

# Epigenetic Instability and *Trans*Silencing Interactions Associated With an *SPT::Ac* T-DNA Locus in Tobacco

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## ABSTRACT

Progeny of tobacco line 2853.6, which carries a *streptomycin phosphotransferase* (*SPT*) gene interrupted by the maize element *Activator* (*Ac*), were selected for streptomycin resistance (Spr) because of germinal *Ac* excision. Some events gave rise to Spr alleles that were unstable and exhibited a mottled phenotype on streptomycin-containing medium due to somatic loss of *SPT* function. This instability was most pronounced in one particular line, Spr12F. Other Spr alleles rarely exhibited silencing of *SPT*. Streptomycin-sensitive, homozygous Spr12F plants were recovered, and crosses were performed with other, more stable Spr lines. A high proportion of the resulting heterozygous progeny were silenced for *SPT* expression. The silenced state was heritable even after the Spr12F allele segregated away. No correlation could be made between silencing and methylation of the *SPT* gene. Structural analysis of allele Spr12F showed that the *SPT* gene from which *Ac* had excised was flanked by direct repeats of *Ac*. A search was carried out among 110 additional Spr alleles for new independent unstable alleles, and four were identified. All of these alleles also carried an *SPT* gene flanked by direct repeats of *Ac*. Thus, there is a strong correlation between this structure and instability of *SPT* expression.

A number of epigenetic phenomena involving interactions between repeated sequences, either unlinked or at allelic positions, result in the silencing or alteration of gene expression in plants. One of the first examples to be described in detail was paramutation involving the *r* locus in maize (Brink 1973). One allele (paramutagenic) could alter the expression of another allele (paramutable) in a directed and heritable way, resulting in a new (paramutant) allele. Paramutation has also been observed at the *b* (Coe 1966) and *pl* (Hollick *et al.* 1995) loci of maize and the *sulfurea* locus of tomato (Hagemann 1969). Other potentially related phenomena include cycling of maize transposable elements between active and inactive phases (Martienssen and Richards 1995); *trans*-silencing interactions between *nivea* alleles in *Antirrhinum majus* (Bollman *et al.* 1991); repeat-induced point mutations (RIP) (Singer and Selker 1995); methylation-induced premeiotically (MIP) (Rossignol and Faugeron 1995); quelling in fungi (Cogoni *et al.* 1996); and various examples of gene silencing in transgenic plants (Jorgensen 1992; Flavell 1994; Matzke and Matzke 1995b).

The introduction of foreign DNA sequences into plants has produced many examples of gene silencing (Jorgensen 1992; Flavell 1994; Matzke and Matzke 1995b). An early study showed a puzzling inverse rela-

tionship between T-DNA copy number and marker gene expression in transgenic petunia plants (Jones *et al.* 1987). Many more recent studies have shown that interactions between repeated sequences can cause gene silencing, also known as *transinactivation* (Matzke and Matzke 1995a; Flavell 1994). The repeated sequences can comprise unlinked T-DNA loci (Matzke and Matzke 1995a; Hobbs *et al.* 1990) or, in the case of cosuppression, an endogenous gene and a homologous introduced T-DNA locus (Napoli *et al.* 1990; van der Krol *et al.* 1990). T-DNA insertions with complex structures may be particularly prone to cosuppression (Jorgensen *et al.* 1996; Cluster *et al.* 1996).

Paramutation at the maize *b* and *r* genes has been studied extensively both at the genetic (Coe 1966; Brink 1973; Kermicle *et al.* 1995) and molecular levels (Patterson *et al.* 1993; Eggleston *et al.* 1995; Patterson and Chandler 1995). A strong correlation has been made between paramutation of the complex *r* locus and the number of repeated *r* genes present (Kermicle *et al.* 1995). In contrast, paramutation of the *b* locus is not associated with the presence of repeated sequences (Patterson and Chandler 1995).

The mechanisms underlying gene silencing are not known. Some examples involve decreased transcription of the affected gene(s) (Pröls and Meyer 1992; Patterson *et al.* 1993; Park *et al.* 1996), while others occur by post-transcriptional mechanisms (Mueller *et al.* 1995; Decarvalho *et al.* 1992; English *et al.* 1996). In general, gene silencing is associated with repeated DNA sequences (Flavell 1994; Cluster *et al.* 1996;

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Hobbs *et al.* 1990), but there are exceptions (Elmayan and Vaucheret 1996).

Here we describe a series of alleles derived from a *SPT::Ac* T-DNA locus in tobacco. Transformed tobacco plants were generated using construct pJJ2853, which contains a T-DNA carrying the bacterial *neomycin phosphotransferase* (*NPT*) gene under the control of the *Agrobacterium tumefaciens nopaline synthase* (*nos*) promoter and the *streptomycin phosphotransferase* (*SPT*) gene under control of the 2' or *mannopine synthase* promoter (Jones *et al.* 1989). One line, 2853.6, carried two copies of the T-DNA, which inserted as an inverted repeat around the right border (Figure 1) (Jones *et al.* 1990). Germinal transpositions of *Ac* were isolated by screening for fully streptomycin-resistant (*Spr*) individuals. The positions of transposed *Ac* (*trAc*) elements were determined genetically relative to the functional *SPT* gene (Jones *et al.* 1990). Somatic loss of *SPT* activity was observed at different frequencies among the different families. This loss of *SPT* function was the subject of the investigation described here.

Most of the 2853.6 *Spr* derivatives exhibited somatic loss of *SPT* function at low frequencies. An exceptional allele, *Spr*12F, showed frequent somatic loss and occasional germinal loss of *SPT* function. Analysis of completely streptomycin-sensitive *Spr*12F (white) plants showed that loss of *SPT* activity was not associated with DNA structural rearrangements. This, and the fact that *Spr*12F (white) plants were homozygous for the *Spr*12F T-DNA, suggested that an epigenetic phenomenon was responsible for loss of *SPT* activity. The *Spr*12F (white) allele was shown to direct silencing *in trans* when crossed to other 2853.6 *Spr* alleles. By Southern blotting and DNA sequence analysis of previously described alleles (Jones *et al.* 1990) and new alleles generated in this study, we show a strong correlation between a particular type of DNA structure and high frequency somatic loss of *SPT* function in 2853.6 *Spr* derivatives. The relationship of this type of gene silencing with other epigenetic phenomena, and possible implications regarding the mechanisms involved, are discussed.

## MATERIALS AND METHODS

**Genetic stocks:** All work was carried out with the streptomycin-sensitive *Nicotiana tabacum* cultivar Petite Havana (PH). Transformant 2853.6, carrying an *Ac* element inserted in the 5' untranslated leader of a chimeric streptomycin phosphotransferase gene (*SPT::Ac*), has been described previously (Jones *et al.* 1989). In this transformant, the T-DNA was inserted as an inverted repeat around its right border (Figure 1). A series of excision alleles of the 2853.6 locus [described previously in Jones *et al.* (1990) and new alleles described in this work] were selected as streptomycin-resistant progeny from testcrosses between untransformed PH and a variegated line that was homozygous for the 2853.6 T-DNA. This is represented as 2853.6/2853.6 X -/-.

