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

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Manuscript received May 19, 1999
Accepted for publication August 31, 1999

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; Bancroft 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, *Zea mays* (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 (Chuck *et al.* 1993), the DRL1 (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

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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% (Schmid *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. Selfing the *Su*/*su* plant results in 1:2:1 segregation ratio of yellow:yellow-green: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 heterozygote 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 Boerhinger 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 (Dellaporta *et al.* 1983). Leaf tissue (~300 mg) was pulverized in liquid N_2 in a 1.5-ml centrifuge tube and incubated at 65° 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 Tris-HCl (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° using 40 units of *Eco*RV before electrophoresis on an agarose gel. Random-primed digoxigenin (DIG; Boerhinger 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 (Boerhinger Mannheim) using Lumi-Phos 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 $NaPO_4$ (pH 7.0) buffer. The DNA was UV-crosslinked at 120 mJ/cm^2 and baked subsequently for 40 min under vacuum at 80°. DNA fragments were radiolabeled using random priming (Decaprime II DNA labeling kit, Ambion Inc., Austin, TX). Hybridization was performed at 65° for 13–17 hr followed by two washes in 1× SSC/0.2% SDS at 42° and two additional washes in 0.2× SSC/0.2% SDS at 62°. 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; Murashige and Skoog 1962) containing 1 μg/ml benzylaminopurine (BAP). Regenerated shoots were excised and placed on MS medium without growth regulators, and rooted plants were transferred to soil and grown to maturity.

Genetics of tobacco lines: The genetic background of transgenic tobacco plants containing either the *Ts* or *Ds* element was described by Fitzmaurice *et al.* (1992). Transformants Ds1.7.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 F_1 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. F_1 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

plants were placed on the regeneration medium containing 0.5 mg/liter Mtx.

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 *Msp*I in a 40-μl reaction volume. Following heat inactivation of the restriction enzyme for 30 min at 65°, a 20-μl aliquot was self-ligated in 4 units of T4 DNA ligase (from Boerhinger 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 DS_P6 (5′-GTTTTTTACCTCGGGTTCGAAATCG-3′) and DS_P8 (5′-TATACAAAACGGTAAACGGAAACGG-3′) primers. The PCR temperature profile was 40 cycles of 1 min at 94°, 1 min at 63°, and 1 min at 72° followed by 1 cycle of 6 min at 72°. 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, 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′-CAGCCCACCCTAATGCAAGG-3′) or DS_P7E (5′-GGAATTCGGTTATACGATAACGG-3′) and SU_P2. The PCR conditions were 40 cycles of 1 min at 94°, 1 min at 60°, and 1 min at 72°.

Cloning of *Ds*-excision footprints: Genomic DNA was extracted from 50–100 mg of tissues of revertant (yellow/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′-CCGGGCCCTCCATAAAACTTCCTCC-3′; underlined bases indicate the *Apa*I recognition site) and SU_P5S (5′-TCCCCCGGGCCTTGTCTTCTCGCTCCTGT-3′; underlined bases indicate the *Sma*I recognition site) primers. The temperature profile was 35 cycles of 1 min at 94°, 45 sec at 56°, 40 sec at 72° followed by 1 cycle of 4 min at 72°. PCR products were extracted with phenol/chloroform and precipitated with ethanol. DNA was cloned subsequently into pBlueScript at the *Apa*I and *Sma*I sites. Sequences of these clones were determined.

RESULTS

Mutagenesis in the yellow-green plant and regeneration of the mutant sector: Green tobacco plants containing *Ts* elements were crossed to heterozygous yellow-green (*Su*/*su*) *Sulfur* tobacco plants carrying *Ds* elements (12 F_1 crosses from various crosses between eight independent *Ts* lines and five independent *Ds* lines; Fitzmaurice *et al.* 1992). No effort was made to choose *Ds* lines with the T-DNA insertion site genetically linked to the *Su* locus. Yellow-green (*Su*/*su*) F_1 plants resistant to kanamycin (selecting for *Ts*) and methotrexate (selecting for *Ds*) 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 F_1 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, or unstable sectors were divided so that part of the sector was placed in tissue culture, and the remaining portion (plus surrounding tissue) was stained histochemically for GUS activity. Some sectors occurred in regions in which both the sector and the surrounding tissue stained completely for GUS activity. This indicates that an earlier somatic excision had occurred in the lineage of cells that generated the portion of the leaf containing the sector. In other sectors (for example, unstable sector 2) the margins of the sector correlated exactly with the area of positive GUS activity (data not shown). One regenerated unstable plant (Spot2a) from this sector was chosen for detailed study. Its parental *Ts* and *Ds* plants are Ts5.8.a (Figure 1A) and Ds1.7.b.1 (Figure 1B), respectively (Fitzmaurice *et al.* 1992). Plant Spot2a showed the unstable phenotype of a green plant exhibiting a high frequency of yellow-green revertant sectors (Figure 1C).

