyledon sector pattern is a record of *Ds* excisions that occur during embryo development. The size and shape of the sectors are, therefore, a consequence of the timing of the *Ds* excision event. The different TPase fusions trigger *Ds* excisions at clearly distinct times during development. The heterogeneity of sector sizes resulting from the *Ac* and *sAc* constructs results, presumably, from continuous expression of TPase activity throughout embryo development. However, a clear temporal sequence is inferred for the time at which each chimeric gene fusion begins to catalyze *Ds* transposition; 35S::TPase causes the earliest excisions, followed by *ocs* and then *nos*. We presume that maximal sector size is an indication of the earliest time in development at which the gene fusions generate sufficient TPase to begin to trigger *Ds* excisions. This level of TPase is probably low because significant somatic variegation results from the extremely low level of transcription from the wild-type *Ac* promoter (Füsswinkel et al., 1991). We are currently trying to gain independent evidence for this interpretation by measuring the accumulation of transcripts from these promoters in developing embryos and by studying the time course of induction of 35S::GUS, *nos*::GUS, and *ocs*::GUS fusions during embryogenesis. The *Ds* excision assay is a very sensitive indication of temporal and spatial expression of promoter activity, and we suggest that fusions to TPase could be a powerful new tool for the analysis of promoter activity during development.

The size and shape of the streptomycin-resistant sectors observed in these experiments are also of interest for understanding patterns of cell division and lineage in cotyledon formation. The *sAc*-triggered sector comprising one-half of the cotyledon shown in Figure 2E is much like the largest chimeric sectors observed in semigametic cotton (Christianson, 1986). In both cases, the cotyledon midrib appears to define a compartmental boundary for cell lineages. Also, a general change in the orientation of cell division is indicated by the transition from large elongated 35S::TPase sectors to small rounded sectors of the *ocs*::TPase and *nos*::TPase fusions.

A curious aspect of the phenotype of the 35S::TPase fusion is that it gives rise to a rather regular pattern of large sectors with very few smaller sectors being observed. Why are there only large sectors and not a continuum of smaller sizes? At least two explanations are possible. The first is the most simple. The CaMV 35S promoter may be transiently activated during cotyledon development and later, after germination, resumes high-level activity in the seedling. A discontinuous pattern of expression could have gone undetected by studies on the 35S promoter fused to reporter genes whose protein products are extremely stable, such as GUS (Jefferson et al., 1987).

A second possible explanation invokes a common basis for the CaMV 35S-conferred phenotype and the inverse relationship usually seen between *Ac* dosage and activity in maize, where increasing *Ac* dosage from one to two copies results in a lowering of transposition frequency and a delay in its timing (McClintock, 1951). In tobacco, however, it has been shown that two doses of *Ac* result in a higher frequency of transposition than one (Jones et al., 1989). It remains a possibility that inverse dosage is an intrinsic property of *Ac* and that the threshold at which it is observed is different in each host plant. Consistent with this interpretation would be the idea that the CaMV 35S fusion triggers some early sectors as it first becomes active, but it quickly reaches levels of expression that are above a certain threshold and subsequent excisions are suppressed. This effect would also provide an explanation for the striking regularity observed for the phenotypes of different transformants carrying the same construct. Dramatic variation between transformants in the expression of transgenes is a common phenomenon in plant systems (Jones et al., 1985). If a self-limiting mechanism is at work, all the transformants with a given construct might exhibit similar phenotypes despite differences in the level of expression, provided that they all exceeded the inhibitory level of TPase expression at about the same time in development. A prediction of the inhibition model is that if seedlings carry both the early-acting 35S::TPase construct and the later acting *ocs*::TPase fusion, then the appearance of small *ocs* sectors will be suppressed by the 35S::TPase fusion. This prediction is being tested.

If the CaMV 35S fusion phenotype has the same basis as the inverse dosage effect in maize, then in the transgenic system we have a unique opportunity to dissect the mechanism in the absence of the large number of heterogeneous *Ds* elements present in maize lines.

Comparison of the Effects of Different TPase Fusions on Germinal Excision Frequencies

The data presented in Figure 3 demonstrate clearly that substitution of promoters has a significant effect on the rate of germinal *Ds* excision. Among the fusion series, only the CaMV 35S fusion showed rates of germinal excision above those measured for the *sAc* or *Ac*::GUS control transformants. On average, the "baseline" germinal excision frequency brought about by a 35S::TPase fusion is about fivefold higher than that of a one locus *sAc* transformant. In a few 35S fusion pods, extremely high rates (26 to 100%) were measured, although most pods showed rates of about 6%. The *ocs* and especially the *nos* fusion showed very low germinal activity. The germinal excision frequencies caused by *sAc* seem rather low in comparison to those of an SPT::*Ac* heterozygote (Jones et al., 1991). It is possible that the complex structure of the 4081D T-DNA may reduce the frequency of streptomycin-resistant sectors or progeny due to *Ds* excision, compared with other T-DNAs carrying *Ds*::SPT.