**Visualization of streptomycin resistance phenotype:** Seedlings were germinated on medium consisting of Murashige

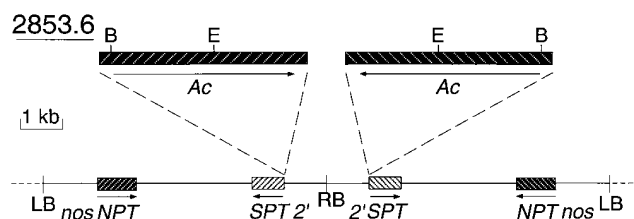


Figure 1.—Structure of the T-DNA insertion in tobacco transformant 2853.6. *SPT*, *streptomycin phosphotransferase*; *NPT*, *neomycin phosphotransferase*; LB, RB, T-DNA left and right borders are indicated. E, *EcoRI*; B, *BamHI* restriction sites are shown to indicate the orientation of *Ac*. Arrows show the direction of transcription of *Ac*, *SPT*, and *NPT*.

and Skoog salts (ICN Biomedicals Inc., Costa Mesa, CA), 0.8% agarose, 1% glucose, and 300  $\mu$ g/ml streptomycin (Jones *et al.* 1989). The streptomycin resistance phenotype was visualized after 10–14 days. The seedlings pictured in Figure 2 represent typical examples obtained from each cross indicated. Each cross was performed 3–5 times and  $\sim$ 200 seedlings were observed for each cross.

**DNA extraction and Southern blot analysis:** DNA extraction was performed as described previously (English *et al.* 1993). For Southern blot analysis, 10  $\mu$ g of genomic DNA was digested with the appropriate restriction enzyme and separated on 1% agarose gels. DNA was transferred to Hybond-N hybridization membranes (Amersham, Arlington Heights, IL) by capillary blotting. The resulting filters were probed with gel-purified DNA fragments which were  $^{32}$ P-labeled by the random priming method (Feinberg and Vogelstein 1983). Probe fragments used were probe A, which contains the *NPT* coding sequence (see Figure 3) and the *octopine synthase* (*ocs*) 3' end, *Ac* 5' (2.5 kb fragment 5' to the *EcoRI* site), and *Ac* 3' (2 kb fragment 3' to the *EcoRI* site).

**Inverse polymerase chain reaction:** Inverse polymerase chain reaction (IPCR) (Ochman *et al.* 1988) was performed as described by Thomas *et al.* (1994). Restriction enzymes *AluI*, *Sau3A*, *TaqI* and *HaeIII* were used. Primers B38 (GATATACCGTAACGAAAACGAACG, *Ac* positions 89–114), B39 (TTT CGTTCCTCCGTCGCCAAGTTAAATA, *Ac* positions 84–58), B34 (ACGGTCGGTACGGGATTTTCCCAT, *Ac* positions 4525–4496) and B35 (TATCGTATAACCGATTTTGTAGTT, *Ac* positions 4526–4549) were used. *Ac* sequence positions are numbered as in Pohlman *et al.* (1984).

**Northern blot analysis:** RNA was extracted from 2-week-old plants (*i.e.*, the same age as visualization of streptomycin resistance) as described previously (Muelier *et al.* 1995). Five  $\mu$ g RNA samples were electrophoresed on 0.8% (w/v) agarose/formaldehyde gels according to Sambrook *et al.* (1989), transferred to Hybond-N membranes (Amersham, Arlington Heights, IL), and hybridized with  $^{32}$ P-labeled DNA probes corresponding to the *SPT* or *NPT* coding sequences.

## RESULTS

**Somatic instability of *SPT* expression in *SPT::Ac* excision alleles:** Germinal excision alleles from the transgenic tobacco line 2853.6 carrying *SPT::Ac* have been characterized previously (Jones *et al.* 1990) with respect to reinsertion of the excised *Ac* element and the genetic distance between the transposed *Ac* (*trAc*) and the functional streptomycin resistance gene (*SPT*).

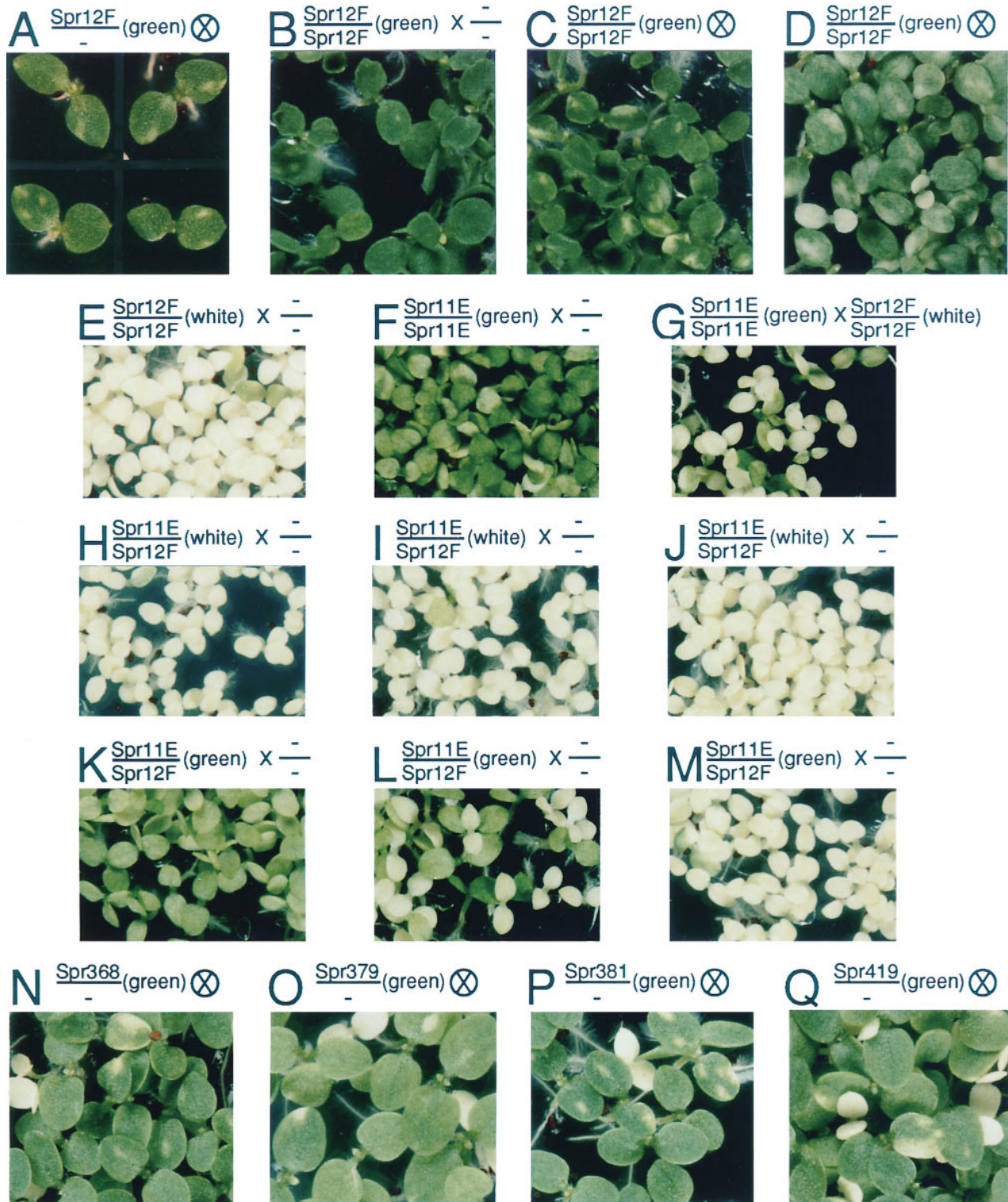


Figure 2.—Streptomycin resistance phenotypes of 2853.6 derivatives. Seedlings were photographed 10–14 days after being sown on streptomycin-containing medium. In each panel, the seedlings shown are progeny from the cross indicated. The genotype of each parent is given and the streptomycin resistance phenotype (*i.e.*, white, streptomycin-sensitive; green, streptomycin-resistant) is shown in parenthesis. (–) represents the locus corresponding to the 2853.6 T-DNA insertion in untransformed tobacco. Thus, –/– represents untransformed tobacco.

