Transposon Tagging of the Sulfur Gene of Tobacco Using Engineered Maize Ac/Ds Elements

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ABSTRACT

The Sulfur gene of tobacco is nuclearly encoded. A Su allele at this locus acts as a dominant semilethal mutation and causes reduced accumulation of chlorophyll, resulting in a yellow color in the plant. An engineered transposon tagging system, based upon the maize element Ac/Ds, was used to mutate the gene. High frequency of transposon excision from the Su locus produced variegated sectors. Plants regenerated from the variegated sector exhibited a similar variegated phenotype. Genetic analyses showed that the variegation was always associated with the transposase construct and the transposon was linked to the Su locus. Sequences surrounding the transposon were isolated, and five revertant sectors possessed typical direct repeats following Ds excisions. These genetic and molecular data are consistent with the tagging of the Su allele by the transposon.

One method for insertional mutagenesis involves interruption of a gene by a T-DNA transferred to the plant cell from the bacterium Agrobacterium tumefaciens. This procedure has been used successfully many times (Koncz et al. 1990; Rerie et al. 1994; Chiang et al. 1995; Takahashi et al. 1995), especially in Arabidopsis. However, T-DNA, once transferred to the plant cell, is immobile. Hence this procedure requires the generation of large numbers of independent transformants to have a reasonable probability of finding a mutation in the gene of interest. This can be technically challenging, especially for plant species that are difficult to transform or with large genomes. Another disadvantage of this method is that one needs to sort out somaclonal variations from T-DNA tagging events.

The mobility of transposable elements provides a valuable alternative. Multiple transposition events will generate multiple mutations from a single original transformation. Additionally, since transposition of Ac/Ds generally occurs to linked sites (Greenblatt and Brink 1963; Greenblatt 1984; Dooner et al. 1988; Jones et al. 1990; Osborne et al. 1991; Bankroft and Dean 1993), transposon tagging of particular genes may be “targeted” by initiating the screens with transposons inserted close to the gene of interest. Finally, the inherent instability of transposable elements provides additional phenotypic evidence that the mutation in the gene of interest is caused by the transposon. With these unique features, transposon tagging is a very attractive alternative method of insertional mutagenesis. Following the tagging of the gene of interest, probes derived from the transposon can be used to identify clones containing an inserted transposon from the genome of the mutant individual. Alternatively, DNA flanking the transposon can be isolated using the inverse polymerase chain reaction (IPCR; Earp et al. 1990). The DNA flanking the transposon should contain sequences from the mutated gene that can then be used to isolate the wild-type gene from a genomic library. Plant transposable elements have already been used to isolate genes from indigenous species, including, for example, Zeamays (Fedoroff et al. 1984) and Antirrhinum majus (Martin et al. 1985).

Development of heterologous transposon tagging systems allows investigations into species where transposable elements have not been isolated and characterized. In the past few years, maize transposable elements have been used successfully to isolate genes in other plant species. For example, maize transposons were used to isolate the petunia Ph6 gene (Chuch et al. 1993), the DR1 (Bancroft et al. 1993), albino (Long et al. 1993), male sterility (Aarts et al. 1993), and PROLIFERA (Springer et al. 1995) genes of Arabidopsis, the tobacco N gene (Whitham et al. 1994), and the tomato Cf-9 gene (Jones et al. 1994).

This article describes the use of an engineered heterologous transposon tagging system (Fitzmaurice et al. 1992) based on using the maize Ac/Ds elements to tag the Sulfur gene in tobacco. The binary transposon tagging system consists of an immobilized Ac encoding the transposase (Ts) and the tag (Ds) elements on separate
vectors. The Ts and Ds constructs carry selectable markers whose products confer resistance to kanamycin (Km) and methotrexate (Mtx), respectively. The transposase gene in the Ts construct is driven either by its own (native Ac) or the cauliflower mosaic virus (CaMV) 35S promoter. However, the terminal sequences of the Ac were removed, rendering it immobile. The Ds element, which can transpose when the transposase is supplied in trans, was inserted between the CaMV 35S promoter and the β-glucuronidase (GUS) coding sequence. Ds excision from the T-DNA construct allows transcriptional activation of the GUS gene that can easily be assayed.