Removal of the *Ts* element stabilizes the green phenotype: It was hypothesized that the unstable phenotype was due to revertant sectors caused by the *Ts* catalyzing *Ds* excision from the *Su* gene. This secondary excision could restore the yellow-green phenotype. This hypothesis leads to the prediction that, when *Ts* 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 *Ts* element loci, the expected ratios of kanamycin resistant to sensitive are 15:1 and 3:1 for selfed and outcrossed progeny, respectively. Progeny that are Mtx^R and Km^R (*i.e.*, possessing both *Ts* and *Ds* elements) have the unstable phenotype (Figure 1, F–G) or are completely yellow-green (presumably due to germinal transmission of a *Ds* excision from the *Su* gene with reintegration of the *Ds* element elsewhere in the genome; Figure 1D). As

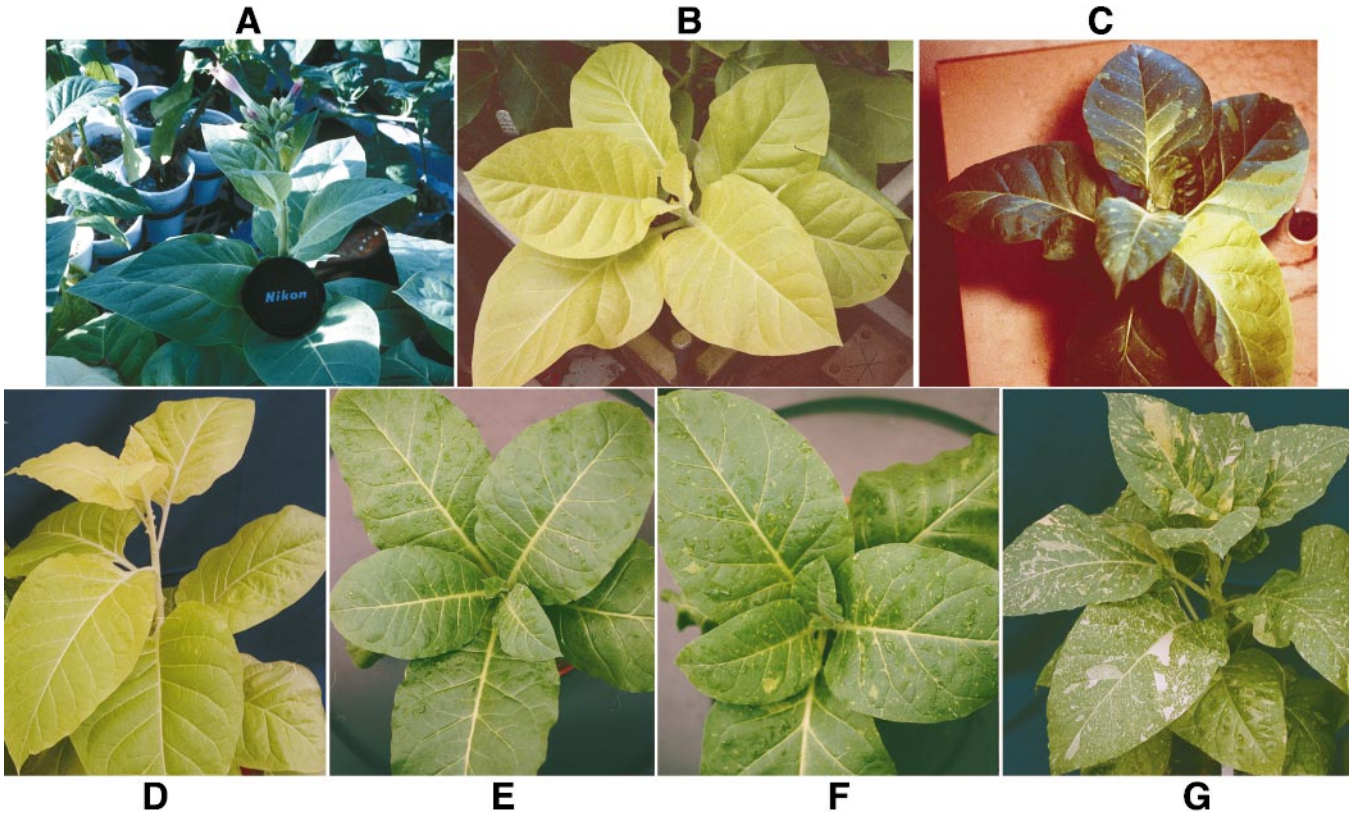


Figure 1.—Photographs of the variegated heterozygous Spot2a plant, its parents and selfed progeny. (A) Plant Ts5.8.a, containing the transposase (*Ts*). (B) Plant Ds1.7.b, heterozygous *Sulfur* containing the tagging *Ds* element. (C) Plant Spot2a, unstable *Ds*-tagged *sulfur* green plant containing 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^R, Km^S), 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^R 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 1

