RESEARCH ARTICLE

A Genetic Analysis of DNA Sequence Requirements for Dissociation State I Activity in Tobacco

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Our objective was to test whether the double Ds structure correlated with Dissociation state I activity (i.e., high frequency of chromosome breakage and low frequency of reversion) in maize exhibited similar properties in tobacco. A genetic assay was established to test double Ds and related structures for their ability to cause loss of the linked marker genes streptomycin phosphotransferase and β-glucuronidase in transgenic tobacco. An engineered double Ds element and a simple Ds element showed behavior consistent with that of state I and state II Ds elements, respectively, as described for maize. DNA structural rearrangements accompanied marker gene loss. Dissection of the double Ds structure showed that a left end and a right end of Ds in direct orientation were sufficient for the instability observed. This result suggested that left and right ends of Ds in direct orientation can participate in aberrant transposition events, consistent with two different models for double Ds-induced chromosome breakage proposed previously. Both models predict that the inversion of a half Ds element accompanies the aberrant transposition event. Such an inversion was detected by polymerase chain reaction experiments in tobacco and maize only when Activator activity was present in the genome.

INTRODUCTION

Members of the maize Activator/Dissociation (Ac/Ds) transposable element family can participate in several types of chromosomal rearrangements in addition to transposition. In response to Ac, certain Ds elements give rise to chromosome breakage associated with the formation of acentric and dicentric chromosomes and to deletions and duplications, inversions, ring chromosomes, and translocations (McClintock, 1947, 1948, 1949, 1953a). The chromosome-breaking activity of a particular Ds element was the first manifestation of transposable activity detected by McClintock (1947). Subsequently, she showed that both Ac and Ds could transpose to new locations in the genome (McClintock, 1948; Fedoroff, 1983).

Different “states” of Ds elements were defined with respect to the relative frequency of chromosome breakage versus reversion (transposition) events associated with them. State I Ds elements give a high frequency of chromosome breaks and a low frequency of reversion when inserted in a known gene. State II Ds elements give little or no chromosome breakage and a high frequency of reversion (McClintock, 1949). The pattern of reversion associated with the state II Ds element at c-m7 was dependent on the state and dose of Ac in the same way as the pattern of chromosome breakage due to state I Ds elements (McClintock, 1951). This result suggested that state I and state II Ds activity were closely related phenomena and might be alternative outcomes of the same process. Whereas transposition is the simple outcome, chromosome breakage and other rearrangements are consequences of aberrant events involving Ds (McClintock, 1949).

Little is known about how these chromosomal rearrangements occur. Several structures involved with chromosome breakage in maize have been characterized. The double Ds element has been identified in several unstable shrunken alleles associated with chromosome breakage (Courage-Tebbe et al., 1983; Weck et al., 1984; Döring et al., 1990). It consists of a 2-kb Ds element inserted, in opposite orientation, into an exact copy of itself, as shown in Figure 1 (Döring et al., 1984). Dooner and Belachew (1991) showed that two Ac elements or an Ac and a Ds located within ~3 centimorgans of each other, one being at the bronze locus, can cause chromosome breakage. Weil and Wessler (1993) characterized two waxy alleles associated with chromosome breakage. Both contain closely linked Ds elements, 0.5 and 1.5 kb apart, respectively. It is not known how these structures cause chromosome breakage, although a feature they all have in common is the presence of multiple transposable element ends in close proximity. Double Ds is the most extreme case; it has four transposable element ends within 4 kb of each other. For this reason, double Ds was chosen for the work presented here.

At least two features of the double Ds structure could potentially give rise to chromosome breakage: (1) double Ds contains long inverted repeats formed by the two pairs of like Ds ends.
that are present, and if these inverted repeats participated in sister chromatid exchanges,acentric and dicentric chromatin would be formed leading to chromosome breakage (Weck et al., 1984); (2) unlike simple Ac/Ds elements, double Ds contains a left end and a right end of Ds in direct orientation. If these directly repeated ends acted as a substrate for transposase, aberrant events leading to chromosome breakage and other rearrangements would be predicted (Döring and Starlinger, 1984; Fedoroff, 1989).

Models for Ac/Ds–induced chromosome breakage have been proposed based on element-induced rearrangements. Double Ds is associated with large and complex insertions in the shrunken alleles sh-m5933 and sh-m6258 (Courage-Tebbe et al., 1983; Döring et al., 1990). These insertions contain double Ds elements in inverted orientation at each end and several additional kilobases of inverted sequences. The structures of these insertions form the basis for a model proposed by Fedoroff (1989) (see below). Excision of a putative "macrotransposon" encompassing two closely linked elements and the DNA between them led to the model of Ralston et al. (1989).

To address how double Ds–induced chromosome breakage occurs, it would be desirable to start with a defined transposable element, to generate rearrangements, and to analyze the resulting structures. This has proved difficult in maize because there are many copies of Ds sequences in most genomes (Geiser et al., 1982; Fedoroff et al., 1983). In maize, the structures that can be observed are limited to those that occur naturally, whereas in heterologous hosts, novel structures can be engineered and tested. For these reasons, it may be advantageous to perform a rigorous analysis of DNA structures sufficient to cause chromosome breakage using a heterologous plant species.

We have established a phenotypic assay to monitor the ability of double Ds and other structures to cause marker gene instability in tobacco. We constructed T-DNA vectors that carry the cell-autonomous markers streptomycin phosphotransferase (SPT) (Maliga et al., 1988; Jones et al., 1989) and 1-glucuronidase (GUS) (Jefferson et al., 1987) and convenient restriction sites into which test structures can be inserted. We have used this system to address whether double Ds can cause loss of adjacent marker genes in the absence of other Ds elements, to dissect the double Ds structure, and to generate double Ds–induced DNA rearrangements. We first characterized this system with respect to marker loss and reversion with double Ds and a simple Ds. Simple Ds behaves like a state I element, and double Ds behaves like a state II element, as described for maize. Dissection of the double Ds structure revealed that a left end and a right end of Ds in direct orientation (i.e., a double Ds) cause marker loss in the same way as double Ds. This result suggests that left and right ends of Ds in direct orientation can participate in aberrant transposition events and is consistent with models for double Ds–induced chromosome breakage proposed by Döring and Starlinger (1984) and by Fedoroff (1989) (see Results). If such an aberrant event occurs, the specific inversion of a half Ds element would be predicted. Polymerase chain reaction (PCR) experiments detected this type of inversion in both tobacco and maize. Further experiments will be required to determine whether Ds-induced chromosome breakage occurs through one or both of the mechanisms proposed.