The Sulfur (Su) gene of tobacco is nuclearly encoded. The Sulfur allele Su is a semidominant, aurea mutation isolated by Burk and Menser (1964). Initial studies show that the heterozygous (Su/su) mutant plant contained only 13% (Schmidt et al. 1966) or 35% (Menser et al. 1965) of the chlorophyll found in the green plant. This reduction in chlorophyll results in a yellow-green color in heterozygous (Su/su) plants. S elfing the Su/su plant results in 1:2:1 segregation ratio of yellow;yellow-green (Burk and Menser 1964). The homozygote (Su/Su) plant is yellow, very photosensitive, and lethal at the seedling stage for plants grown in soil. The homozygote exhibits a relatively moderate frequency of spontaneous green or yellow mutant sectors on a yellow-green background. Sulfur is an enticing gene for transposon tagging. Our prediction is that inactivation of either the Su or wild-type (su) allele may result in a somatic sector that can be distinguished visually. Since this is a somatic event, a single tobacco leaf may provide up to thousands of tagging events that can be recovered via regeneration of plants, reducing the number of plants needed for screening. In this article, we present both genetic and molecular evidence to demonstrate that the Sulfur gene has been tagged by the transposon.

**MATERIALS AND METHODS**

**General DNA manipulation:** Enzymes were obtained from either Boehringer Mannheim (Indianapolis) or Promega (Madison, WI). Most techniques were performed according to Sambrook et al. (1989). DNA sequence was determined by dideoxynucleotide chain termination according to the manufacturer’s procedure (Sequenase 2.0; United States Biochemical, Cleveland). Genomic DNA was isolated as follows (Delaporta et al. 1983). Leaf tissue (~300 mg) was pulverized in liquid N2 in a 1.5-ml centrifuge tube and incubated at 65°C for 30 min in 0.4 ml of grinding buffer [80 mM NaCl, 160 mM sucrose, 58 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), and 124 mM TrishCl (pH 8.5)]. A total of 133 μl of 3 M potassium-acetate (pH 4.7) was added to each tube and incubated on ice for 30 min. The tube was centrifuged for 10 min at maximum speed in a tabletop microfuge. The supernatant was collected and nucleic acids were precipitated using 0.7 ml of 95% ethanol.

**DNA blot analyses:** For the genomic DNA blot, 10 μg of DNA was digested for 7 hr at 37°C using 40 units of EcoRV before electrophoresis on an agarose gel. Random-primed digoxigenin (DIG; Boehringer Mannheim) dhfr probes were prepared according to Fitzmaurice et al. (1992). A DNA blot was performed as described (Fitzmaurice et al. 1992) and detection of signal was performed, as recommended by the supplier (Boehringer Mannheim) using Lumiphos 530. In the confirmation of the inverse PCR product, DNA blot analyses were performed as described (Church and Gilbert 1984). DNA in the gel was transferred to Magnagraph nylon membrane (Micron Separations, Westborough, MA) by capillary action in 25 mm NaPO4 (pH 7.0) buffer. The DNA was UV-crosslinked at 120 ml/cm2 and baked subsequently for 40 min under vacuum at 80°C. DNA fragments were radiolabeled using random priming (Decaprime II DNA labeling kit, Ambion Inc., Austin, TX). Hybridization was performed at 65°C for 13-17 hr followed by two washes in 0.1 SSC, 0.2% SDS at 42°C and two additional washes in 0.2× SSC, 0.2% SDS at 62°C. The 1× SSC solution contains 150 mM NaCl and 15 mM Na-citrate.

**Plant regeneration from mutant sectors:** Leaves containing the sectors of interest were surface-sterilized by washing in 0.1% Triton X-100 and rinsing briefly in 70% ethanol. They were then soaked in 1% sodium hypochlorite for 20 min, followed by three washes in sterile water. Sectors were excised from the leaves and placed on MS regeneration medium (0.8% agar, 3% sucrose, 58 mM NaCl and 15 mM Na-citrate) supplemented with 100 mg/liter kanamycin (Km) and 0.5 mg/liter methotrexate (Mtx), respectively. The transgenic tobacco plants containing either the Ts or Ds element was described by Fitzmaurice et al. (1992). Transforms DsL7.b.1 and Ts5.8.a carry the pWPF147 Ds vector and the pWPF130 Ts vector, respectively (Fitzmaurice et al. 1992). Nicotiana tabacum cv. Petite Havana, strain SR1 was used as a wild-type green (su/su) parent for segregation analysis. Tobacco line 1719, derived from the strain Red Russian, is heterozygous (Su/Su) for Sulfur.