Segregation of *Ts* stabilizes the *Ds* inactivation of *Su*

Cross	Mtx selection[a]		Km selection[b]		Color phenotype[c]	
	Observed (Mtx^R:Mtx^S)	Expected ratio	Observed (Km^R:Km^S)	Expected ratio	Km resistant	Km sensitive
Spot2a selfed	Selected	—	37:2	15:1	35 unstable 2 YG[d]	2 stable green
SR1 × Spot2a	27:23	1:1	20:7	3:1	19 unstable 1 YG[d]	7 stable green

[a] F_1 progeny were germinated on plates containing 1 μm 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

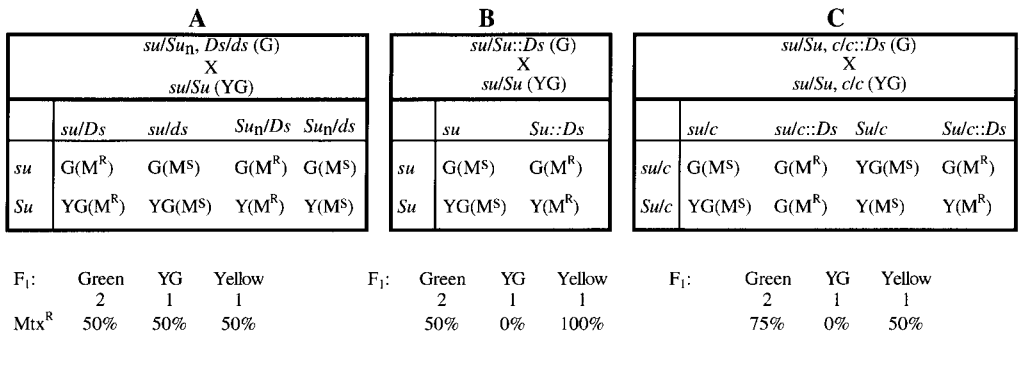
Reactivation of the unstable phenotype in stable green segregants by backcross to *Ts* plants

	Phenotypes of F_1 plants[a]			
Crosses	Km^R,Mtx^R	Phenotype	Km^R,Mtx^S	Phenotype
Ts5.8.a:1 × 22	15	15 unstable	12	12 stable green
35STs4.5.a:3 × 22	9	9 unstable	17	17 stable green

[a] 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 Km or Mtx.

the transposase. A *Ts* plant containing the native *Ac* (Ts5.8.a:1) or the CaMV 35S (35STs4.5.a:3) promoter was used as a source of the *Ts* element. Both *Ts* parents were homozygous and so all F_1 progeny received *Ts* (Km^R). The unstable phenotype was reactivated in all of the F_1 progeny that received the tagging element (Mtx^R).