RESULTS

Ds Elements

Simple Ds elements used in this work were Ds4135 and Ds4081. Ds4135 was derived from Ac by deleting the internal 1.6-kb HindIII fragment. Ds4081 was derived from Ac by filling in the 5' HindIII site (Jones et al., 1990). An engineered double Ds (eDDs) element was constructed by cloning, in the opposite orientation, the 2961-bp Ds4135 between the Nrul and Xhol sites of another Ds. This resulted in a structure that has the same configuration of Ds ends as double Ds (Figure 1). The elements eDDs and Ds4135 were cloned adjacent to the marker genes SPT (Maliga et al., 1988; Jones et al., 1989) and GUS (Jefferson et al., 1987) to test their ability to cause Ac-dependent instability of nearby genes. The resulting constructs were designated eDDs/SPT and Ds4135/SPT, respectively, and are shown in Figure 2. To assess their relative rates of excision, eDDs and Ds4081 were cloned into the 5' untranslated leader of SPT, resulting in constructs SPT::eDDs and SPT::Ds (Jones et al., 1990), respectively (Figure 2). Tobacco plants were transformed with these constructs via Agrobacterium and crossed to untransformed tobacco and to Ac transposase (Ac TPase)–expressing lines.

Ac TPase Sources

Three different sources of Ac TPase were used in this work. The first, saC, carries a 177-bp deletion of Ac at its 3' end and provides transposase under control of the Ac promoter, but cannot transpose itself (Scofield et al., 1992). Construct 35S:TPase contains a cauliflower mosaic virus (CaMV) 35S...
promoter fused to the Ac TPase gene (Scofield et al., 1992). A 35S:TPase cDNA construct (35S:AcDNA) was made by fusing a CaMV 35S promoter to a PCR-generated Ac cDNA (J. English, unpublished data). Transformed tobacco lines were generated containing each of these constructs and crossed to the SPT::Ds tester line (Jones et al., 1990) to assess the Ac activity associated with them. Each Ac TPase source gave rise to a unique pattern of $D_s$ excision due to differences in the timing and level of Ac TPase expression during embryo development (Scofield et al., 1992; S. Scofield, J. English, and J. Jones, manuscript in preparation). In the cases of sAc and 35S:TPase, all transformants gave patterns of $D_s$ excision similar to those previously described (Scofield et al., 1992). Transformants that had single T-DNA insertions were chosen, and homozygous lines were generated from them. In the case of 35S:AcDNA, a single locus transformant giving the characteristic pattern of $D_s$ excision was chosen. The pattern of $D_s$

Figure 2. Schematic Diagrams of T-DNA Regions of Constructs Used to Assay the Behavior of eDDs and Simple $D_s$ Elements.

Constructs eDDs/SPT have eDDs cloned adjacent to SPT. Two different versions were used. pSLJ1336 is shown here. Construct pSLJ4123 is similar, the only difference being that eDDs was cloned into the dBS+ polylinker of pSLJ4061. Construct DeSPT has the 3-kb $D_s$4135 cloned into a dBS+ polylinker adjacent to SPT. Construct SPT::eDDs has eDDs cloned into the 5' untranslated leader of SPT. Construct SPT::Ds has the 4.6-kb $D_s$4081 cloned into the 5' untranslated leader of SPT. NPTII, neomycin phosphotransferase gene; HPTII, hygromycin phosphotransferase gene; 35S, cauliflower mosaic virus 35S promoter; B, BamHI; H, HindIII; E, EcoRI. T-DNA left and right borders (LB and RB, respectively) are indicated. Open and solid arrowheads are as given in Figure 1.

Figure 3. Somatic Variegation Resulting from Transactivation of eDDs/SPT and SPT::$D_s$ by Different Ac TPase Sources.

(A) sAc × SPT::$D_s$.
(B) sAc × eDDs/SPT.
(C) 35S:TPase × SPT::$D_s$.
(D) 35S:TPase × eDDs/SPT. 
(E) 35S:AcDNA × SPT::$D_s$.
(F) 35S:AcDNA × eDDs/SPT.

eDDs Causes Instability of Adjacent Marker Genes Dependent on Ac Activity

Eight independent eDDs/SPT-transformed tobacco lines were generated and crossed to Ac TPase–containing tobacco lines and to untransformed tobacco. The resulting progeny were germinated on streptomycin-containing medium. Seven of these transformed lines gave rise to green, streptomycin-resistant progeny, which exhibited white sectors when Ac TPase was present in the genome. The remaining line did not produce
white-sectored progeny. In the absence of Ac TPase, all of the eDDs/SPT transformants gave rise to fully green progeny. Typical phenotypes of sectored seedlings are shown in Figures 3B, 3D, and 3F. The white, streptomycin-sensitive sectors are clonal in nature, with sharp boundaries.

Four independent Ds/SPT-transformed tobacco lines were generated. These transformants were crossed to Ac TPase-containing tobacco lines and to untransformed tobacco. Progeny from two plants were fully green when germinated on streptomycin-containing media both in the presence and absence of an Ac TPase source. Loss of SPT function dependent on Ac activity was observed at very low frequencies (seven to nine sectors per 200 seedlings) in progeny of the other two Ds/SPT transformants.

Pattern of Marker Loss Due to eDDs Is Similar to the Pattern of Reversion Due to Ds4081

To compare the pattern of instability caused by eDDs to the pattern of reversion due to a simple Ds, eDDs/SPT and SPT::Ds transformants were crossed to each of three different Ac TPase sources. Typical progeny from these crosses are shown in Figure 3. The pattern of Ds excision in response to sAc is a continuous range of sector sizes from large to small (Figure 3A). This pattern is also observed in the white sectors on a green background of eDDs/SPT x sAc seedlings (Figure 3B). The pattern of Ds excision promoted by 35S:TPase is fairly regular and consists mostly of medium-sized sectors (Figure 3C). The white sectors on green of eDDs/SPT x 35S:TPase seedlings show a similar pattern (Figure 3D). The pattern of Ds excision promoted by 35S:AcDNA gives rise to a high frequency of medium to small sectors (Figure 3E). This pattern is also reflected in the pattern of white sectors on green of eDDs/SPT x 35S:AcDNA seedlings (Figure 3F). Thus, the pattern of eDDs-associated marker loss is similar to the pattern of reversion due to excision of Ds4081 with each Ac TPase source tested.