**Germination and selection techniques:** Surface-sterilized F1 seeds from crosses of the Ts and Ds transgenic lines were germinated on MS medium supplemented with 100 mg/liter kanamycin (Km) and 0.5 mg/liter methotrexate (Mtx). Resistant yellow-green plants were grown to maturity and screened for the unstable sectors.

In the segregation analysis to stabilize the mutant phenotype, surface-sterilized seeds were germinated on the MS medium supplemented with 0.5 mg/liter methotrexate. To examine the presence of the Ts construct, cotyledons from resistant seedlings were excised and placed on the regeneration medium (MS + BAP) supplemented with 100 mg/liter kanamycin. The yellow phenotype was determined on all mature plants that were resistant to methotrexate.

In reactivation analyses of the unstable phenotype, surface-sterilized seeds from the appropriate crosses were germinated on MS medium. Cotyledons from the seedlings were excised and placed separately on each regeneration medium (MS + BAP) containing either 100 mg/liter kanamycin or 0.5 mg/liter methotrexate for determining the presence of Ts- and Ds-elements, respectively. Seedlings were subsequently transplanted to soil and scored for the variegated phenotype.

To determine allelism between the Ds-tagged and the Sulfur genes, pollen from yellow-green plant 1719 (Su/su) was used to pollinate flowers of stable green plants. These green plants, resulting from a cross of Spot2a and SR1, contained the Ds element but not the Ts element. F1 seeds were surface-sterilized and germinated on MS medium. Seedlings were scored for the yellow, yellow-green, and green phenotype. To determine the presence of the Ds, leaf discs from these three classes of
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plants were placed on the regeneration medium containing 0.5 mg/liter Mx.

**IPCR and PCR of flanking DNA:** DNA templates for IPCR were prepared according to Earp et al. (1990). Genomic DNA (~2 μg) was digested to completion with MspI in a 40-μl reaction volume. Following heat inactivation of the restriction enzyme for 30 min at 65°C, a 20-μl aliquot was self-ligated in 4 units of T4 DNA ligase (from Boehringer Mannheim) at a DNA concentration of 4 μg/ml. Ligated DNA was extracted with phenol/chloroform and DNA was precipitated with ethanol in the presence of 50 μg/ml tRNA. DNA was then resuspended in 10 μl of water. PCR amplification was performed using 1 μl of resuspended DNA in a 50-μl reaction volume containing 2.5 units of Taq DNA polymerase (Promega), 100 μM each deoxynucleotide triphosphate (dNTP), and 0.4 μM each primer. The PCR temperature profile was 40 cycles of 1 min at 94°C, 1 min at 63°C, and 1 min at 72°C followed by 1 cycle of 6 min at 94°C. Nested PCR amplification (35 cycles) was achieved using 1/1000 of the first PCR reaction. Substrate concentrations and PCR conditions were similar to that above except nested primers. IPCR and PCR conditions were similar to that above except nested primers, Ds_P10 (5'-GAGCTAGTTTCCCGACCGTTT-CACC-3') and Ds_P11 (5'-TTATACGATAACGGTCGGTA CGGG-3') were used. The IPCR product was cloned subsequently into the pBlueScript plasmid (Stratagene, La Jolla, CA) and its sequence was determined.

The linkage between the newly isolated IPCR product and DsB was confirmed by PCR amplification of the flanking DNA. About 120 ng of each genomic DNA was used as the template in a 25-μl reaction containing similar concentrations of dNTP and primers as described above. The primers used here are either Ds_P11 and SU_P2 (5'-GAGCTAGTTTCCCGACCGTTT-CACC-3') or Ds_P7E (5'-GAATTCGGTTATACGATAACGG-3') and SU_P2. The PCR conditions were 40 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C.

**Cloning of Ds excision footprints:** Genomic DNA was extracted from 50-100 mg of tissues of revertant (yellow/green) sectors of Spot2a's progeny. The isolated DNA was subjected to PCR amplification in a 25-μl reaction volume containing 100 μM each dNTP, and 0.2 μM each SU_P4A (5'-CCGGGGGCTCCATATGCGAAGG-3') or Ds_P7E (5'-GAATTCGGTTATACGATAACGG-3') and SU_P2. The PCR conditions were 40 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C.