Fourteen fully streptomycin-resistant (Spr) plants, hemizygous for different 2853.6 excision alleles, were self-pollinated. When the resulting progeny were germinated on streptomycin-containing media, white (strep-

tomycin-sensitive) sectors were observed on a background of green (streptomycin-resistant) cotyledon cells. This mottled phenotype occurred at different frequencies among the different families. Typical sectors

**TABLE 1**  
**Frequency of white-sectored seedlings vs genetic distance of trAc from SPT**

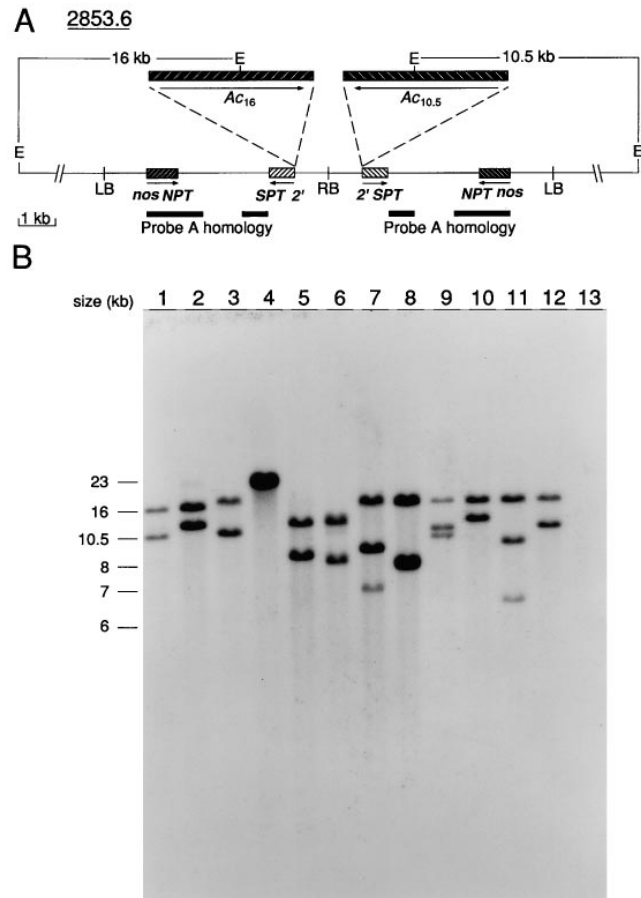
Allele	Recombination frequency between trAc and <i>SPT</i> (%)	Frequency of white-sectored seedlings in selfed progeny of hemizygotes (% of greens)
Spr1A	0.2	0
Spr1E	0.4	2
Spr2	0.2	3
Spr6A	0.2	17
Spr6F	0.8	1
Spr10B	43 (unlinked)	2
Spr10C	53 (unlinked)	1
Spr10D	0.3	1
Spr10N	0.4	6
Spr11B	2.2	2
Spr11E	0.6	16
Spr12C	44 (unlinked)	1
Spr12D	1	2
Spr12F	0.5	60
Spr368	0.6	40
Spr379	0.3	42
Spr381	0.5	48
Spr419	2.5	43

Plants hemizygous for each allele were self-pollinated. Progeny were germinated on streptomycin-containing media and evaluated for *SPT* expression. Recombination frequencies between trAc and *SPT* (for previously described alleles) are from Jones *et al.* 1990. Recombination frequencies between trAc and *SPT* for new alleles were calculated in the same way.

are shown in Figure 2A. Table 1 lists the families that were examined for white sectors, the frequency with which white-sectored seedlings were observed, and the percentage of recombination between trAc and the *SPT* gene. Most families exhibited white-sectored seedlings at frequencies ranging from 0-6%. In two families, Spr6A and Spr11E, 16-17% of green seedlings had white sectors. One exceptional family, Spr12F, exhibited a much higher frequency of white-sectored seedlings than the others, with 60% of green seedlings having white sectors.

The percentage recombination expressed in Table 1 can only be interpreted as unlinked (in the cases of Spr10B, Spr10C, and Spr12C) or very closely linked (in the rest). This is because secondary transpositions of *Ac* can contribute to the apparent recombinant class (as described in Jones *et al.* 1990). Thus, the values given here are maximum values for percent recombination. There is no correlation between the frequency of white-sectored seedlings and the position of trAc at this level of resolution.

**Molecular analysis of *SPT::Ac* excision alleles:** To address whether the structures resulting from *Ac* transposition in the *SPT::Ac* excision alleles might provide information about the mechanism by which *SPT* activity



**Figure 3.**—Southern blot analysis of DNA from 2853.6 derivatives. (A) Diagram of the 2853.6 locus. *EcoRI* restriction sites (E) are indicated. The extent of homology to probe fragment A (*NPT* coding sequence and *ocs* 3' end) is indicated. The two *Ac* elements are distinguished by which *EcoRI* fragment they are associated with and have been named *Ac*<sub>10.5</sub> and *Ac*<sub>16</sub>, accordingly. (B) DNA from 2853.6 and derivatives was digested with *EcoRI*, Southern blotted, and hybridized with probe A. Lane 1, 2853.6; lane 2, Spr11B; lane 3, Spr12D; lane 4, Spr1A; lane 5, Spr10D; lane 6, Spr2; lane 7, Spr1E; lane 8, Spr12F; lane 9, Spr368; lane 10, Spr379; lane 11, Spr381; lane 12, Spr419; lane 13, untransformed tobacco.

was being lost, a molecular analysis of these alleles was undertaken. Southern blot analysis was performed on *EcoRI*-digested DNA from transformant 2853.6 and each of the excision alleles. A typical Southern blot, including each type of transposition event observed, is shown in Figure 3. There are two *EcoRI* sites within the 2853.6 T-DNA, one in each *Ac* element. Probe A hybridizes to bands of 16 and 10.5 kb in DNA from transformant 2853.6 (Figure 3, lane 1). The two *Ac* elements can be identified by the band to which they correspond and have been designated *Ac*<sub>10.5</sub> and *Ac*<sub>16</sub>, accordingly. In the *SPT::Ac* excision alleles, the *Ac* element that excised can be determined from analysis of which probe A-hybridizing band has been altered. If one of the *Ac* elements excises, the probe A-hybridizing fragment extends to the *EcoRI* site in the other *Ac* element (as long as the fragment is not disrupted by transposition of *Ac*

into it). This results in a predictable 1-kb increase in size.

In allele Spr11B (lane 2),  $Ac_{10.5}$  excised, and an 11.5-kb band resulted. The same result was obtained with alleles Spr6A and Spr10N (data not shown). In allele Spr12D (lane 3),  $Ac_{16}$  excised, and a 17-kb band appeared. The same result was obtained with alleles Spr6F and Spr11E (data not shown). Both *Ac* elements excised in allele Spr1A (lane 4), giving rise to one band of 23 kb. Alleles Spr10D (lane 5) and Spr2 (lane 6) have the 11.5-kb band predicted for excision of  $Ac_{10.5}$ . The 16 kb band is no longer present in these individuals because  $Ac_{10.5}$  transposed into this fragment, giving rise to bands of 8.7 and 8.3-kb, respectively. Alleles Spr1E (lane 7) and Spr12F (lane 8) retain the 16-kb band, indicating that  $Ac_{16}$  has not excised. Transposition of  $Ac_{10.5}$  into the region of probe A homology disrupted the 10.5-kb band and two new bands were produced in each. Allele Spr1E has bands of 7 and 9.6 kb. Bands of 8.2 and 8.4 in allele Spr12F were not resolved on this blot.

Southern blot analysis using *Hind*III (for which there are two recognition sequences in each *Ac* element and not elsewhere in the T-DNA) and probed with *Ac* 5' and *Ac* 3' probes confirmed which *Ac* had transposed and the positions and orientations of tr*Ac* elements within the T-DNA (data not shown). Additional confirmation came from analysis of sequences flanking the tr*Ac* elements in alleles Spr1E, Spr10D, and Spr12F (data not shown). Figure 4 shows schematic representations of the structures of the *SPT::Ac* excision alleles.

