The Plant Journal (1996) **10**(4), 721–732

TECHNICAL ADVANCE

Characterization and mapping of *Ds*–GUS-T-DNA lines for targeted insertional mutagenesis

D. Smith[1,†], Y. Yanai[2], Y.-G. Liu [2], S. Ishiguro[3,‡], K. Okada[3,‡], D. Shibata[2], R. F. Whittier[2] and N. V. Fedoroff[1,*,§]

[1]*Carnegie Institution of Washington, Department of Embryology, 115 West University Parkway, Baltimore, MD 21210, USA,*

[2]*Mitsui Plant Biotechnology Research Institute, Sengen 2-1-6 TCI-D21 Tsukuba, Ibaraki 305, Japan, and*

[3]*National Institute for Basic Biology, 38 Nishigonaka, Myodaijicho, Okazaki 444, Japan*

Summary

The transposition patterns of the *Ds*–GUS transposon T-DNA in 23 independent single-copy lines have been characterized and the map positions of 10 of them on three of the five *Arabidopsis* chromosomes are reported. Using overexpressed *Activator* (*Ac*) elements as a transposase source, it was found that the primary determinant of transposition frequency is the insertion site of the *Ac*-T-DNA. Neither the structure of the transposon T-DNA nor, in most cases, its insertion site have a significant effect on transposition frequency. Both the frequency and timing of transposition are influenced by the parent through which the transposon and transposase T-DNAs are transmitted. Overall, nearly 75% of plants in which excision has occurred bear a reinserted element and very short-range transpositions predominate, underlining the advantage of using mapped transposons for insertional mutagenesis.

Introduction

Transposon mutagenesis in plants has become an increasingly useful tool for gene discovery with the development of well-marked, versatile transposons. Most of the transposon tagging systems that have been developed for plants other than maize are based on the maize *Activator* (*Ac*) transposable element family (Fedoroff, 1989; McClintock, 1951). Following its initial isolation, *Ac* was shown to transpose in dicots, as well as other monocots (Baker *et al.*, 1986; Fedoroff *et al.*, 1983; Houba-Herin *et al.*, 1990; Izawa *et al.*, 1991; Laufs *et al.*, 1990; Shimamoto *et al.*, 1993; Van Sluys *et al.*, 1987; Yoder *et al.*, 1988) and used as the basis for transposon tagging systems in a variety of plants (Hehl, 1994). We and others have described *Ac*-based transposon tagging systems for *Arabidopsis* (Altmann *et al.*, 1992; Bancroft *et al.*, 1992; Fedoroff and Smith, 1993; Honma *et al.*, 1993; Long *et al.*, 1993a; Sundaresan *et al.*, 1995). Transposon tagging provides several advantages over T-DNAs mutagenesis (Walbot, 1992). Introduction of the insertional mutagen can be separated from mutagenesis by controlling the supply of transposase to the transposon. Remobilization of the element provides a rapid means of establishing that a mutation is caused by the insertion. Because excision is imprecise, transposition of an element out of a gene often yields further mutations (Wessler *et al.*, 1986). Finally, transposons of the *Ac* family exhibit a marked preference for short-range transposition, potentially reducing the labor of identifying an insertion in a target gene by using a closely linked transposon (Dooner and Belachew, 1989; Greenblatt, 1984; James *et al.*, 1995; Jones *et al.*, 1990; Osborne *et al.*, 1991).

The efficiency of transposon tagging systems has been limited by several factors. Heritable mutations unlinked to transposon markers have been reported, primarily in systems in which plants containing both the transposon and transposase gene are propagated for multiple generations (Altmann *et al.*, 1995). This permits serial transposition, yielding mutations that are unproductive for gene isolation because imprecise excision can result in a mutation caused by, but no longer associated with, the transposon (Wessler *et al.*, 1986). The efficiency of transposon tagging has been improved by using transposons that (i) are immobile and maintained in a separate lines from the transposase, (ii) carry selectable markers, and (iii) disrupt a selectable marker gene whose function is restored upon excision, providing a means of identifying plants in which transposition has occurred (Hehl, 1994). The source of transposase is either an immobile *Ac* element or cDNA for the element-encoded ORFA protein (Feldmar and Kunze, 1991; Kunze and Starlinger, 1989; Kunze *et al.*, 1987). Recent transposons carry reporter genes which are activated upon insertion into a gene or promoter, facilitating analysis of the gene's expression pattern (Fedoroff and Smith, 1993; Sundaresan *et al.*, 1995). The utility of transposon mutagenesis is evidenced by the growing number of genes

Received 11 April 1996; revised 20 May 1996; accepted 28 June 1996.
*For correspondence (fax +1 814 863 1357, e-mail nvf1@psu.edu).
†Present address: USDA-ARS, Horticultural Crops Quality Laboratory, Building 002, 10300 Baltimore Avenue, Beltsville, MD 20705, USA.
‡Present address: Department of Botany, Graduate School of Science, Kyoto University, Kyoto 606-01, Japan.
§Present address: Biotechnology Institute, The Pennsylvania State University, University Park, PA 16802, USA.

identified by transposon tagging (Bancroft *et al.*, 1993; Chuck *et al.*, 1993; Long *et al.*, 1993a; Smith and Fedoroff, 1995; Springer *et al.*, 1995).