**RESULTS**

**Mutagenesis in the yellow-green plant and regeneration of the mutant sector:** Green tobacco plants containing T-DNA elements were crossed to heterozygous yellow-green (Su/su) plants. However, when Su/su plants were crossed to Su Su plants and methotrexate (selecting for Su) were screened for Ds excision activity by assay of a leaf for GUS activity. [The Ds element is flanked by a CaMV 35S promoter and the β-glucuronidase (GUS) coding sequence such that GUS expression occurs upon excision of the Ds element (Fitzmaurice et al. 1992).] Nine individuals (from three different crosses) that exhibited relatively high excision activity (many GUS spots or sectors) were grown to maturity and their leaves examined for somatic Sulfur sectors. Twenty-three unstable sectors were observed in five flux progeny resulting from two crosses [Ds1.7.b.1 × Ts5.8.a and Ds1.2.a × Ts5.8.b (Fitzmaurice et al. 1992)]. These unstable green sectors were excised and placed in tissue culture to regenerate whole plants. Since somatic transposition of the Ds element is necessary for tagging the Su gene, it is possible that a sector of GUS activity will exactly correlate with the borders of a yellow, green, or unstable sector (assuming this was the first transposition of the Ds element from the initial T-DNA integration site). Yellow, green, and unstable sectors were divided into sectors containing both green and GUS activity and sectors containing only green or only GUS activity.

**Removal of the T-DNA element stabilizes the green phenotype:** It was hypothesized that the unstable phenotype was due to revertant sectors caused by the T-DNA catalyzing Ds excision from the Su gene. This secondary excision could restore the yellow-green phenotype. This hypothesis leads to the prediction that, when Su segregates from the tagging Ds element (by self-pollination or outcrossing to a wild-type green plant), the Ds would be stable and the phenotype of the plant would be solid green. Table 1 presents results consistent with this hypothesis. Plant Spot2a was either selfed or outcrossed to a wild-type green SR1 plant (Havana petite SR1). Since the Spot2a plant has two unlinked T-DNA element loci, the expected ratios of kanamycin resistant sensitive were 15:1 and 3:1 for selfed and outcrossed progeny, respectively. Progeny that are Mtx2 and Km2 (i.e., possessing both Ts and Ds elements) have the unstable phenotype (Figure 1, F-G) or are completely yellow-green (presumably due to recombination of a Ds excision from the Su gene with reintegration of the Ds element elsewhere in the genome; Figure 1D). As
Figure 1.—Photographs of the variegated heterozygous Spot2a plant, its parents and selfed progeny. (A) Plant Ts5.8.a, containing the transposase (Ts). (B) Plant Ds1.7.b, heterozygous Sulfur containing the tagging Ds element. (C) Plant Spot2a, unstable Ds-tagged sulfur green plant containing the Ts. (D–G) Selfed progeny of plant Spot2a. (D) A germinal revertant in which Ds had reexcised from the Su locus, resulting in restoration of the yellow-green phenotype. (E) A plant in which Ts had been lost by segregation, resulting in stabilization of the green phenotype caused by Ds tagging of the Su gene. (F and G) Examples of progeny retaining the unstable phenotype. Excision of the Ds element results in yellow-green sectors in green background. Levels of excision seen in the progeny vary widely.

predicted, progeny that received the Ds element, but not the Ts element (Mtx\(^a\), Km\(^b\)), always exhibited a stable green phenotype (Figure 1E). These results indicate that segregation of the Ts elements results in stabilization of the phenotype. Three of the seven stable green plants from the outcross (plants numbered 22, 17, and 37) were chosen for further analysis described below.

**Reactivation of transposition:** Introduction of an active transposase into a stable green plant that retains the tagging Ds element should restore the unstable phenotype. Table 2 presents the results of such a test. A stable green Mtx\(^a\) F\(_1\) plant (plant 22) from the cross of plant Spot2a with SR1 was chosen for an attempt to reactivate the unstable phenotype by reintroduction of

<table>
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<th>TABLE 1</th>
<th>Segregation of Ts stabilizes the Ds inactivation of Su</th>
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<td><strong>Cross</strong></td>
<td><strong>Mtx selection</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Observed (Mtx(^a):Mtx(^b))</strong></td>
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<tr>
<td>Spot2a selfed</td>
<td>Selected</td>
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<tr>
<td>SR1 × Spot2a</td>
<td>27:23</td>
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\(^a\) F\(_1\) progeny were germinated on plates containing 1 \(\mu\)m methotrexate (Mtx).

\(^b\) Those F\(_1\) resistant to Mtx were tested subsequently for resistance to kanamycin (Km).