Loss of GUS Activity Is Correlated with Loss of SPT Activity

Loss of GUS activity is correlated with loss of SPT activity in eDDs/SPT x Ac TPase seedlings. Figure 4A shows an eDDs/SPT x sAc seedling with a white, streptomycin-sensitive sector, which was stained for GUS activity. When the chlorophyll was extracted with 70% ethanol, it was clear that the sector which lacked GUS activity coincided with the streptomycin-sensitive sector (Figure 4B). Even and reproducible staining of seedlings for GUS activity proved to be difficult. To confirm that loss of SPT function was correlated with loss of GUS function, a population of eDDs/SPT homologous individuals was generated that had lost the SPT gene germinally. Progeny from three different eDDs/SPT transformants, homozygous for the eDDs/SPT T-DNA and with Ac TPase in the genome, were crossed to untransformed tobacco. Progeny were germinated on streptomycin-containing medium. In addition to the majority of green individuals with white sectors, there was a small number of fully white seedlings (0.5 to 2%), as shown in Figure 4C. These individuals had lost SPT function germinally. Histochemical staining for GUS activity was performed on 33 fully white individuals and, of these, 29 had lost GUS activity. Coincidental loss of GUS and SPT activity rules out insertional inactivation of SPT. The relatively high frequency argues against the occurrence of small adjacent deletions, which has been shown to be a low-frequency event in maize (Dooner et al., 1988). These points suggest that other types of rearrangements are giving rise to loss of SPT activity in most cases.

Loss of Marker Genes Is Associated with DNA Structural Rearrangements

When the 29 eDDs/SPT individuals that had lost both SPT and GUS activity were large enough, DNA was extracted and
subjected to gel blot analysis to determine whether DNA structural rearrangements accompanied the marker loss observed. A restriction map of the relevant part of the eDDs/SPT T-DNA is shown in Figure 5A. DNA samples were digested with SstI and probed with fragment A (SPT and 3' sequence). None of these individuals had homology to fragment A (data not shown).

This blot was stripped and reprobed with fragment B (Ds4135) (Figure 5A). Of 29 individuals, 23 had no homology to fragment B (data not shown), except for background hybridization to the Ac TPase source (of which there were three T-DNA loci segregating in this population). The observation that 23 of 29 individuals no longer had homology to Ds could be explained by asymmetric breakage of an eDDs-induced dicentric chromosome. Such an individual should be viable, even as a haploid gamete, because tobacco is a tetraploid and would have another copy of the sequence to provide function. The availability of a complete set of tobacco monosomic lines (Clausen and Cameron, 1944) is consistent with this idea.

The remaining six individuals had homology to fragment B, but the 4-kb SstI band of the progenitors (Figure 5B, lanes 7 and 9) was now absent, and one or more new Ds-hybridizing bands had appeared (Figure 5B). The individual represented in lane 2 no longer has Ds sequences, but two of the Ac TPase bands can be seen. Individuals in lanes 3, 4, 5, and 6 of Figure 5B have lost the 4-kb progenitor band and each has a new Ds-hybridizing band. Individuals in lanes 1 and 8 have also lost the progenitor band, and they now show two and three new bands, respectively. These results show that DNA rearrangements involving Ds are associated with loss of SPT and GUS function. The individuals with multiple new Ds-hybridizing bands may indicate that complex DNA rearrangements have occurred, or since the Ac TPase source is still present, they could be due to somatic events.

**Figure 5. DNA Gel Blot Analysis of eDDs/SPT Plants Selected for Germinal Loss of SPT and GUS Function.**

(A) Map of the relevant part of the eDDs/SPT T-DNA. Probe fragments A and B are indicated. SstI (Ss), HindIII (H), and BamHI (B) restriction sites are indicated. T-DNA right border is indicated as RB. Open and solid arrowheads are as given in Figure 1.

(B) Each DNA sample was digested with SstI and probed with fragment B. Lane 10 contains DNA from untransformed tobacco. Lane 7 contains DNA from primary transformant eDDs/SPT-1, and lanes 1 to 6 contain DNA from its streptomycin-sensitive progeny. Lanes 9 and 8 contain DNA from primary transformant eDDs/SPT-2 and a streptomycin-sensitive progeny plant, respectively. Three Ac TPase T-DNAs were segregating in this population. The Ac TPase source is an Ac cDNA fusion, so fragment B hybridizes less to it than to eDDs. The sample in lane 2 is an example of a streptomycin-sensitive plant that no longer has eDDs sequences. Two bands corresponding to Ac TPase source T-DNAs can be seen. The 4-kb progenitor band (present in lanes 7 and 9) is indicated. New Ds-hybridizing bands are indicated by asterisks.

To compare somatic reversion rates due to excision of eDDs and a simple Ds, four independent SPT::eDDs transformants and four independent SPT::Ds transformants were crossed to 35S:TPase and sAc tobacco lines. Progeny were germinated on streptomycin-containing medium, and the average frequency of green sectors was determined for each transformed line. Typical seedlings are shown in Figures 4D and 4E. The frequencies of sectors observed with 35S:TPase are listed in Table 1. Variability between transformants was observed in the rates of eDDs and Ds excision. Green sectors occurred at average frequencies of 22 to 66 per seedling for SPT::Ds x 35S:TPase seedlings. Green sectors occurred at average frequencies ranging from 0.02 to 0.83 sectors per seedling in SPT::eDDs x 35S:TPase seedlings. Despite the vast decrease in excision frequency exhibited by eDDs, the characteristic patterns of sector sizes are retained with both 35S:TPase and sAc transposase sources.

PCR analysis showed that both the internal Ds element of eDDs and the entire eDDs element can excise (data not shown). To permit SPT function in SPT::eDDs seedlings, the outer Ds element must excise, either as the entire eDDs or subsequent to excision of the inner Ds element. We do not have a direct estimate of the relative frequencies of excision of the internal and external Ds elements of eDDs. The data presented in Table 1 suggest that the outer Ds element excises at a low frequency relative to a simple Ds. If the inner Ds element
Table 1. Frequency of Somatic Reversion of SPT in SPT::eDDs and SPT::Ds Tobacco Lines

<table>
<thead>
<tr>
<th>Plant</th>
<th>Sectors per Seedling</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPT::eDDs-1</td>
<td>0.24</td>
</tr>
<tr>
<td>SPT::eDDs-2</td>
<td>0.02</td>
</tr>
<tr>
<td>SPT::eDDs-3</td>
<td>0.02</td>
</tr>
<tr>
<td>SPT::eDDs-4</td>
<td>0.63</td>
</tr>
<tr>
<td>SPT::Ds-1</td>
<td>32</td>
</tr>
<tr>
<td>SPT::Ds-2</td>
<td>64</td>
</tr>
<tr>
<td>SPT::Ds-3</td>
<td>66</td>
</tr>
<tr>
<td>SPT::Ds-4</td>
<td>22</td>
</tr>
</tbody>
</table>

Each plant listed was crossed to a 35S:TPase source. Progeny were germinated on streptomycin-containing media. Green revertant sectors were counted. Sectors per seedling are average values for 100 seedlings in the cases of SPT::eDDs-1 and SPT::eDDs-4; ~700 seedlings in the cases of SPT::eDDs-2 and SPT::eDDs-3; 15 seedlings in the SPT::Ds transformants.

excised, it would leave a 1.6-kb simple Ds inserted in SPT. If such an event occurred early, it would produce a large sector carrying a 1.6-kb simple Ds element in SPT, which we would expect to see as a sector with a high frequency of green spots. Large green sectors corresponding to excision of the outer Ds occur at a low frequency in SPT::eDDs x sAc seedlings. However, sectors with a high frequency of green spots, corresponding to an early excision of the internal Ds element, have not been seen in more than 2000 SPT::eDDs x sAc seedlings screened so far, suggesting that excision of the internal Ds element of eDDs is a low-frequency event.