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 × 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 Mtx^R progeny from the test crosses can be used to distinguish among the three scenarios. The observed Mtx^R 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



D

Cross	Number of Plants (ratio and % of Mtx^r progeny tested)		
	Green	Yellow-green	Yellow
1719 x 17	138 (45/91; 49.5%)	71 (0/21; 0%)	77 (49/49; 100%)
1719 x 37	126 (58/108; 53.7%)	70 (0/29; 0%)	71 (43/43; 100%)
Totals	264 (51.6%)	141 (0%)	148 (100%)

Figure 2.—Genetic analysis of the linkage of *Su* and the *Ds*-tagged gene. (A) Crossing a *Su*-null mutant green plant to a heterozygous *Sulfur* plant results in F_1 progeny that segregate 2:1:1, green : yellow-green : yellow. (B) Su::*Ds* indicates that the *Ds* element directly interrupts the *Su* gene [*i.e.*, *Ds* (Mtx^R) is linked to *Su*]. In this case, crossing a stabilized green plant to a heterozygous *Sulfur* plant results in F_1 progeny that segregate 2:1:1, green:yellow-green:yellow. The percentage of segregants receiving the Mtx^R marker contained within the *Ds* element is also indicated (M^R, Mtx resistant; M^S, Mtx sensitive). (C) In the third case, a hypothetical "controlling gene" required for the expression of *Su* is represented by *c*. *Ds* influence on *c* results in no expression of the *Su* allele. A heterozygous *Sulfur* containing the tagged *c* (*c*::*Ds*) would be green instead of yellow-green. The phenotype of a homozygous *Sulfur* plant containing the *c*::*Ds* gene is speculative (see text), but if the phenotype is yellow, crossing a stabilized green plant to a heterozygous *Sulfur* plant also results in F_1 progeny that segregate 2:1:1, green:yellow-green:yellow. G, green; YG, yellow-green; Y, yellow; Su_n, mutation resulting in null of *Su* allele. (D) Results of the testcross between yellow-green (*su*/*Su*) and the stable green segregants (plant nos. 17 and 37) carrying the methotrexate-resistant phenotype. F_1 seeds were germinated in the absence of selection. Leaf discs from seedlings were examined subsequently for resistance to methotrexate.

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^R 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 in which the *Ds* element would be unlinked to the *Su* gene could result in several different segregation patterns, depending upon the phenotype of a segregant inheriting two *Su* alleles (normally yellow) but also the *Ds*-tagged gene. If such a segregant were green, the segregation ratio would be 5:2:1, G:YG:Y. If the segregant were yellow-green, then the segregation ratio would be 4:3:1. These segregation ratios are inconsistent with those observed (Figure 2D). However, if the segregant were yellow, the segregation ratio would be 2:1:1, G:YG:Y (Figure 2C) as observed in the test crosses. This case can be distinguished from the *Su*-tagged case by analyzing the Mtx^R status of segregants. If the *Ds* element and the *Su* were unlinked, 75% of the green segregants would be Mtx^R, while none of the yellow-green and 50% of the yellow segregants would be resistant, which is inconsistent with the observed result.

Analysis of the segregation of Mtx^R in two crosses with two different stable green plants containing *Ds* (plants 17 and 37) showed methotrexate resistance ratios of ~50, 0, and 100% for green, yellow-green, and yellow, respectively (Figure 2D). These results demonstrate that the *Ds* element is either in the *Su* gene or very tightly linked to it.

Different sectors possess different restriction fragment length polymorphisms: To test whether *Ds* had excised from the donor locus in the variegated sector, restriction fragment length polymorphisms (RFLPs) were examined. Figure 3A shows the results of a Southern analysis of genomic DNA from various sectors of the putatively tagged unstable plant. Genomic DNA was digested with *Eco*RV that cleaves once in the *Ds* element such that the other site in the fragment liberating *dhfr* was in the flanking genomic DNA (Figure 3B). Therefore, the size of the *dhfr*-hybridizing DNA fragment would depend on the position of the *Ds* element in the genome. Figure 3A, lane 1, shows a band of 2.7 kbp for the T-DNA *Ds* construct. The primary *Ds* transformant