DNA gel blot analysis of CaMV 35S germinal excision progeny demonstrates two important points. First, excisions triggered by this fusion are associated with reinsertion of *Ds*. Second, eight of 12 progeny from the same pod appear to contain the same germinal *Ds* transposition (Figure 4). This observation is likely to explain the high degree of variation in germinal excision frequencies observed from pod to pod. Sectors arising at different times before the formation of pollen or egg cells could have significant effects on the observed numbers of germinal, prezygotic, *Ds*::SPT excisions. It remains to be determined whether the CaMV 35S fusion will be useful in tagging applications. For it to be advantageous, the acceleration in germinal excision frequency will need to be great enough to counterbalance the tendency for clonal *Ds* insertion.

The mRNA measurements show that despite the fact that *ocs* and *nos* promoter fusions express approximately 100 and 10 times the level of TPase mRNA, respectively, in leaves as the *sAc* transformants, they exhibit almost no ability to trigger germinal excisions of *Ds*. Perhaps this indicates that the *ocs* and *nos* promoters have little activity in the L2 histogenic layer that gives rise to germ cells in plant development (Dawe and Freeling, 1990). Our findings suggest that to significantly improve the efficiency of germinal transposition triggered by wild-type *Ac* elements in tobacco, TPase sources will need to be carefully engineered not only in terms of abundance but also in timing and cell specificity of expression.

METHODS

DNA Constructions

The recombinant plasmids (Figure 1) were constructed by standard techniques (Maniatis et al., 1982). Full details of the constructions will be supplied upon request. The DNA fragment carrying the CaMV 35S

promoter in SLJ1111 consists of the BglII-XhoI fragment that spans the sequences −198 to +4, as described elsewhere (Harpster et al., 1988). An XhoI site was introduced into the *ocs* promoter at position +18 (J. Jones and S. Scofield, unpublished data) by site-directed mutagenesis using the method described by Kunkel (1985). This promoter was then incorporated into SLJ114B12 as a BamHI-XhoI fragment spanning nucleotides −122 to +18 (DeGreve et al., 1983). The *nos* promoter fragment was generated by introducing an XhoI site at +5 in the *nos* transcript sequence (Depicker et al., 1982). This resulting fragment was then incorporated as a BclI-XhoI fragment, spanning nucleotides −266 to +5, into SLJ1081.

The fragment synthesized to provide the 5′ untranslated leader of TMV Ω has the following sequence: 5′-TCGAGTATAAGAGCTCTATTTTTACAACAATTACCAACAACAACAAACAACAAACAACATTACAATTACTATTTACAATTAC-3′. DNA sequences determined in the final constructs indicated that the T at position 70 was missing in all three of the TPase fusions and the T at position 17 was missing in the 35S::TPase construct. We believe these 1-base deletions occurred during synthesis of the oligonucleotides.

Plant Transformation

All transformations were performed with the streptomycin-sensitive *Nicotiana tabacum* cv Petite Havana. Binary T-DNA constructs were mobilized into *Agrobacterium tumefaciens* LBA4404 (Hoekema et al., 1983). Transgenic tobacco plants were regenerated as described by Horsch et al. (1985).

Ds::SPT Excision Assay

Transgenic seeds were harvested and plated on medium consisting of Murashige and Skoog salts (ICN Biomedicals Inc., Costa Mesa, CA), 0.8% agar, 1% glucose, and 300 μg/mL streptomycin (Maliga et al., 1988; Jones et al., 1989). Variegation could be observed within 2 weeks for all transformed lines, although the *nos* fusion pattern became more obvious after another 1 to 2 weeks, during which time further cotyledon expansion occurs.

Isolation of Genomic DNA

Genomic DNA was isolated from young, unexpanded tobacco leaves. Half gram leaf pieces were homogenized using a Meku (Wennigsen, Germany) sap-extraction press by the protocol described by Clarke et al. (1989). The homogenates were extracted with an equal volume of phenol/chloroform (1:1), and ethanol precipitated. The pellets were dissolved in 300 μL of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 and then were mixed with an equal volume of 0.2 M Tris-HCl, pH 7.5, 0.05 M EDTA, 2.0 M NaCl, 2% CTAB (hexadecyltrimethylammonium) and incubated at 65°C for 10 min. The samples were then extracted with an equal volume of chloroform and precipitated with isopropanol. Approximately 50 μg of DNA was obtained from each leaf piece.

Analysis of Genomic DNA

Ten micrograms of genomic DNA was digested with EcoRI and separated on 0.8% agarose gels. The DNA was transferred by alkaline vacuum blotting to GeneScreen Plus (New England Nuclear, Boston, MA) membranes. The resulting filters were probed with gel-purified restriction fragments that were radiolabeled by the random priming method (Feinberg and Vogelstein, 1983).

Isolation of Leaf RNA

Total RNA was prepared from leaves of plants heterozygous for both *Ds*::SPT and one of the different TPase sources by a standard method (Harpster et al., 1988).

RNase Protection Analysis

The RNase A + T_1 protection studies were performed as described in Sambrook et al. (1989). Aliquots of total leaf RNA were hybridized with an SP6-synthesized probe complementary to the coding strand of the 906-bp EcoRI-HindIII fragment of *Ac*. To generate probes of high specific activity, transcriptions were performed in 15-μL reactions containing 150 μCi of α-^{32}P-UTP (800 Ci/mmol) and supplemented with cold UTP to give a final concentration of 24 μM UTP. These probes routinely gave detectable signals after 48 hr of autoradiography when hybridized to 50 μg of total RNA from *sAc* transformants. Untransformed tobacco RNA was added to serially diluted samples to maintain 50 μg of RNA in each hybridization.

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