Dissection of the Double Ds Structure

The system described above was used to dissect double Ds to determine which feature of its structure gives rise to the marker gene instability that was observed. A series of 11 constructs was made based on pSLJ4061, shown in Figure 6A, which has GUS and SPT as markers for chromosome breakage and the neomycin phosphotransferase gene as a selection for transformation. A darkBluescript (dBS+) lacZ region (Jones et al., 1992) adjacent to the SPT gene provided convenient restriction sites and a blue/white screen for inserting test structures. Between three and eight transformed tobacco plants were generated for each construct. Transformants were crossed to untransformed, sAc and 35S:TPase tobacco lines. Progeny were germinated on streptomycin-containing medium and observed for SPT expression.

The structures that were assayed and a summary of the results that were obtained are shown in Figure 6B. Construct Ds::Ac/SPT (Figure 6B, construct 1) contains an Ac element inserted in a Ds element, in opposite orientation, a structure that is essentially an autonomous double Ds. Two Ac/SPT constructs (Figure 6B, constructs 2 and 3) were generated carrying eDDs adjacent to SPT, in both possible orientations. Construct eDDs/SPT (Figure 6B, construct 4) contains an eDDs element adjacent to SPT. Construct Ds/SPT (Figure 6B, construct 5) carries the simple Ds element, Ds4135, adjacent to SPT. Construct

Figure 6. Summary of Experiments Designed to Dissect the Double Ds Structure.

(A) Schematic diagram of the T-DNA region of pSLJ4061. Structures to be assayed were inserted into a dBS+ lacZ region, which is situated adjacent to the SPT gene. X, Xhol; E, EcoRI; S, SstI; LB and RB, T-DNA left and right borders, respectively; NPTII, neomycin phosphotransferase gene. The SPT, NPTII, and GUS genes are indicated.

(B) Chart showing phenotypic classes of individual tobacco transformants for each construct based on pSLJ4061. Schematic representations show structures that were cloned into the dBS+ polylinker in each construct: 1, Ds::Ac/SPT; 2, Ac/SPT; 3, Ac/SPT; 4, eDDs/SPT; 5, Ds/SPT; 6, RE, RE/SPT; 7, LE, LE/SPT; 8, LE, RE/SPT; 9, RE, LE/SPT; 10, LE/SPT; 11, RE/SPT (described in detail in Methods). Elements were oriented as shown relative to the rest of the T-DNA. BamHI (B) sites are indicated. Open and solid arrowheads are as given in Figure 1. The number of independent transformants in each phenotypic class is shown next to each structure. Phenotypic classes are described in the text. Superscript A, individuals that acquire the 35S:TPase phenotype when 35S:TPase is present; superscript E, one of three transformants giving 10 to 15 sectors per 200 seedlings; superscript C, two of four transformants giving seven to nine sectors per 200 seedlings when Ac TPase was present; superscript D, one of five transformants giving two sectors per 200 seedlings with or without Ac TPase present.
Figure 6B, construct 6) carries two right Ds ends as an inverted repeat adjacent to SPT. Construct LE, LEISPT (Figure 6B, construct 7) carries two left Ds ends and is analogous to construct RE, REISPT. Constructs LE, REISPT and RE, LEISPT (Figure 6, constructs 8 and 9, respectively) carry left and right Ds ends in direct orientation, equivalent to one-half or the other of eDDs. Constructs LEISPT and REISPT (Figure 6B, constructs 10 and 11, respectively) carry one end or the other of Ds.

Three different classes of transformants were observed. In one class, seedlings were fully green and had no white sectors in the absence of Ac TPase, but in the presence of Ac TPase, there were white sectors in the characteristic pattern of the Ac TPase source used. Ac induced a range of sector sizes, and 35S:TPase induced medium-sized sectors, as shown in Figures 3B and 3D, respectively. Thus, transformants in this class gave rise to progeny in which SPT expression was unstable dependent on the presence of Ac activity.

Structures that caused instability of SPT expression dependent on the presence of Ac activity all had a common feature, namely, left and right Ds ends in direct orientation. These included Ds::AcSPT, eDDsISPT, LE, REISPT, and RE, LEISPT. Of eight eDDsISPT transformants (Figure 6B, construct 4), all but one exhibited instability of SPT expression dependent on the presence of Ac activity. The frequency of white sectors on eDDsISPT seedlings was two- to threefold lower than the frequency of green sectors on SPT::Ds seedlings. 35S:TPase induced ~10 to 20 white sectors per eDDsISPT seedling, compared to 20 to 66 green sectors in SPT::Ds seedlings (Table 1).

Four of five Ds::AcSPT transformants (Figure 6B, construct 1) exhibited SPT expression that was unstable dependent on the presence of Ac activity. Of course, Ac is always present in these individuals, so nothing can be said about their behavior in the absence of Ac activity. However, they are put in this category because they exhibit the 35S:TPase phenotype when 35S:TPase is present. This result is consistent with the observation that SPT::Ds seedlings containing both Ac and SPT; whereas in LEISPT, they are oriented toward SPT The other of Ds. In LE, REISPT, the element ends are oriented away from SPT, whereas in RE, LEISPT, they are oriented toward SPT. The observation that both of these structures produce the same phenotype implies that their effect is bidirectional.

The possibility existed that streptomycin-sensitive sectors could be due to interactions between T-DNA sequences leading to gene silencing (Matzke et al., 1989; Hobbs et al., 1990; Napoli et al., 1990). As a control for this type of interaction, each plant in which SPT function was unstable dependent on the presence of the Ac TPase T-DNA was crossed to SPT::Ds carrying plants from two independent lines. Plants in this category never had white sectors in the presence of an SPT::Ds T-DNA, confirming the interpretation that Ac activity was responsible for the loss of SPT function that was observed.

In a second class of transformants, expression of SPT was relatively stable whether or not an Ac TPase source was present. All of the constructs that did not have left and right Ds ends in direct orientation gave rise to transformants in this category. Both sets of Ac::SPT transformants (Figure 6B, constructs 2 and 3) showed relatively stable SPT expression in three to three cases each. Each set had one individual with white sectors at a frequency of 10 to 15 per 200 seedlings. This was 200-fold fewer sectors than was observed with transformants categorized as having SPT expression that was unstable dependent on the presence of Ac activity. Similar results were obtained with two of the Ds::SPT transformants.