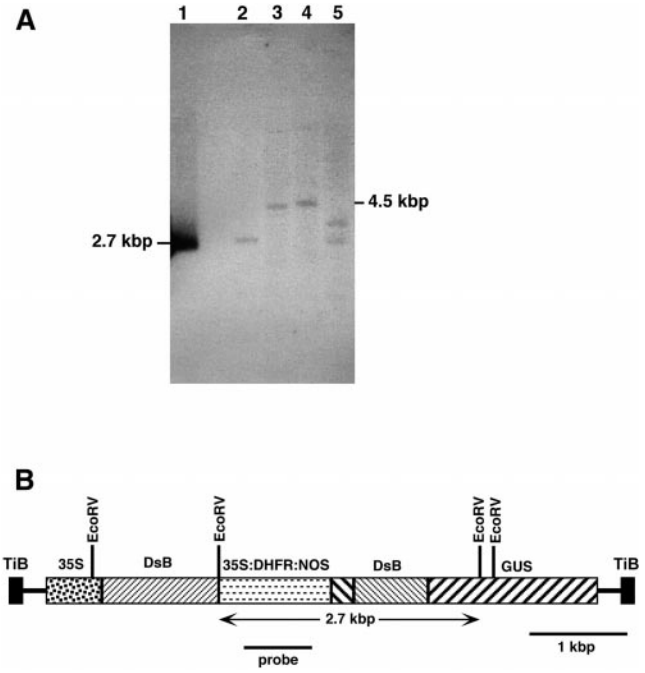


Figure 3.—Southern analysis correlating *Ds* movement with *Sulfur* phenotype. (A) Autoradiogram of a genomic DNA blot, probed with DIG-labeled *dhfr* coding sequence. Plasmid and genomic DNAs were digested with *Eco*RV. Lane 1, 20 pg of pWPF147 plasmid DNA (*Ds* construct); lane 2, primary *Ds* transformant; lane 3, a green portion of a reactivated unstable plant; lane 4, a revertant yellow-green sector of the same unstable plant; lane 5, tissue from the original F_1 plant containing *Ts* and *Ds* elements. For each genomic sample, 10 μg of DNA was loaded. (B) Diagram showing the T-DNA region of the *Ds* construct, pWPF147. TiB, T-DNA border; 35S, cauliflower mosaic virus 35S promoter; DsB, *Ds* border; GUS, *uidA* gene.

also shows a band at 2.7 kbp, lane 2. DNA from a green sector of the reactivated plant showed a new band at 4.5 kbp with the loss of the 2.7-kbp band (lane 3). This observation correlates with the plant's phenotype and with our prediction that *Ds* had excised from the original T-DNA locus and reinserted into the *Su* gene. However, a revertant yellow-green sector of the same reactivated plant showed a band slightly larger than the 4.5-kbp band (lane 4), indicating that *Ds* had transposed a second time, presumably from the *sulfur* locus. DNA of the original variegated F_1 plant (Spot2a) possessed two bands, one of which is the 2.7-kbp band (lane 5) and an additional band suggesting that *Ds* transposed in this tissue. These data indicate that *Ds* movement is associated with the unstable *Su* phenotype.

Isolation of the DNA flanking the transposon using IPCR: The genotype of the stable green segregant, plant 17, is *su*/*Su*::*Ds* (the *Ts* has been segregated away). IPCR was performed using DNA isolated from plant 17 to obtain 424 bp of DNA flanking one end of the transposon (partial sequence is listed under GenBank accession no. AF050164). To confirm that this 424-bp IPCR product is linked to the *Ds* border, we performed DNA ampli-