\(^c\) All F\(_1\) plants resistant to Mtx were examined for the color phenotype.

\(^d\) We hypothesize that these YG (yellow-green) plants represent germinal excision events.
TABLE 2

Reactivation of the unstable phenotype in stable green segregants by backcross to \( T_s \) plants

<table>
<thead>
<tr>
<th>Crosses</th>
<th>Phenotypes of ( F_1 ) plants*</th>
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<tr>
<td>( T_s5.8.a:1 \times 22 )</td>
<td>( K_m^a,M_bx^b ) Phenotype</td>
</tr>
<tr>
<td>15</td>
<td>15 unstable</td>
</tr>
<tr>
<td>9</td>
<td>9 unstable</td>
</tr>
<tr>
<td>( 35STs4.5.a:3 \times 22 )</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>22 unstable</td>
</tr>
<tr>
<td>9</td>
<td>9 unstable</td>
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* \( F_1 \) progeny were germinated in the absence of selection. Leaf discs from the seedlings were placed separately on regeneration media (MS + BAP) containing either \( K_m \) or \( M_bx \).

The transposase. \( A \) \( T_s \) plant containing the native Ac (\( T_s5.8.a:1 \)) or the CaMV 35S (\( 35STs4.5.a:3 \)) promoter was used as a source of the \( T_s \) element. Both \( T_s \) parents were homozygous and so all \( F_1 \) progeny received \( T_s \) (\( K_m^b \)). The unstable phenotype was reactivated in all of the \( F_1 \) progeny that received the tagging element (\( M_bx^b \)).

**Linkage analysis of the Sulfur and \( Ds \)-tagged gene:**

To determine whether the \( Ds \) element had tagged \( Su \), cosegregation analyses were performed (Figure 2). Stable green segregants of the \( SR1 \times Spot2a \) (plants 17 and 37; heterozygous for the \( Ds \) element) were crossed to an untransformed heterozygous \((su/Su)\) yellow-green plant (1719). The expected result when crossing a wild-type green plant \((su/su)\) with a heterozygous Sulfur plant \((su/Su)\) would be a segregation ratio of 1:1:0 (green:yellow-green:yellow; \( G:YG:Y \)) among the progeny. The observed result (Figure 2D) was a segregation ratio of 2:1:1, \( G:YG:Y \). Three scenarios are described in Figure 2, A, B, and C, that are consistent with this observed ratio. The percentage of \( M_bx^b \) progeny from the test crosses can be used to distinguish among the three scenarios. The observed \( M_bx^b \) was 51.6, 0, and 100% for green, yellow-green, and yellow, respectively (Figure 2D). In the scenario described in Figure 2A the \( Su \) allele has a spontaneous null mutation resulting in a green sector on a yellow-green background. Crossing a spontaneous null mutant of the Sulfur allele with the test-cross

![Figure 2](image-url)
plant would result in a ratio of 2:1:1, G:YG:Y. However, if there is an unlinked heterozygous Ds element in the spontaneous mutant background, then the Mtx\(^{a}\) conferred by the Ds would be present in 50\% of the progeny irrespective of the Sulfur content (Figure 2A), which is inconsistent with the observed result. In addition, this scenario is unlikely since the original green sector was unstable, indicative of linkage to a transposable element. The second and third possibilities are that the unstable phenotype could be caused by the Ds inserting into the Sulfur gene or into another gene that controls expression of the Sulfur phenotype. Crossing a heterozygous sulfur plant (su/ Su) with a stable green (su/ Su::Ds) plant having Ds tagging sulfur would result in the observed segregation ratio of 2:1:1, G:YG:Y (Figure 2B). Of these, half of the green progeny, none of the yellow-green progeny, and all of the yellow progeny would be resistant to Mtx (Figure 2B), which is also consistent with the observed result. Tagging of a regulatory gene with observed segregation ratio of 2:1:1, G:YG:Y (Figure 2B). Of these, half of the green progeny, none of the yellow-green progeny, and all of the yellow progeny would be resistant to Mtx (Figure 2B), which is also consistent with the observed result. Tagging of a regulatory gene with observed segregation ratio of 2:1:1, G:YG:Y (Figure 2B). Of these, half of the green progeny, none of the yellow-green progeny, and all of the yellow progeny would be resistant to Mtx (Figure 2B), which is also consistent with the observed result. Tagging of a regulatory gene with observed segregation ratio of 2:1:1, G:YG:Y (Figure 2B). Of these, half of the green progeny, none of the yellow-green progeny, and all of the yellow progeny would be resistant to Mtx (Figure 2B), which is also consistent with the observed result. Tagging of a regulatory gene with observed segregation ratio of 2:1:1, G:YG:Y (Figure 2B). Of these, half of the green progeny, none of the yellow-green progeny, and all of the yellow progeny would be resistant to Mtx (Figure 2B), which is also consistent with the observed result. Tagging of a regulatory gene
Figure 4.—PCR and Southern analysis of the DNA flanking the Ds border. Total genomic DNA was subjected to PCR amplification using SU_P2 primer with either DS_P11 (lanes 1-4) or DS_P7E (lanes 5-8) primer. The predicted PCR products for DS_P7E/SU_P2 and DS_P11/SU_P2 are 440 and 450 bp, respectively. Template genomic DNA was extracted from primary Ds-transformant (plant Ds1.7b), lanes 1 and 5; wild-type (su/su) green plant (lane 1098), lanes 2 and 6; heterozygous yellow-green (su/su) line 1719 plant, lanes 3 and 7; stable Ds-tagged Su green plant 17 (su/Su::Ds), lanes 4 and 8. Each lane contains a fifth of the total PCR product. (A) Ethidium bromide-stained gel. (B) Southern blot using probes derived from the IPCR product. (C) Structure of the Ds element along with primers and probe used in A and B.