Transformants RE, REISPT and LE, LEISPT (Figure 6B, constructs 6 and 7, respectively) contain long inverted repeats formed by inverted like ends of Ds. These individuals had stable SPT expression in each case. Finally, transformants LEISPT and REISPT (Figure 6B, constructs 10 and 11, respectively) showed stable SPT expression in each case.

In the third category of transformants, SPT was expressed poorly whether or not an Ac TPase source was present. The phenotype was an overall mottling, rather than being comprised of clonal sectors, which are characteristic of Ac-induced events. In four lines, SPT expression decreased when the Ac TPase T-DNA was present. This decrease in expression could be due to gene silencing (Matzke et al., 1989; Hobbs et al., 1990; Napoli et al., 1990). These individuals were crossed to SPT::Ds-carrying plants. In this case, SPT expression was decreased in the presence of the SPT::Ds T-DNA for each of the four lines, supporting the notion that an interaction between T-DNA loci was responsible for the mottling observed. Individuals in this category were uninterpretable with respect to Ac TPase-dependent instability of SPT expression.

Structures Predicted by Models for Double Ds-Induced Chromosome Breakage

The observation that a left end and a right end of Ds in direct orientation are sufficient to give marker gene instability in this system suggests that this structure can act as a substrate for Ac TPase. The implication is that aberrant transposition events involving the directly repeated left and right Ds ends in double Ds lead to chromosome breakage and other rearrangements. This is consistent with two different models for double Ds-induced chromosome breakage.

The first, shown in Figure 7, is similar to the model presented by Döring and Starlinger (1984). This model predicts that the directly repeated left and right ends of Ds, which serve as a substrate for transposase, are on the same DNA strand. For simplicity, only aberrant transpositions from one sister chromatid to the other are shown in Figure 7. If this type of aberrant transposition event occurs, it can result in the formation of acentric and dicentric chromosomes, leading to a chromatid-type breakage-fusion-bridge cycle (Figure 7). The alternative outcome occurs if reinsertion is in the opposite orientation and gives rise to a sister chromatid exchange. This would result
**Figure 7. Model for Double Ds-Induced Chromosome Breakage.**

(A) to (E) Simple representation of Ac or simple Ds transposition to a sister chromatid.
- **(A)** Shows a Ds element in the starting position and the sister chromatid.
- **(B)** The transposase recognizes left and right ends of the element and excises them.
- **(C)** Shows the ligation of flanking sequences forming an “empty site.”
- **(D)** Shows the insertion of Ds at a new position in one orientation.
- **(E)** Shows the insertion of Ds at a new position in the other orientation.

(F) to (J) Model for aberrant transposition of double Ds to a sister chromatid.
- **(F)** Represents the double Ds element in the starting position and the sister chromatid.
- **(G)** The double Ds element has been twisted around so directly repeated left and right ends look like a simple Ds element.
- **(H)** The transposase recognizes directly repeated Ds ends 2 and 4 and excises them.
- **(I)** Shows the ligation of flanking sequences 1 and 3 to form an “empty site,” resulting in the inversion of a half-Ds (3,2) relative to flanking sequence 1. Insertion of element ends 2 and 4 in this orientation forms U-shaped acentric and dicentric chromatids.
- **(J)** The ligation of flanking sequences 1 and 3 results in an inversion. Insertion in this orientation results in an exchange between sister chromatids.

Boxes (open and shaded) represent the 8-bp sequences flanking transposable element termini. Open and solid arrowheads are as given in Figure 1. This model is similar to the one proposed by Döring and Starlinger (1984).

In a deletion and a duplication (Figure 7J). In either case, a half Ds (extending from 2 to 3 in the Figure 7) will be inverted if the sequences flanking the excised Ds ends are ligated together to form an empty site. This would be analogous to the formation of an empty site resulting from a simple excision event.

The “strand selectivity” model (Fedoroff, 1989), shown in Figure 8, is based on the complicated insertions present in the maize shrunken alleles sh-m5933 and sh-m6258. This model predicts that the double Ds structure disrupts a strand selectivity mechanism associated with Ac TPase, such that directly repeated left and right ends of Ds located on sister chromatids act as a substrate for transposition. An aberrant transposition event involving directly repeated element ends on sister chromatids would produce an empty site (Figure 8C), resulting in the inversion of a half Ds element (extending from 2’ to 3’) relative to the flanking sequence (designated 1). In this case, formation of the empty site would give rise to an acentric or dicentric chromosome. Reinsertion of element ends 2 and 4’ could result in complex structures, such as the insertion at sh-m5933, acentric or dicentric chromosomes, or other complicated structures.

**PCR Experiments Detect Inversion of a Half Ds in Tobacco and Maize**

PCR experiments were designed to test whether the type of inversion predicted by these models occurs. Oligonucleotide primers were made to internal Ds sequences and sequences...
flanking Ds in constructs LE, RE/SP and RE, LE/SP such that PCR products would be made if the predicted inversion of a half Ds occurred, as shown in Figure 9A (see Methods for primer sequences). DNA was isolated from LE, RE/SP and RE, LE/SP plants with or without 35S:TPase present. PCR was performed on the RE, LE/SP DNA samples using primers M13+ and J1, which would yield a product of ~278 bp if the inversion occurred. PCR was performed on LE, RE/SP DNA samples using primers M13− and J2, which would yield a product of ~220 bp if the inversion occurred. PCR products of the predicted sizes were generated only when Ac TPase was present (data not shown).

PCR products were cut from gels, cloned, and sequenced. Sequences around the junction point of the inversions are shown in Figure 9B. The junction point of the inversion is analogous to an empty site formed by excision of a Ds or Ac element. The “predicted” sequences would result if the inversion was exact, i.e., if the flanking sequences joined precisely, without inserting or deleting any bases. The eight bases flanking the junction points are the same, because the left and right Ds ends in these constructs were derived from the same Ac element (see Methods). The sequences obtained have small deletions at the junction point of the inversion, consistent with Ds and Ac empty site sequences that have been reported previously (Coen et al., 1989; Fedoroff, 1989).

Similar PCR experiments were performed to determine whether the same type of inversion occurs in maize lines carrying a double Ds element. DNA was extracted from maize seeds carrying the sh-m5933 allele with and without Ac present. PCR was performed with primers D82 and D83 and with primers D84 and D85, which would amplify -490- and -237-bp fragments, respectively, if the inversion occurred. PCR products of the predicted sizes were obtained only when Ac was present in the genome (data not shown). PCR was performed with primers M13− and J2, which would yield a product of ~220 bp if the inversion occurred. PCR products of the predicted sizes were generated only when Ac TPase was present (data not shown).

Figure 9. PCR Experiments to Detect Inversion of a Half Ds in Tobacco.

(A) Schematic representation of the double Ds-like structures in LE, RE/SP and RE, LE/SP before and after inversion. PCR primers are indicated by small arrowheads. Open boxes represent the 8-bp sequences flanking transposable element termini. Open and solid arrowheads are as given in Figure 1. Specific element ends and flanking sequences are identified by number.