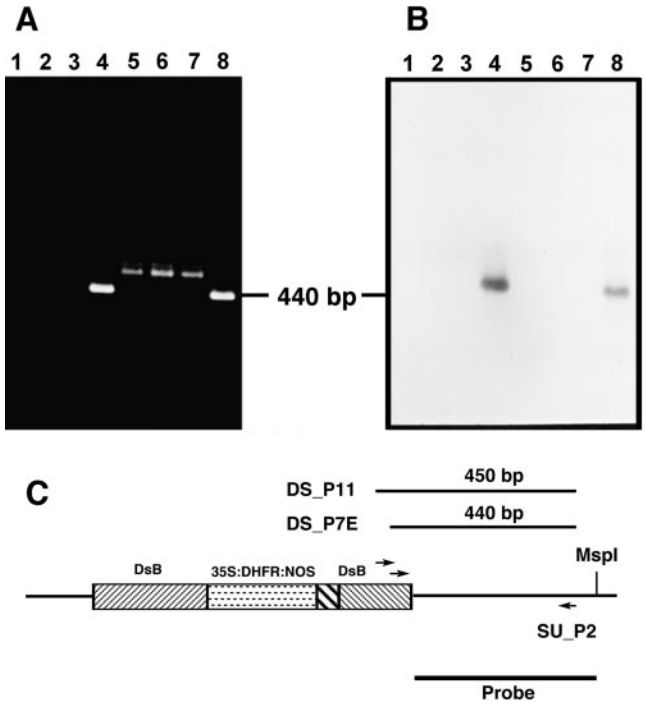


Figure 4.—PCR and Southern analysis of the DNA flanking the *Ds* border. Total genomic DNA was subjected to PCR amplification using SU_P2 primer with either DS_P11 (lanes 1–4) or DS_P7E (lanes 5–8) primer. The predicted PCR products for DS_P7E/SU_P2 and DS_P11/SU_P2 are 440 and 450 bp, respectively. Template genomic DNA was extracted from primary *Ds*-transformant (plant Ds1.7.b), lanes 1 and 5; wild-type (*su*/*su*) green plant (line 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.

fications 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 (Ds1.7.b; 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

A

B

Sequence name	Sequences bordering the *Ds* insertion			Phenotype	Base change
Ds tagged *Su*	CAGTTCAATA	**ATTTTAAA** ▽ **ATTTTAAA**	GCTAAGT T	Green	+8
Genomic clone 39C1	CAGTTCAATA	**ATTTTAAA**	GCTAAGT T		0
Wildtype S7	CAGTTCAATA	**ATTTTAAA**	GCTAAGT T	Green	0
Revertant S8	CAGTTCAATA	**ATTTTAA***	***TTTTAAA** GCTAAGT T	YG	+6
Revertant S10	CAGTTCAATA	**ATTTTAA***	**T**TTTTAAA GCTAAGT T	YG	+7
Revertant S13	CAGTTCAATA	**ATTTTAA***	**T**TTTTAAA GCTAAGT T	YG	+7
Revertant S14	CAGTTCAATA	**ATTTTAA***	****TTTAAA** GCTAAGTT	YG	+5
Revertant 30	CAGTTCAATA	**ATTTTAA***	**T**TTTTAAA GCTAAGTT	YG	+7

Figure 5.—The genomic sequence surrounding the *Ds*-element insertion site and the footprint analysis of revertant sectors. (A) Partial sequence of the genomic clone 39C1 that contains sequences surrounding the *Ds* insertion site. Primers used in the footprint analysis are shown above or below arrows. The site of *Ds*-element insertion is shown as a triangle. (B) Genomic DNA of revertant yellow-green (YG) sectors or a wild-type green region in selfed progeny of Spot2a plant were used as templates for PCR amplification using SU_P4A and SU_P5S primers. With the exception the genomic clone 39C1, others are sequences obtained from PCR amplification of either a wild-type control green portion of a leaf (Wildtype S7) or yellow-green (YG) revertant sectors (S8, S10, S13, S14, and 30). Boldface nucleotides represent the direct repeat generated by the *Ds* insertion. The triangle indicates the *Ds* insertion site. Asterisks represent deleted nucleotides. Nucleotides that differ from the known sequence are underlined.

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 F_1 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 of *Su*. Southern analysis confirmed the correlation of transposition of *Ds* and mutation of *Su*, both in the original unstable plant and in revertant sectors. Regeneration of a plant from the unstable sector on selective medium containing Mtx showed that the *Ds* element had reintegrated after excision (*i.e.*, transposition). Segregation of *Ts* from the *Ds* resulted in stabilization of the green phenotype. Furthermore, the unstable phenotype

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 phenotypic 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 germinal 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 (Osborne *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.

We thank Marvin Williams for his assistance in making photographs of some figures. We thank the Southeastern Plant Environmental Laboratory at N.C.S.U. for providing the greenhouse facility for this project. W.P.F. and L.V.N. were supported by a grant from the McKnight Foundation.

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Communicating editor: V. L. Chandler