**Structures of *SPT::Ac* excision alleles:** It is clear from the structures of the *SPT::Ac* excision alleles that *Ac* can frequently transpose very short distances in tobacco. Of fourteen transposition events characterized (Jones *et al.* 1990 and this study), four were to positions within 10.5 kb of the original starting position. This raises the possibility that the streptomycin-sensitive sectors observed may be because of reinsertion of *Ac* into *SPT*. This appeared to be the case in three out of five streptomycin-sensitive hemizygous plants derived from Spr1E and Spr10D testcrosses (data not shown).

An interesting comparison can be made between the structures present in the Spr1E and Spr12F alleles. The tr*Ac* in Spr1E (tr*Ac*<sub>1E</sub>) transposed 1.4 kb to a position downstream of *SPT* and is in the same orientation as in the 2853.6 progenitor (Figure 4). The tr*Ac*<sub>12F</sub> transposed 2.1 kb, also to a position downstream of *SPT*, but the orientation has changed so that the two *Ac* elements are in direct orientation (Figure 4). The only differences detected between Spr1E and Spr12F are the 700 bp difference in the insertion sites of the tr*Ac* elements and the orientations of the tr*Ac* elements. Nonetheless, line Spr12F exhibits a very high frequency of white-sectored seedlings, while line Spr1E gives a very low frequency of white-sectored seedlings (Table 1).

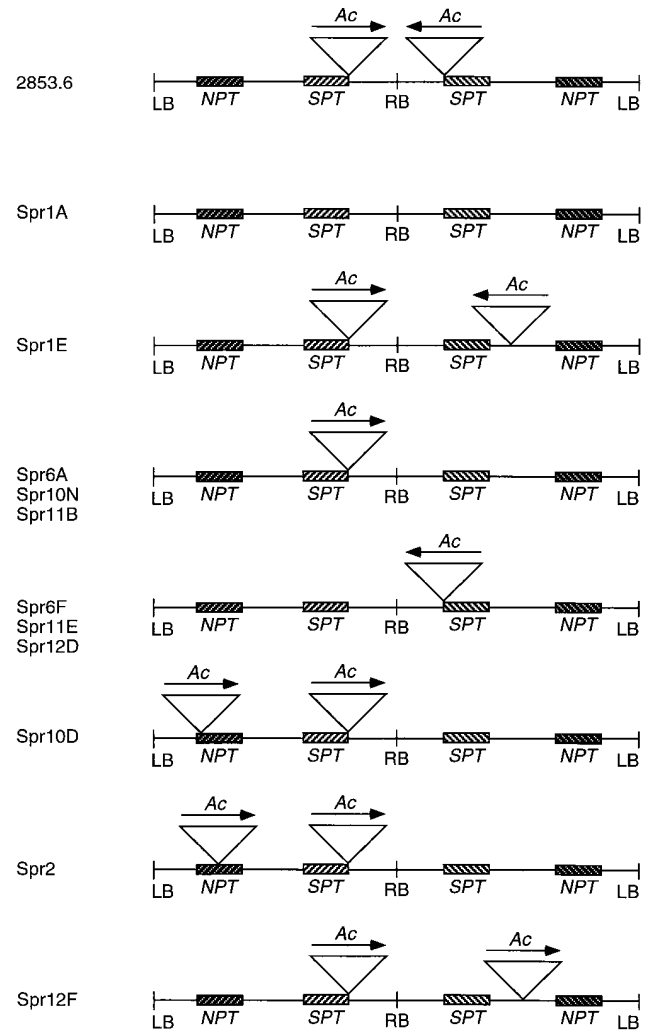


Figure 4.—Structures of the 2853.6 locus in 2853.6 parent and derivatives. Orientation of *Ac* elements is indicated by an arrow that points in the direction of *Ac* transcription. LB, RB—T-DNA left and right borders, respectively; *NPT*, neomycin phosphotransferase; *SPT*, streptomycin phosphotransferase are indicated.

Several types of rearrangements involving the two *Ac* elements in direct orientation in allele Spr12F could lead to loss of *SPT*. For example, homologous recombination between the directly repeated *Ac* elements in Spr12F would cause loss of *SPT*. If the two *Ac* elements in Spr12F acted as a *macrotransposon* (Ralston *et al.* 1990) and were excised, the DNA between them (which includes *SPT*) would be deleted. Also, chromosome breakage induced by the closely linked *Ac* elements could result in loss of *SPT* (Ralston *et al.* 1989; Dooner and Belachew 1991). However, as the following sections explain, DNA structural rearrangements are unlikely to account for the majority of *SPT* marker gene loss described here.

**Comparison of the frequency of white-sectored seedlings in Spr12F homozygotes and hemizygotes:** Five plants

homozygous for the *Spr12F* allele were self-pollinated and crossed to untransformed tobacco. Progeny seed, homozygous and hemizygous for the *Spr12F* allele, respectively, were germinated on streptomycin-containing medium. A high proportion of seedlings homozygous for the *Spr12F* allele had white sectors (Figure 2C). The frequency of white sectors was drastically reduced in *Spr12F* hemizygous seedlings (Figure 2B). The phenotype of hemizygous seedlings was similar whether the *Spr12F* allele was transmitted through male or female gametes (data not shown). Similar results were obtained with progeny from each of the four *Spr12F* parents used. No increase in the frequency of white-sectored seedlings in homozygotes versus hemizygotes was observed with any of the other 2853.6 *Spr* alleles (data not shown). These results argue against the mechanisms described above in which DNA structural rearrangements would cause loss of *SPT* function in *Spr12F* seedlings.

**Recovery of streptomycin-sensitive *Spr12F* homozygous plants:** In addition to the majority of white-sectored

seedlings in self progeny of *Spr12F* homozygous plants, fully white seedlings were observed at a low frequency (zero to a few percent), as shown in Figure 2D. Four streptomycin-sensitive *Spr12F* homozygous plants were transferred to antibiotic-free medium and grown to maturity. Southern blot analysis was performed using 7 different digests with restriction enzymes cutting within and outside of the *Ac* elements and an *SPT* probe, *Ac* 5', and *Ac* 3' probes. An example is shown in Figure 5. No differences were detected between streptomycin-sensitive and streptomycin-resistant *Spr12F* homozygous plants at the Southern blot level (Figure 5; data not shown). This result provides additional support for the idea that DNA structural rearrangements were not involved with loss of *SPT* function in *Spr12F* plants.

The streptomycin-sensitive phenotype was heritable in self (data not shown) and testcross (Figure 2E) progeny from *Spr12F* (white) homozygous plants. Reversion to streptomycin resistance was observed in 1–2% of testcross progeny. Reversion was not always complete; intermediate phenotypes were often observed. In addition to *SPT*, the markers *NPT* and *Ac* were also tested for activity. The *Spr12F* streptomycin-sensitive seedlings were fully kanamycin resistant (data not shown). To test for *Ac* activity, streptomycin-sensitive *Spr12F* plants were crossed to an *SPT::Ds* tester line (Jones *et al.* 1990). In each case, these crosses produced seedlings that were highly variegated due to *Ds* excision, suggesting that at least one *Ac* element was active (data not shown).