ifications using a primer (SU_P2) located within the 424 bp DNA going toward the transposon and a Ds border primer (DS_P7E or DS_P11, Figure 4C). If the IPCR product is linked to the Ds border, then DNA from plant 17 should generate PCR products of 440 bp and 450 bp for DS_P7E and DS_p11, respectively. Results of this experiment are shown in Figure 4. DNA from plant 17 produced the predicted PCR products of ~450 bp (Figure 4A, lane 4) and 440 bp (Figure 4A, lane 8) using DS_P11 and DS_P7E, respectively. DNA from the primary Ds transformant (Ds1.7b; Figure 4A, lanes 1 and 5) did not serve as a template for amplification nor did DNA from untransformed wild-type green (su/su) and yellow-green (su/Su) (Figure 4A, lanes 2, 3, 6, and 7). When DS_P7E and SU_P2 primers were used, some products in the 570-bp range were visible (Figure 4A, lanes 5, 6, and 7). To determine if these products are specific to the IPCR product, a DNA blot of this gel was hybridized to probes derived from the IPCR fragment (Figure 4B). As expected, only the predicted products of plant 17 DNA hybridized to the IPCR probe (Figure 4B, lanes 4 and 8). These data indicated that the 424-bp IPCR DNA is adjacent to the Ds border.

Ds excision created footprints in revertant sectors: A genomic library was constructed by CLONTECH Laboratory, Inc. (Palo Alto, CA) using DNA isolated from the stable green segregant, plant 29. Using DNA of the dhfr gene within the transposon as the probe, we screened a total of 2 million plaques without any positives. The genomic library was rescreened using probes made from the 424-bp of IPCR product. We isolated two phage clones, 29C1 and 39C1, that hybridized to the flanking DNA. All 424 bp of the IPCR product are identical to sequences within clone 39C1. Since clone 39C1 does not contain the Ds element, we believe that it contains the wild-type sulfur gene (su). The genomic clone 29C1 also does not contain the Ds element. Clone 29C1 is not identical to 39C1 or the 424-bp IPCR product, with mismatches on both sides of the Ds insertion site (data not shown). The high degree of similarity between the two clones indicates that clone 29C1 may contain a homeologous gene of sulfur.