(B) Sequences around the junction point of inversions. Progenitor sequences show junctions of element ends and flanking sequences. Element ends (underlined by arrows) and flanking sequences (underlined) are numbered as given in (A). Positions and orientations of primers are indicated. Predicted sequences would result if the flanking sequences were joined exactly. Sequences obtained are shown below.
The flanking sequences were joined exactly. Sequences recovered are shown below; bases in italics are insertions. Predicted sequences would result if element ends and flanking sequences are identified by number. Underlined by arrows and flanking sequences (underlined) are numbered as given in Figure 1. Specific element ends and filled arrowheads are as given in Figure 1. Progenitor sequences show junctions of element (E) and flanking transposable element termini. Open arrows are homologous to the shrunken sequence. Primer D84 is after both possible inversions. Primers are shown as small arrowheads.

**Figure 10.** PCR Experiments to Detect Inversion of a Half Ds in the Maize sh-m5933 Allele.

(A) Schematic representation of double Ds at sh-m5933 before and after both possible inversions. Primers are shown as small arrowheads. Primer D82 is homologous to the shrunken sequence. Primer D84 is homologous to a sequence within the 30-kb insert. Open boxes represent the 8-bp sequences flanking transposable element termini. Open and filled arrowheads are as given in Figure 1. Specific element ends and flanking sequences are identified by number.

(B) Sequences around the junction points of inversions. Element ends (underlined by arrows) and flanking sequences (underlined) are numbered as given in (A). Progenitor sequences show junctions of element ends and flanking sequences. Predicted sequences would result if the flanking sequences were joined exactly. Sequences recovered are shown below; bases in italics are insertions.

al., 1989; Fedoroff, 1989). Again, there are small deletions around the junction points. In products 5601, 5581, and 5571, there are additional complementary bases inserted at the junction, a characteristic feature of transposable element empty sites (Coen et al., 1989).

**DISCUSSION**

We have established a system for assaying double Ds and other related structures for their ability to cause marker gene instability in tobacco. T-DNA vectors carrying the cell-autonomous markers SPT and GUS were constructed. Structures to be assayed were cloned adjacent to SPT, and the resulting constructs were used to generate transgenic tobacco lines. We have characterized this system with respect to marker loss and marker gene reversion due to a double Ds element (eDDs) and simple Ds elements (Ds4081 and Ds4135). The eDDs element causes instability of SPT and GUS expression when Ac activity is present. Simple Ds does not. Simple Ds produces an ~100-fold higher rate of reversion of SPT than does eDDs when inserted in the 5′ untranslated leader of SPT (Table 1). Thus, eDDs behaves like a chromosome-breaking (state I) Ds element, as has been defined in maize, and the simple Ds elements behave like nonbreaking (state II) elements (McClintock, 1949).

If the different promoter fusions to Ac TPase act as different states or doses of Ac, the different patterns of somatic excision of Ds observed with the SPT assay (Scofield et al., 1992) should be reflected in the pattern of instability of SPT expression caused by eDDs. In other words, the white sectors on a green background in eDDs/SPT seedlings would have the same pattern as the green sectors on a white background in SPT::Ds seedlings with each Ac TPase fusion. This was indeed the case (Figure 3) and is a parallel between our system and the observations of state I and state II Ds behavior in c-mt alleles described by McClintock (1951).

Our results strongly support the inference from work with maize that double Ds is involved in specific chromosome breakage (Courage-Tebbe et al., 1983; Böring et al., 1989). In maize, where there are many (30 to 50) copies of Ds sequences present in most genomes (Geiser et al., 1982; Fedoroff et al., 1983), it is difficult to prove that a particular Ds element confers a given phenotype. The allele sh-m5933 is associated with chromosome breakage (Courage-Tebbe et al., 1983). Several derivatives of sh-m5933 have been isolated that give an altered (later, less frequent) pattern of chromosome breakage. Molecular analysis of these derivatives showed that they no longer carried a double Ds-like structure. They are presumed to be responsible for chromosome breakage. The interpretation of these experiments was that double Ds is responsible for chromosome breakage. We have shown that a half Ds element does not induce marker gene instability in tobacco. This is in agreement with the finding of Ralston et al. (1989), who showed that a 2.5-kb terminally deleted Ac element at the bronze locus in maize was not involved in chromosome breakage unless a closely linked, intact Ac element was present. These data suggest that the half Ds in the sh-m5933 derivatives could be interacting with a nearby, undetected element.

Experiments in which we dissected double Ds showed that the structural feature responsible for the marker loss observed was a left end and a right end of Ds in direct orientation. Marker loss was not dependent on how the Ds ends were oriented relative to the marker gene. In LE, REISPT, the Ds is in direct orientation away from the SPT gene. In RE, LEISPT, the Ds ends point towards the SPT gene. Thus, if adjacent deletions extending out of the transposon were responsible for the instability observed, LE, REISPT would give marker loss and LE, REISPT would not. This suggests that more complex events, such as the formation of acentric and dicentric chromatids leading to breakage-fusion-bridge cycles, are responsible for the marker loss observed.

Twenty independent transformants that carried left and right ends of Ds in direct orientation and were interpretable with...
respect to Ac-dependent instability of SPT expression were analyzed. In 18 of these, SPT expression was unstable when Ac activity was present. It is unlikely that all 18 had the SPT marker gene distal to the Ds-derived elements that were being assayed. This suggests that markers proximal as well as distal to the element can be lost, consistent with the idea that breakage-fusion-bridge cycles may be involved with the marker loss observed.

The frequency with which eDDS:SPT gave rise to white sectors in response to Ac TPase was two- to threefold lower than the frequency of green sectors with SPT::Ds. This could mean that not all events give rise to marker loss. For example, only half of the events that caused a deletion and a duplication would give rise to white sectors.

The observation that a left end and a right end of Ds in direct orientation can induce marker gene instability dependent on the presence of Ac activity suggests that this type of structure can participate in aberrant transposition events. DNA gel blot analysis of eDDS:SPT plants that had lost SPT terminally (Figure 5) showed that DNA rearrangements accompanied marker loss, thereby supporting this idea.

Two models for double Ds-induced chromosome breakage based on aberrant transposition of directly repeated left and right Ds ends have been proposed previously. The first, shown in Figure 7, is similar to the one proposed by Döring and Starlinger (1984). It predicts that a left end and a right end of Ds in direct orientation, in unreplicated DNA or on the same chromatid, can participate in aberrant transposition events. The version presented here extends this idea, showing an insertion event associated with the aberrant transposition. One outcome would be formation of acentric and dicentric chromatids leading to chromosome breakage. An alternative outcome is a sister chromatid exchange that would result in a deletion in one strand and a duplication in the other. If the element ends were inserted somewhere other than a sister chromatid, more complicated outcomes, including translocations and ring chromosomes, would be predicted.