Expression of the *SPT* and *NPT* marker genes was analyzed by Northern blot analysis. Total RNA was extracted from pooled *Spr12F* homozygous seedlings from a streptomycin-resistant line (as in Figure 2C) and a streptomycin-sensitive line (similar to Figure 2E). A Northern blot was made and probed with the *SPT* coding sequence as shown in Figure 6. No *SPT* transcript was detected in the streptomycin-sensitive line, while the resistant line gave a strong signal at 0.7 kb, the expected size. After stripping and reprobing with a frag-

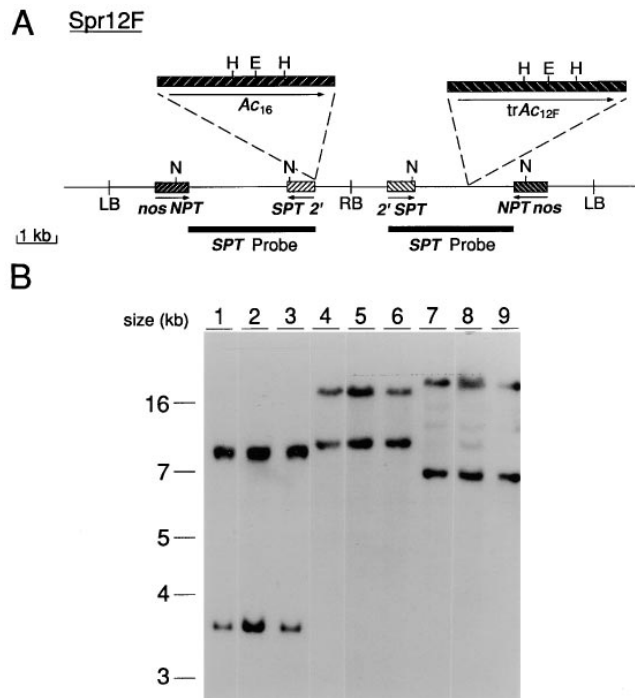


Figure 5.—Southern blot analysis of streptomycin-resistant and streptomycin-sensitive *Spr12F* homozygous plants. (A) Diagram of the *Spr12F* allele with *NcoI* (N), *HindIII* (H), and *EcoRI* (E) restriction sites indicated. The *SPT* probe fragment is represented by a bar. (B) DNA samples from a homozygous *Spr12F* plant selected as streptomycin-resistant (lanes 1, 4, and 7) and two *Spr12F* plants selected as streptomycin-sensitive (lanes 2, 5, and 8 and lanes 3, 6, and 9) were subjected to Southern blot analysis using the *SPT* probe. Samples were digested with *NcoI* (lanes 1 to 3), *EcoRI* (lanes 4 to 6), or *HindIII* (lanes 7 to 9). Note: the *NcoI* digest is predicted to produce three *SPT*-hybridizing fragments. Two fragments at 7 kb were not resolved on this blot.

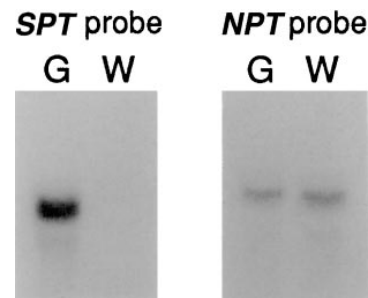


Figure 6.—Northern blot analysis of *Spr12F* homozygous seedlings from a streptomycin-resistant line (G) and a streptomycin-sensitive line (W). 20  $\mu$ g of total RNA was electrophoresed, blotted, and probed with the *SPT* coding sequence or the *NPT* coding sequence.

ment corresponding to the *NPT* coding sequence, signals of similar intensity were detected in both lines. This is in agreement with the kanamycin resistance phenotype exhibited by the Spr12F streptomycin-sensitive line and provides further evidence that silencing does not spread beyond the *SPT* gene. Ethidium bromide staining and reprobing of the blot with a RUBISCO fragment both indicated that the samples had been equally loaded (data not shown).

**Trans-silencing directed by allele Spr12F:** The observation that Spr12F homozygous seedlings exhibited a higher frequency of streptomycin-sensitive sectors than Spr12F hemizygous seedlings suggested that an interaction between alleles might be involved. The availability of streptomycin-sensitive Spr12F homozygous lines provided a useful tool for testing this idea. Four streptomycin-sensitive Spr12F homozygous plants were crossed to streptomycin-resistant Spr11E plants. Each plant was also crossed to untransformed tobacco. Typical progeny seedlings resulting from each of these crosses are shown in Figure 2, E–G. Nearly 100% of progeny hemizygous for the Spr12F (white) allele were streptomycin-sensitive (Figure 2E). Nearly 100% of progeny hemizygous for the Spr11E (green) allele were streptomycin-resistant (Figure 2F). Approximately 60% of heterozygous Spr12F (white)/Spr11E (green) seedlings were streptomycin-sensitive (Figure 2G). Spr1E and Spr1A alleles gave similar results when crossed to Spr12F (white) alleles (data not shown). The results of these crosses were the same whether the Spr12F allele was transmitted from the male or the female parent (data not shown).

Streptomycin-resistant and streptomycin-sensitive Spr11E/Spr12F heterozygous plants (green seedlings and white seedlings, respectively, in Figure 2G) were grown to maturity and crossed to untransformed tobacco. The results were the same whether the Spr12F/Spr11E heterozygous parent was used as male or female. Testcross progeny from three streptomycin-sensitive plants (*i.e.*, *white* seedlings in Figure 2G) are shown in Figures 2, H–J. These progeny are predicted to segregate 50% Spr12F hemizygous and 50% Spr11E hemizygous because the two alleles are linked in repulsion. Southern blot analysis of twelve progeny was consistent with this prediction: seven individuals received the Spr12F allele and five received the Spr11E allele (data not shown). Nearly all of the seedlings observed were streptomycin-sensitive (Figures 2, H–J). This shows that Spr11E hemizygous seedlings inherited the streptomycin sensitivity phenotype after the Spr12F allele had segregated away. Streptomycin-sensitive plants hemizygous for the Spr11E allele were recovered and grown to maturity. Progeny from these plants were germinated on streptomycin-containing medium. The streptomycin sensitivity phenotype persisted in this generation, although the reversion to streptomycin resistance in these seedlings increased to ~5% (data not shown).

Three streptomycin-resistant Spr11E/Spr12F heterozygous plants (*i.e.*, green seedlings in Figure 2G) were grown to maturity and crossed to untransformed tobacco. One plant gave rise to 100% streptomycin-resistant progeny (Figure 2K). Another produced 99% streptomycin-sensitive progeny (Figure 2M). The third produced 50% sensitive and 50% resistant progeny (Figure 2L). From this latter family, five resistant progeny and five sensitive progeny were grown and subjected to Southern blot analysis. This analysis showed that all five streptomycin-sensitive individuals carried the Spr12F allele and all five resistant individuals carried the Spr11E allele (data not shown). Thus, in this particular family, expression of the two alleles was unaffected after passage through the heterozygous condition.

**Isolation of new *SPT::Ac* excision alleles:** We sought to address whether the high frequency loss of *SPT* gene function associated with the Spr12F allele was caused by the presence of closely linked, directly repeated 4.6 kb *Ac* sequences flanking the *SPT* gene (Figure 4). A series of new excision alleles from the 2853.6 locus was generated. A 2853.6 homozygous plant was crossed to untransformed tobacco, progeny were germinated on streptomycin-containing media, and 110 fully green seedlings were selected, as described in materials and methods. These individuals were grown to maturity, self pollinated, and their progeny were germinated on streptomycin-containing media. Four individuals, Spr368, Spr379, Spr381 and Spr419 gave rise to progeny with a high frequency of *white*-sectored seedlings similar to the Spr12F allele. In all four of these families, about 40–50% of the green seedlings had white sectors (Table 1, Figures 2, N–Q). The percentage recombination between the *trAc* and the functional *SPT* gene was determined for each of the new unstable 2853.6 excision alleles. The results, shown in Table 1, indicated that in each of these alleles the *trAc* is very closely linked to *SPT*.