Sequence analysis of clone 39C1 around the area of the Ds element insertion site (as determined from the junction in the IPCR product) allowed the design of PCR primers to examine the footprints left by the Ds element excision in revertant sectors (Figure 5A). Ac/Ds elements generally create a direct repeat of eight nucleotides upon insertion. These elements would leave a footprint at the target site following their excision. If Ds excises perfectly, it would leave eight extra nucleotides at the target site. The extra eight nucleotides would cause a frameshift in the protein if the Ds had inserted into the coding region of the Sulfur gene. Revertant yellow-green sectors appeared frequently in Spot2a or its selfed progeny (Figure 1, C, F, and G), indicating that the Ds element may have inserted into a noncoding region of the Sulfur gene. Primers SU_P4A and SU_P5S (Figure 5A), flanking the Ds insertion site, were used to amplify genomic DNA of revertant yellow-green sectors from a selfed progeny of Spot2a. The PCR products of 210 bp were subcloned and their sequences determined. Generally, sequences were determined in more than one subclone of the amplification because of the presence of a wild-type (su) allele within these sectors. Genomic clone 39C1 sequence is shown as the reference to the wild-type sequence (Figure 5B). As expected, a green portion of the leaf, wild-type S7, contains similar sequence to the genomic clone 39C1. However, sequences of five yellow-green revertant sectors show between five and seven extra nucleotides at the target site (Figure 5B). Except for revertant sector S8, other sectors possessed either five or seven extra nucleotides, suggesting that the Ds insertion site may be confined to a noncoding region of the Sulfur gene. These results indicate that Ds excision from the Su allele is always associated with the revertant yellow-green phenotype.
and that Ds's target site is probably located within a noncoding region of the gene.

**DISCUSSION**

The presence of spontaneous sectors in the heterozygous Su background gave us confidence that we would be able to detect somatic sectors in which the gene had been disrupted. However, their presence also posed a potential problem of background events not linked with the Ds element. Therefore, attention was focused on somatic sectors with characteristics of a transposon-induced mutation. We detected several unstable green sectors showing islands of reversion to yellow-green within their borders. Such unstable sectors were never observed in the wild-type su/Su plants, or in plants with the Ds element alone. The high number of unstable sectors (23 sectors) found in five F1 plants reflects the advantage of our strategy of targeted disruption of a gene where mutant phenotype can be easily screened at somatic level.

Several lines of evidence demonstrate that the Su has been tagged by the engineered Ds element. Genetic analysis demonstrated the linkage of Su with the Ds-tagged gene. A major concern was to distinguish between tagging of the Su gene directly and disruption of a controlling gene (i.e., a second-site suppressor). Both phenotypic ratio and methotrexate resistance were employed to distinguish between these possibilities. Molecular evidence also supported the assertion of tagging the Ds element. Therefore, attention was focused on Southern analysis confirmed the correlation of somatic sectors with characteristics of a transposon transposition of Ds and mutation of Su, both in the induced mutation. We detected several unstable green sectors showing islands of reversion to yellow-green within their borders. Such unstable sectors were never observed in the wild-type su/Su plants, or in plants with the Ds element alone. The high number of unstable sectors (23 sectors) found in five F1 plants reflects the advantage of our strategy of targeted disruption of a gene where mutant phenotype can be easily screened at somatic level.

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could be reactivated by crossing the stabilized green plant to a source of Ts, driven either by the native or the CaMV 35S promoters. Histochemical staining was used to demonstrate that the borders of an unstable sector on a heterozygous sulfur plant containing Ds and Ts corresponded to the boundaries of an area of GUS expression indicative of Ds excision.

Results from PCR amplification of the flanking DNA established that the 424-bp IPCR DNA fragment is indeed adjacent to a Ds border (Figure 4). Based on pheno-type observations of frequent revertant sectors in Spot2a plant, the Ds element was hypothesized to have inserted into a noncoding region of the gene. Results from the Ds-excision footprint analysis were consistent with this hypothesis. Footprints recovered following Ds excision from the sulfur gene contain between five and seven additional nucleotides (Figure 5B). These numbers are within the range reported by other investigators working with Ac/Ds elements in endogenous (Pohlman et al. 1984; Sutton et al. 1984) and heterologous (Baker et al. 1986; Laufs et al. 1990; Bancroft et al. 1993) systems.

Occasionally, yellow-green germinial revertants among the selfed progeny of unstable plant Spot2a were observed that showed an extremely high rate of new green or unstable sectors. We hypothesize that the Ds had transposed to a position closely linked to the Su gene, and it was subsequently transposing back into the linked Su gene. The Ac transposon has been shown to transpose preferentially to linked sites in Arabidopsis (Bancroft and Dean 1993), maize (Dooner et al. 1994), tobacco (Jones et al. 1990), and tomato (Osbourn et al. 1991). This property of Ac/Ds would provide the opportunity to generate many independent mutations in Su for study of the range of possible mutant phenotypes of Su, as has been done with genes in maize (Peterson 1990; Athma et al. 1992; Alleman and Kermicle 1993).

The successful tagging of Su should facilitate the determination of the complete sequence of the Su and su alleles. This information may provide insight into the nature of the semidominant Su mutation.

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