The second model, which is shown in Figure 8, assumes that there is an active strand selectivity mechanism associated with Ac TPase that can distinguish between the asymmetric DNA strands immediately following replication. Strand asymmetry could be mediated by hemimethylation of newly replicated DNA on opposite strands. In vitro experiments have shown that Ac TPase binds more strongly to hexamer motifs (AAACGG) methylated on a particular strand (Kunze and Starlinger, 1989). However, it is not known if this is of biological significance. Moreover, genomic sequencing experiments suggest that cytosine residues between positions 73 and 220 (at the 5' end) of Ac are not methylated in transgenic tobacco (Ott et al., 1992).

A strand selectivity mechanism would ensure that, when the substrate for transposition is a simple element, only element ends on the same DNA strand could be used. This would be consistent with genetic (Greenblatt, 1968, 1984) and molecular (Chen et al., 1987, 1992) data from the P locus in maize, which also suggest that Ac transposition follows DNA replication. Directly repeated left and right Ds ends would be predicted to disrupt the strand selectivity of Ac TPase such that Ds ends on different DNA strands could act as a substrate for transposition. This would result in aberrant transposition events that would lead to chromosome breakage and the other types of chromosomal rearrangements mentioned above.

If aberrant transposition events involving directly repeated left and right Ds ends occurred, the formation of an empty site due to ligation of flanking sequences after excision would be predicted. A consequence of this would be inversion of a half Ds element. PCR experiments detected this type of inversion in both tobacco and maize only when Ac activity was present. These results suggest that aberrant transposition events involving directly repeated left and right ends occur in both systems.

It is not clear why marker gene loss due to these aberrant transposition events occurs more frequently than simple excision when the double Ds structure is present. Apparently, directly repeated left and right Ds ends are preferred to ends in normal orientation as substrates for Ac TPase when they are present together in double Ds elements.

Dooner and Belachew (1991) have provided strong genetic evidence showing that closely linked Ac and Ds elements can cause breakage of maize chromosome 9S. A model based on transposition of a macrotransposon spanning two closely linked elements and the intervening DNA explains these results (Ralston et al., 1989).

The models presented here can also be applied to closely linked Ac and Ds elements. Both models can be applied to closely linked elements in opposite orientation. Two Ds elements that are closely linked and in opposite orientation contain left and right ends of Ds in direct orientation. If these Ds ends acted as a substrate for Ac TPase, either model would predict the same types of outcomes as for double Ds.

The strand selectivity model would not apply to closely linked elements in direct orientation, because there are no left and right ends of Ds in direct orientation present to disrupt the strand selectivity of Ac TPase. However, a variation of the model illustrated in Figure 7 could be applied to this type of structure. If the internal left and right ends of a pair of elements in direct orientation served as a substrate for Ac TPase, acentic and dicentric chromatids could be formed leading to chromosome breakage. Instead of inverting a half Ds, formation of an empty site would produce a circle including the sequence between the two elements.

None of the models described here is mutually exclusive. However, they do make different predictions about the structures that may give rise to chromosome breakage and about the structures that may result. The recovery of "post breakage" or "post rearrangement" structures from known starting structures would permit further testing of these models.

Experiments designed to elucidate the mechanism of double Ds-induced chromosome breakage should be very interesting. In addition to answering a historically interesting question, these experiments may have broader implications with regard to the mechanism of normal Ac/Ds transposition.
The aberrant events that result in chromosome breakage and other rearrangements probably follow the same rules as simple transposition. When these rules are applied to the directly repeated left and right Ds ends present in double Ds, some fundamental property of the transposition process is altered such that aberrant outcomes result. The models described here provide possible explanations for how this might happen, but they make different assumptions about the rules of normal transposition. The strand selectivity model requires that AcDs transposition is intimately associated with DNA replication and that Ac TPase can distinguish between the newly replicated strands in some way. If we can deduce the mechanism of double Ds-induced chromosomal rearrangements, it will allow us to address these fundamental aspects of AcDs behavior from a new and different point of view; this may help us to better understand normal AcDs transposition.

METHODS

DNA Constructions

Recombinant plasmids were constructed by standard techniques (Sambrook et al., 1989).

Activator Transposase Sources

pSLJ1804 (sac) was derived from pSLJ10512 (Scofield et al., 1992) by digesting with Clal and recircularizing to remove the 1-glucuronidase (GUS) coding sequence. A cauliflower mosaic virus (CaMV) 35S promoter fusion to the Activator (Ac) transposase gene pSLJ1811 (35S:Tpase) was derived from pSLJ1111 (Scofield et al., 1992) in the same way. A polymerase chain reaction (PCR)–generated cDNA copy of the Ac transposase gene (J. English, unpublished data) was cloned as a 2.5-kb SalI-BglII fragment behind a CaMV 35S promoter in pSLJ532 (Jones et al., 1992) digested with BamHI and SalI, resulting in pSLJ77B2 (35S:Ac DNA).

Dissociaion Elements

A 3-kb Dissociation (Ds) element, pSLJ1231, was made by digesting pSLJ7C3 with HindIII and recircularizing. This Ds element was cloned as a 3-kb EcoRI (Klenow fragment of DNA polymerase I fill-in)–SalI fragment into pSLJ7C3 between NruI (Klenow fragment fill-in) and XhoI to yield pSLJ1324 (engineered double Ds, eDs). pSLJ13019 was made by inserting the GUS coding sequence from pSLJ4J8 (Jones et al., 1992) as a Clal fragment into pCL0101 (Dean et al., 1992) behind the Agrobacterium tumefaciens 2' promoter (Velten et al., 1984). pSLJ1336 (eDStreptomycin phosphotransferase [SPT]) was constructed by cloning eDs from pSLJ1324 as an EcoRI–SalI fragment (Klenow fragment fill-in) into the Hpal site of pSLJ13019. pSLJ1856 (SPT:eDs) was made by cloning eDs as an SstI-SalI fragment into pSLJ1502 (Jones et al., 1992) digested with SstI and XhoI. pJ4081 (SPT: Ds) has been described previously (Jones et al., 1990).