White-sectored plants from each of the new *SPT::Ac* excision lines were crossed to green Spr11E and Spr1A plants. When the heterozygous progeny from these crosses were germinated on streptomycin-containing media, a high frequency of white-sectored seedlings was observed, suggesting that the new alleles could interact with the Spr1A and Spr11E alleles in the same way as allele Spr12F (data not shown).

**Molecular analysis of new *SPT::Ac* excision alleles:** The newly isolated *SPT::Ac* excision alleles, selected solely based on their phenotypic similarity to the Spr12F allele, were subjected to Southern blot analysis to test whether they also had similar DNA structures. Figure 3 (lanes 9–12) shows an *EcoRI* Southern blot hybridized with probe A. Like Spr12F, all four new alleles have the 16-kb band intact, suggesting that *Ac*<sub>16</sub> has not excised. Alleles Spr368 (lane 9) and Spr381 (lane 11) have lost the 10.5-kb band and they each have two new

bands, suggesting that  $Ac_{10.5}$  has transposed downstream of *SPT* into the region spanned by probe A homology. Allele Spr379 (lane 10) has the 11.5 kb band predicted if  $Ac_{10.5}$  has excised. Allele Spr419 (lane 12) has a band of similar size to the  $Ac_{10.5}$  resident band. This Southern blot suggested that  $Ac_{10.5}$  had excised in each of the new *SPT::Ac* excision lines. In addition,  $trAc_{368}$  and  $trAc_{381}$  appeared to have inserted a few kilobases downstream of *SPT*, similar to  $trAc_{12F}$ .

Figure 7A shows a map of the 2853.6 locus, including *XhoI* and *MluI* restriction sites. If a transposition event occurs to within 13 kb of  $Ac_{10.5}$  or greater than 23 kb of  $Ac_{16}$ , new probe A-hybridizing bands should be produced in a Southern blot of *XhoI*-digested DNA. If the same DNA samples are subjected to Southern blotting after digestion with *XhoI* plus *MluI*, the orientation of the *trAc* may be revealed. If the *trAc* was inserted in the same orientation as  $trAc_{12F}$ , the *MluI* site will be between the *XhoI* sites and the probe A-hybridizing fragment will be reduced in size by  $\sim 3$  kb.

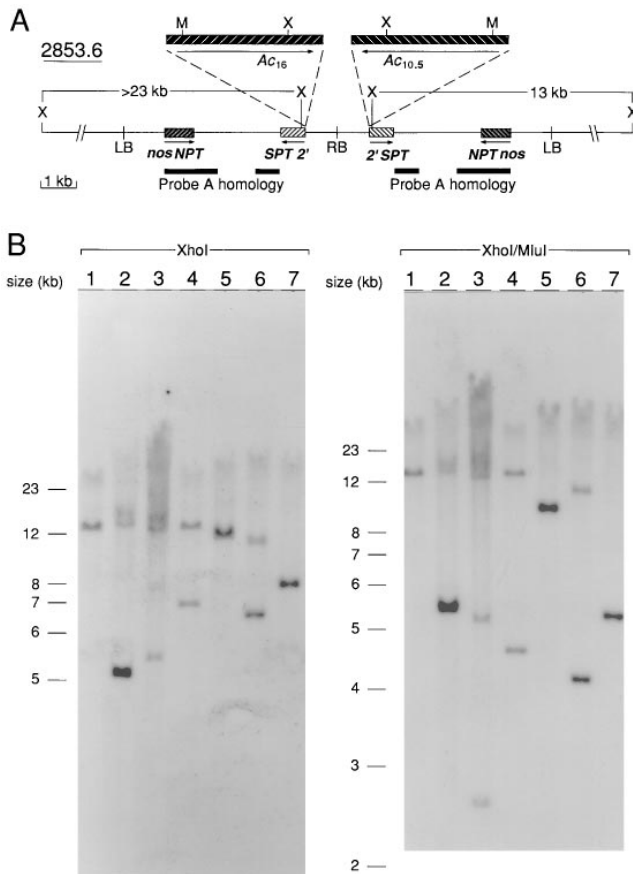


Figure 7.—Southern blot analysis of 2853.6 parent and new alleles. (A) Diagram of the 2853.6 locus with *XhoI* (X) and *MluI* (M) restriction sites indicated. The extent of homology to probe fragment A is indicated. (B) Sample 1, 2853.6; sample 2, Spr10D; sample 3, Spr12F; sample 4, Spr368; sample 5, Spr379; sample 6, Spr381; and sample 7, Spr419 were digested with *XhoI* or with *XhoI* plus *MluI*, blotted, and hybridized with probe A.

Figure 7B shows the results of such Southern blots. Sample 1 is the 2853.6 parent, which has a 13-kb band and a  $>23$ -kb smear with both digests. Allele Spr10D (sample 2) has had a transposition which disrupts the  $>23$ -kb *XhoI* fragment, resulting in a new 5.2-kb band. The  $trAc_{10D}$  is inserted so that the *MluI* site is not between the two *XhoI* sites, a prediction which was confirmed by the *XhoI* plus *MluI* Southern blot. Allele Spr12F (sample 3) has undergone a transposition of  $Ac_{10.5}$  to a position 2.1 kb away from the donor site (Figure 4). This results in two new probe A-hybridizing fragments in the *XhoI* digest: one at 5.5 kb and the other at  $\sim 12$  kb. There is also a faint band of 8 kb, which is not predicted. This band is probably because of an early somatic transposition event in the material from which this particular DNA preparation was made. The  $trAc_{12F}$  has inserted in an orientation so that the *MluI* site in *Ac* is between the *XhoI* sites that give rise to the 5.5 kb band. Thus, in the *XhoI* plus *MluI* blot, a 2.5 kb band is produced. The band at 5 kb suggests that the *trAc* which resulted from a somatic transposition event was in the same orientation.

Allele Spr368 (sample 4) has bands of 7 and 13 kb in the *XhoI* blot. The total size of these two bands is predicted to be  $\sim 17.5$  kb (as in Spr 12F, 5.5-kb + 12-kb). A careful examination of the Spr368 sample in the *EcoRI* Southern blot shown in Figure 3 reveals that this digest also produced two bands whose total size was greater than predicted. Thus, these blots are consistent and suggest that allele Spr368 has undergone an additional rearrangement that inserted  $\sim 2.5$  kb of DNA somewhere between the insertion site of  $trAc_{368}$  and the *XhoI* site. In the *XhoI* plus *MluI* blot, the 13-kb band remains unchanged, while the 7-kb band is reduced to  $\sim 4$  kb. This shows that  $trAc_{368}$  transposed  $\sim 3.5$  kb downstream of *SPT* and inserted in the same orientation as  $trAc_{12F}$ . Allele Spr379 (sample 5) has a 12-kb band in the *XhoI* blot. The *XhoI* plus *MluI* blot has a band of 9 kb, which shows that  $trAc_{379}$  has inserted  $\sim 8$  kb downstream in the same orientation as  $trAc_{12F}$ . Allele Spr381 (sample 6) has bands of 6.7 and 11 kb in the *XhoI* blot. The 6.7-kb band is reduced to 3.7 kb in the *XhoI* plus *MluI* blot, which shows that  $trAc_{381}$  has inserted  $\sim 2.7$  kb downstream in the same orientation as  $trAc_{12F}$ . Allele Spr419 (sample 7) has an 8-kb band in the *XhoI* blot, which is reduced to 5 kb in the *XhoI* plus *MluI* blot. This shows that  $trAc_{419}$  has inserted  $\sim 4$  kb downstream in the same orientation as  $trAc_{12F}$ . The positions and orientations of the *trAc* elements in the new *SPT::Ac* excision alleles deduced from these Southern blots have been confirmed by reprobing the *EcoRI* blot shown in Figure 3 with *Ac* 5' and *Ac* 3' probes (data not shown).

Alleles Spr1A, Spr6A, Spr6F, Spr10N, Spr11B, Spr11E, and Spr12D were analyzed on an *XhoI* Southern blot similar to the one shown in Figure 8B. Each of these alleles had the 13-kb band and the 23-kb smear intact (data not shown). This result suggested that the

trAc had transposed to a position outside of the *XhoI* sites in each of these alleles.

**Structures of new *SPT::Ac* excision alleles:** Schematic representations of the new *SPT::Ac* excision alleles deduced from Southern blot analysis are shown in Figure 8. The selection of these new alleles was based solely on their phenotypic similarity to allele Spr12F. Their DNA structures are also remarkably similar to that of allele Spr12F. The new alleles have all had transpositions of *Ac*<sub>10.5</sub> to positions downstream of *SPT* so that they have two *Ac* elements in direct orientation flanking the functional *SPT* gene. TrAc<sub>381</sub> moved the shortest distance to a position ~2.7 kb from the starting position. TrAc<sub>368</sub> and trAc<sub>419</sub> transposed ~3.5- and ~4 kb, respectively. TrAc<sub>379</sub> transposed the farthest to a position ~8 kb from the starting position. These data provide a strong correlation between high frequency loss of *SPT* function and the presence of *Ac* elements in direct orientation flanking the *SPT* gene.

## DISCUSSION

The work described here was initiated to characterize the loss of *SPT* gene function in tobacco plants carrying a series of *SPT::Ac* excision alleles. The first step was to characterize the structures of the different alleles. The allelic series resulted from 14 transposition events, four of which were to genetically unlinked positions and 11 of which were to very closely linked posi-

tions. Four of the closely linked transpositions were to positions less than 10 kb from the starting position (Spr1E, Spr2, Spr10D, and Spr12F) (Figure 4). Thus, *Ac* has the potential to transpose very short distances in tobacco. The ability of *Ac* and *Ds* elements to transpose very short distances has also been observed in maize (Weil *et al.* 1992) and tomato (Carroll *et al.* 1995).

The observation that *Ac* could transpose very short distances at significant frequencies suggested that loss of *SPT* function could be due to reinsertion of the trAc into *SPT*. While this appeared to be the case for one Spr1E and two Spr10D streptomycin-sensitive progeny examined (data not shown), the involvement of DNA structural rearrangements was ruled out for streptomycin-sensitive Spr12F homozygous plants examined (Figure 5; data not shown). Consistent with this finding was the observation that loss of *SPT* function occurred at a much higher frequency in Spr12F homozygous plants than in Spr12F hemizygous plants (Figure 2). There are other examples of gene silencing in which the effect is much stronger when the gene of interest is homozygous rather than heterozygous or hemizygous (Decarvalho *et al.* 1992; Hollick *et al.* 1995; Elmayan and Vaucheret 1996; Kermicle 1996).

Southern blot analysis (Figure 3; data not shown) and DNA sequencing (data not shown) indicated that allele Spr12F carried a functional *SPT* gene flanked by two *Ac* elements in direct orientation. To address whether this particular structure was involved with loss

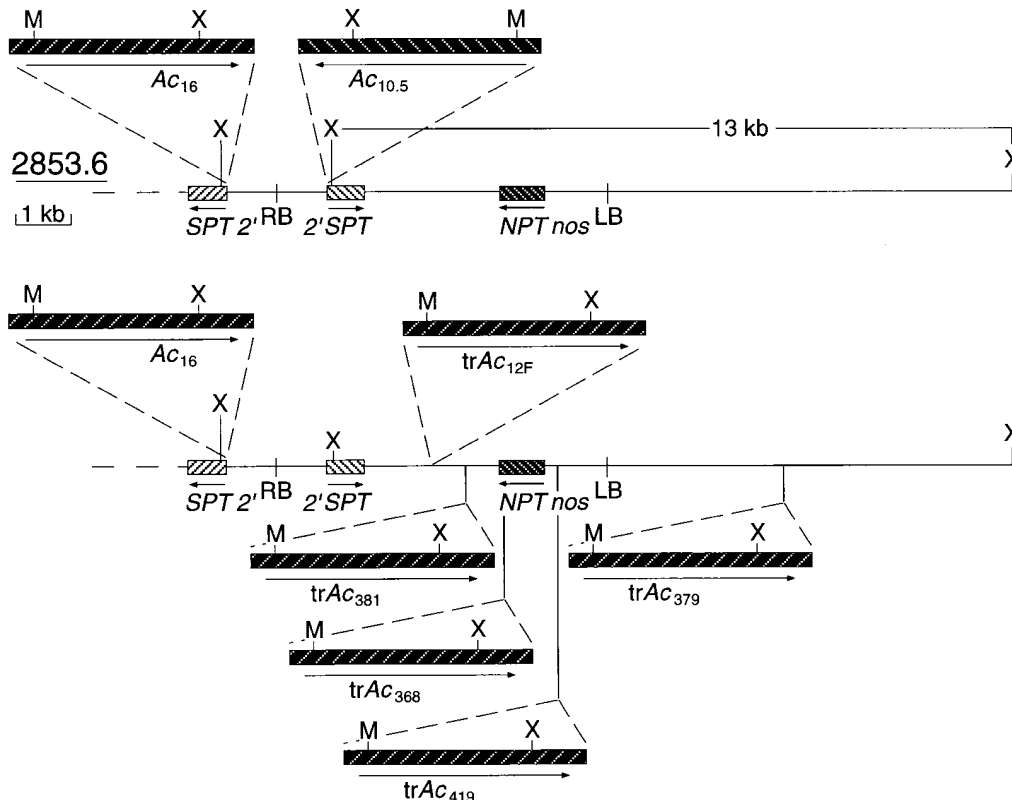


Figure 8.—Structures of the 2853.6 locus in 2853.6 parent and new alleles. Orientation of *Ac* elements is indicated by an arrow which points in the direction of *Ac* transcription. *XhoI* (X) and *MluI* (M) restriction sites; LB, RB, T-DNA left and right borders, respectively; *NPT*, neomycin phosphotransferase; *SPT*, streptomycin phosphotransferase are indicated.

of marker gene function, 110 new transpositions from line 2853.6 were generated. Four events gave rise to families that had similar phenotypes to Spr12F (Figures 2, N–Q and Table 1). Remarkably, all four new alleles also had *Ac* elements in direct orientation flanking the functional *SPT* gene (Figure 8). Thus, a strong correlation was established between this particular structure and high frequency loss of *SPT* marker gene function.

It is possible that *Ac* orientation after transposition from the 2853.6 locus may not be random. However, it seems more likely that the recovery of *trAc* elements all in the same orientation was due to the selection that was used in this experiment. In support of this idea, allele Spr1E, in which *Ac* inserted in the opposite orientation, does not exhibit a high frequency of white-sectored seedlings and therefore would not have been selected in this screen.

It is interesting that only the *SPT* gene is silenced. The other marker genes on the 2853.6 T-DNA, *Ac*, and *NPT* remain active in all the Spr12F (white) and Spr11E (white) progeny that have been examined (Figure 6; data not shown). This suggests that the effect is specific and that gene silencing does not spread outward from the directly repeated *Ac* elements. This interpretation is consistent with observations of the Spr2 and Spr10D alleles, which have *Ac* elements in direct orientation with the functional *SPT* gene ~1 kb away from one of the *Ac* elements (Figure 4). If marker genes adjacent to the directly repeated *Ac* elements were subject to silencing, a higher frequency of *SPT* loss would be expected in lines Spr2 and Spr10D.

Remarkably, the *SPT* gene silencing can be transferred from Spr12F to other 2853.6 alleles such as Spr1A, and furthermore, this silencing of Spr1A can be maintained in the absence of the Spr12F allele.