Constructs Used to Dissect Double Ds

pSLJ4061 was made by digesting pSLJ13019 with SstI and XhoI, treating with T4 DNA polymerase to remove those restriction sites, and recircularizing; a darkBluescript (dBS+) lacZ region (Jones et al., 1992) was then cloned as a 680-bp HaeII (T4 DNA polymerase plus deoxyribonucleotide triphosphates) fragment into the Hpal site. The series of constructs based on pSLJ4061 was made by cloning fragments of Ac and Ds, as indicated below, into the appropriate sites of the dBS+)

pSLJ4074 (LE:LE/SPT) was made by a three-way ligation of a pJ4368 (Jones et al., 1992) SstI-MluI (Klenow fill-in) 0.5-kb fragment and a pJ4361 (Jones et al., 1992) Sali-PvuII 1.3-kb fragment into pSLJ4061 between the SstI and XhoI sites. pSLJ40613 (RE:RE/SPT) was made by a three-way ligation of a pJ4368 Sall-EcoRI (Klenow fill-in) 2-kb fragment and a pJ4361 Sali-PvuII 0.8-kb fragment into pSLJ4061 between the SstI and XhoI sites. pSLJ4092 (Ds:Ac/SPT) was made by inserting the 6.2-kb SstI–SalI fragment carrying Ac in Ds from pSLJ13510, which is analogous to pSLJ1324, into pSLJ4061 between the SstI and XhoI sites. pSLJ41018 (Ac/SPT) was made by inserting the 4.6-kb SstI–SalI fragment from pJ4368 into pSLJ4061 between the SstI and XhoI sites. pSLJ4123 (eDs/SPT) was made by inserting the 4.6-kb SstI–SalI fragment from pSLJ324 into pSLJ4061 between the SstI and XhoI sites. pSLJ4135 (Ds/SPT) was made by inserting the 3-kb SstI–SalI fragment from pSLJ1231 into pSLJ4061 between the SstI and XhoI sites. pSLJ4146 (LE,RE/SPT) was made by inserting the 3.5-kb EcoRI–SstI fragment from pSLJ13510 into pSLJ4061 between the SstI and EcoRI sites. pSLJ4156 (RE,LE/SPT) was made by inserting the 2.6-kb EcoRI–SalI fragment from pSLJ13510 into pSLJ4061 between the EcoRI and XhoI sites. pSLJ4168 (RE/SP) was made by inserting the 2-kb EcoRI–SalI fragment from pJ4368 into pSLJ4061 between the EcoRI and XhoI sites. pSLJ4177 (LE) was made by inserting the 2.6-kb EcoRI–SalI fragment from pJ4368 into pSLJ4061 between the EcoRI and XhoI sites.

Plant Transformation

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

Visualization of the Streptomycin Resistance Phenotype

Transgenic seed were germinated on medium consisting of Murashige and Skoog salts (ICN Biomedicals Inc., Costa Mesa, CA), 0.8% agar with 1% glucose, and 300 μg/mL streptomycin (Maliga et al., 1988; Jones et al., 1989). Variegation was visualized 10 to 14 days after plating of seed.

GUS Staining

Histochemical staining of seedlings for GUS activity was performed using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as described by Jefferson et al. (1987).

DNA Gel Blot Analysis

Large-scale DNA preparations were performed as described below (E. Ralston, personal communication). Leaf material (10 to 15 g) was
harvested and immersed in liquid nitrogen. The frozen leaves were then ground in a coffee grinder in the presence of dry ice and transferred to an ice-cold beaker; 15 to 20 mL of ice-cold extraction buffer was added (100 mM Tris- HCl, pH 9.0, 100 mM NaCl, 10 mM MgCl2, 0.5 M sucrose, 0.1% [v/v] 2-mercaptoethanol, and 0.4% [w/v] sodium diethyldithiocarbamate). The sample was then transferred to a plastic centrifuge tube and centrifuged for 2 min at 10,000 rpm in a Sorvall SS34 rotor at 4°C. The supernatant was discarded and the pellet resuspended in 1 to 2 mL of extraction buffer before the addition of 7 mL of lysis buffer (100 mM Tris-HCl, pH 8.3, 100 mM NaCl, 50 mM EDTA, 1.5% [w/v] SDS, and 15% [v/v] phenol). After a 5-min incubation at 55°C, 3 mL of 5 M potassium acetate was added, and the tubes were immersed in ice for 10 min. After a 10-min centrifugation at 4°C in a bench top centrifuge, 1 mL of 10 M ammonium acetate and 5 mL of chloroform/isoamyl alcohol were added to the supernatant. The tubes were extensively vortexed and the phases were separated by centrifugation (5 min in a bench top centrifuge). Nucleic acids were precipitated by the addition of an equal volume of isopropanol, spooled out with a heat-sealed Pasteur pipette, washed in 80% ethanol, and dissolved in 4 mL of water. RNA was removed by adding 2 mL of 10 M ammonium acetate, incubating on ice for 10 min, and centrifuging in a bench top centrifuge (5 min at 4°C). DNA was precipitated from the supernatant with isopropanol, spooled out, washed in 80% ethanol, and finally dissolved in 10 mM Tris, pH 8, 1 mM EDTA.

Genomic DNA (10 µg) was digested with the appropriate restriction enzyme and separated on 1% agarose gels. The resulting gels were probed with gel-purified DNA fragments that were labeled with phosphorus-32 by the random priming method (Feinberg and Vogelstein, 1983).

PCR Analysis

To isolate DNA for PCR analysis, the protocol of Lassner et al. (1989) was followed. PCR was performed in a thermocycler using the conditions recommended by the manufacturer (Perkin-Elmer-Cetus). A thermal profile of 35 cycles of 94°C for 15 sec, 55°C for 15 sec, and 72°C for 1 min followed by 10 min at 72°C was used. PCR primers used are as follows: D82, CAGGGTGCCTCAACAGTGGC (Ac nucleotides 4002 to 4020); D84, GCAGAAAGGTGTCATTGG (Ds flanking sequence nucleotides 3986 to 3984 as numbered in Döring et al., 1984); D85, GAGAGGTGACACTCAGG (Ac nucleotides 991 to 1011); M13+, M13 "forward" sequencing primer; J1, GCAGACCGCAACAGCCCATG (Ac nucleotides 863 to 881); M13, M13 "reverse" sequencing primer; J2, GCAGACGCCGCCATCGAC (Ac nucleotides 4002 to 4020).

Maize Stocks

Maize seed carrying the shrunken (sh)–m5933 allele (McClintock, 1953b; Courage-Tebbe et al., 1983) was kindly provided by Peter Starlinger, University of Cologne, Germany.

ACKNOWLEDGMENTS

This paper is dedicated to the memory of Barbara McClintock in honor of her incomparable contribution to this and other fields. We thank Steve Scofield, Cliff Weil, Sue Wessler, and Enrico Coen for helpful discussion; Hugo Dooner for training and helpful discussions; and Advanced Genetic Sciences (now DNA Plant Technology, Oakland, CA) for its generous help in making available DNA plasmids made while J.D.G.J. was an employee. We also thank Barry Allen, Andrew Davies, Peter Scott, and Nigel Hannant for their excellent photographic work. Research at the Sainsbury Laboratory is supported by a grant from the Gatsby Charitable Foundation.

Received January 19, 1993; accepted March 8, 1993.

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Plant Cell 1993;5:501-514
DOI 10.1105/tpc.5.5.501

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