Repeated DNA sequences are associated with many examples of gene silencing in plants and other organisms. Unlinked repeats formed by an introduced T-DNA and an endogenous gene are associated with cosuppression in plants (Napoli *et al.* 1990; van der Krol *et al.* 1990). Repeats present in different T-DNA insertions in sequentially transformed tobacco plants have also been shown to participate in gene silencing (Matzke *et al.* 1989). If repeated sequences are present because of introduction of foreign DNA into the fungus *Neurospora crassa*, there is a rapid and efficient reaction by the host cell, which causes the sequence to be highly mutated, probably via a methyl-cytosine intermediate (Singer and Selker 1995). Repeated DNA sequences in the fungi *Ascobolus immersus* (Rossignol and Faugeron 1995) and *Coprinus cinereus* (Freedman and Pukkila 1993) quickly become methylated and silenced. Complex T-DNA insertions, which carry repeated sequences are associated with poor expression of introduced genes (Jones *et al.* 1987), gene inactivation (Hobbs *et al.* 1990), and a high frequency of cosuppression (Jorgensen *et al.* 1996; Cluster *et al.*

1996). Also, increasing numbers of *r* genes within the complex *R-st* allele of maize are correlated with increasing paramutagenic strength (Eggleston *et al.* 1995; Kermicle *et al.* 1995). In all of the above examples, the repeated sequences that are associated with gene silencing include the promoter and/or coding sequence of the silenced gene(s). Curiously, in the case of the silenced 2853.6 alleles, the repeats that appear to induce silencing do not include the silenced transgene itself. In this example, we have separated the determinant of silencing from the gene whose expression is affected.

A recent review (Matzke *et al.* 1996) argued that some cases of paramutation and transgene silencing may be due to the action of a genomic defense system that inactivates invasive DNA such as transposons and multicopy transgenes. The presence of *doppia* transposon sequences in the *r* gene repeats of the complex maize *R-st* allele supports this idea. The *doppia* sequences provide the only homology within the promoter regions of the paramutagenic components of the *R-st* allele and paramutable components of the *R-r* allele. Moreover, the *P* gene of the complex *R-r* allele lacks a *doppia* element and is relatively insensitive to paramutation (Walker *et al.* 1995). However, after several generations of paramutation, *P* expression is heritably reduced, thus adding support to the argument that *doppia* elements are not required for heritable silencing (Brink 1973).

Transposable elements have also been postulated to be involved with silencing interactions between *nivea* alleles in *Antirrhinum majus*. The *niv-44* allele carries a *Tam2* element within the *nivea* gene (Upadhyaya *et al.* 1985). The *niv-53* allele carries a *Tam1* element in the promoter of *nivea* (Bonas *et al.* 1984). Krebbers *et al.* (1987) proposed that an interaction between these transposons could be involved in the transfer of epigenetic information between alleles. However, a derivative of the *niv-44* allele, *niv-4432*, was recovered that no longer had *Tam2* at *nivea*, but that was still able to influence expression of *niv-53 in trans*. This result may indicate that transposon *Tam2* is not important in this example of silencing. Alternatively, it may be that the initial process that gave rise to the *transsilencing* allele involved *Tam2*, but that the transposon was not required for its maintenance. For example, if the *niv-44* allele had an altered chromatin structure due to the presence of *Tam2*, the altered chromatin structure might persist even after excision of *Tam2*.

The maize *b* locus has been studied extensively with regard to paramutation. The sequences required *in cis* for paramutation have been mapped within 0.1 cM (1–150 kb) upstream of the transcription start site in the paramutagenic allele *B'* (Patterson *et al.* 1995). Transposon sequences have been detected within this region in both paramutagenic and paramutable alleles, suggesting that transposons are not the determinant of paramutation in this case.

Dorer and Henikoff (1994) proposed that silencing of *mini-white* transgenes in *Drosophila* was because of the formation of heterochromatin as in position-effect variegation (PEV). Repeated sequences are also involved in this example, and increasing numbers of repeats are inversely correlated with *mini-white* expression. In *Drosophila*, known modifiers of PEV exist that can be crossed in to the silencing stock to test their effect on silencing. These tests indicated that the silencing of *mini-white* transgenes was indeed because of heterochromatin formation at the transgene loci. The favored model is that the multiple, closely linked copies of the *mini-white* transgenes can pair somatically, and the resulting folded structures are recognized by heterochromatin-specific proteins (Dorer and Henikoff 1994). Curiously, silencing of *mini-white* transgenes in *Drosophila* appears to be dependent on the orientation of the repeated sequences relative to each other and relative to the heterochromatin/euchromatin transitions (Sabl and Henikoff 1996). It is striking that in our study no Spr12F-like derivatives were recovered in which *Ac*<sub>16</sub> had moved into direct orientation with *Ac*<sub>10</sub>. This may indicate that the orientation of the *Ac* elements relative to the centromere, or other chromosomal features, is important.

Localized heterochromatin formation could explain the results reported here. This process might be facilitated if *Ac* transposase binding to its target sites in the *Ac* subterminal repeats (Kunze and Starlinger 1989) stabilized any folded structures that formed. It is known that *Ac* elements can interact over distances of >18 kb (*i.e.*, greater than the distances between *Ac* elements in any of the unstable 2853.6 Spr alleles described here) leading to excision (Ralston *et al.* 1989). It is also known that the choice of which element ends participate as a transposition substrate depends on their relative orientations (English *et al.* 1993). This could explain the phenotypic differences between the unstable 2853.6 excision alleles and the stable allele Spr1E. Heterochromatin-specific proteins would recognize and bind to the folded structures as proposed for silencing of *mini-white* transgenes in *Drosophila* (Dorer and Henikoff 1994). Transfer of the heterochromatic state from one allele to another, possibly by transfer of heterochromatin-specific proteins, would lead to silencing *in trans*.

If the silencing of *SPT* in the 2853.6 excision alleles is due to heterochromatin formation, it is curious that it does not spread to neighboring genes. There is no decrease in *NPT* mRNA accumulation in Spr12F (white) plants (Figure 6), and the kanamycin resistance phenotype is maintained for at least three generations of selfing after loss of *SPT* function (data not shown). These results would indicate that if heterochromatin formation is involved, it can be very localized.

Binding of *Ac* transposase to the subterminal repeats of *Ac* could potentially have more direct effects on *SPT* expression. Steric hindrance because of the binding of

*Ac* transposase molecules near to the 2' promoter could prevent transcription of *SPT*. Fridlender *et al.* (1996) showed that *Ac* transposase could repress expression from the *Ac* transposase gene promoter (which overlaps the 5' subterminal repeat region) or chimeric promoters containing part of the 5' subterminal repeat region. Alternatively, binding of *Ac* transposase molecules could produce topological alterations in the DNA which would affect transcription. For example, if DNA nicking associated with attempted *Ac* transposition released DNA supercoiling locally, transcription of intervening DNA might be affected. However, it is not clear why this "supercoiling release" mechanism would have a more pronounced effect between direct copies of *Ac* than between inverted copies.

Topological constraints could be particularly relevant in the case of Spr12F and the other unstable 2853.6 alleles. It is possible that the directly oriented *Ac* elements promote, and act as boundaries for, the formation of a small loop domain which would be stabilized by binding of *Ac* transposase. This could explain why only the *SPT* gene is affected because it would be the only gene contained in such a loop.

In summary, we have provided a strong correlation between a particular structure, comprised of two directly repeated *Ac* elements flanking a *SPT* marker gene and silencing a *SPT* marker gene. In this system, the directly repeated *Ac* elements appear to be responsible for silencing of *SPT in cis* and *in trans*. Thus, we have separated the determinant of gene silencing from the affected gene. This was made possible by the exploitation of a transposon-carrying T-DNA insertion to generate an allelic series. These results provide new insights into the related areas of gene silencing and paramutation.

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