Although it is known that the *Ac* family of transposons moves preferentially to linked sites, no systematic effort has been made to use this property of transposons to target mutagenesis by mapping a large number of donor T-DNA insertion sites (Dooner and Belachew, 1989; Greenblatt, 1984; Greenblatt and Brink, 1963; Jones *et al.*, 1990; Osborne *et al.*, 1991). We have embarked on such an effort. In the present publication, we assess the primary factors that determine how frequently a transposon moves and where it reinserts for a set of *Arabidopsis* lines, each containing a single *Ds* transposon or *Ac* transposase T-DNA insert and report the map positions of 10 transposon T-DNAs that can be used to initiate local mutagenesis.

Results and Discussion

Assessment of excision/transposition frequency

Crosses between Ac- *and* Ds-*GUS-T-DNA lines.* The structure of the T-DNAs containing the tagging transposons, designated *Ds*-GUS 1-6, and the modified *Ac* elements carrying a complete transposase gene used here has been described previously (Fedoroff and Smith, 1993) and is reproduced in Figure 1. Twelve different single-copy *Ac*-T-DNA insertion lines and 23 different single-copy *Ds*-GUS transposon T-DNA lines were intercrossed. Each line contained a single, complete T-DNA (Fedoroff and Smith, 1993). F_1 plants were grown from 87 *Ac* × *Ds* crosses and 1263 individual F_2 progenies were analyzed. In the majority of crosses, the *Ds*-GUS transposon T-DNA line was used as the male and a homozygous *Ac*-T-DNA plant was used as the female. F_1 seeds were plated on hygromycin to ensure that all F_2 progenies contained both the transposon and the transposase gene. Hygromycin-resistant (H^r) F_1 plants were grown and allowed to self-pollinate. Excision and transposition were assessed in F_2 seeds collected from these plants.

Detecting germinal excision and transposition. Empty donor sites (EDS) and transposed elements (TrE) were detected by plating 200–500 F_2 seeds on medium containing chlorsulfuron and hygromycin. Seedlings were scored for acquisition of resistance to chlorsulfuron consequent on excision of the transposon from the leader region of the chlorsulfuron-resistant ALS gene, as well as the presence of the transposon, as judged by resistance to hygromycin (Figure 1; Fedoroff and Smith, 1993). Segregation of the EDS chlorsulfuron-resistance (C^r) marker from the transposon-borne H^r marker is observed among F_2 progeny and depends on the linkage between them, as discussed below. A fraction of progeny show evidence of excision, but do not exhibit co-segregation of the transposon marker with the EDS marker, indicating that C^r EDS plants do not contain a TrE. The present marker system is quite reliable in detecting germinal transmission of excision and transposition events that have occurred during development of the F_1 plant (Fedoroff and Smith, 1993). As is evident from the data shown in Table 1, however, the fraction of F_1 plants in which excision and transposition events occurred varied widely. The sources of this variation are analyzed in subsequent sections.

Measuring transposition frequency. The most commonly used measure of germinal transposition frequency is the percentage of F_2 progeny with a transposed element (Schmidt and Willmitzer, 1989; Altmann *et al.*, 1992; Honma *et al.*, 1993). However, this is not a valid measure of germinal transposition frequency because most transposition events occur sufficiently early in development to give rise to multiple progeny carrying the same transposed element. Indeed, a single transposition event commonly occurs much earlier in development than all others, so that progeny arising from the plant sector in which it occurred predominate numerically (Fedoroff and Smith, 1993; Honma *et al.*, 1993). We examined two to six sibling plants from 19 progenies for the location of the TrE by blot hybridization and found that only two progenies (11%) gave evidence of having a different element in one or more of the siblings examined. To avoid erroneous over-reporting, we use the fraction of F_1 plants showing at least one germinally transmissible excision/transposition event as the measure of the germinal transposition frequency. Because transposition commences only after fusion of the gametes bearing the *Ds*-GUS- and *Ac*-T-DNAs, independent transposition events occur in each F_1 plant. While our assumption of a single transposition event per F_1 plant that yields C^r F_2 progeny underestimates the true germinal transposition frequency, it is the number that is important for the experimentalist seeking to minimize the time and effort involved in selecting lines with independent transpositions. The simplest way to assure that transposition events are independent is to sample each F_2 progeny once. The most important consideration is therefore the number of independent F_2 progeny that must be sampled per element isolated. Thus if the transposition frequency is 50%, then two progenies must be sampled per transposition event recovered, while if it is 5%, 20 progenies must be sampled and if it is 0.5%, 200 progenies must be sampled (the actual number is fractionally higher because of lines in which all C^r progeny are H^s).

Using the most active transposase lines identified, we detect transposition in up to 97% of the F_1 plants tested (Table 2). The fraction of C^r sibling F_2 progeny varies widely between plants from the smallest fraction detectable in the seeds sample tested to as much as 50–60%. Plants in

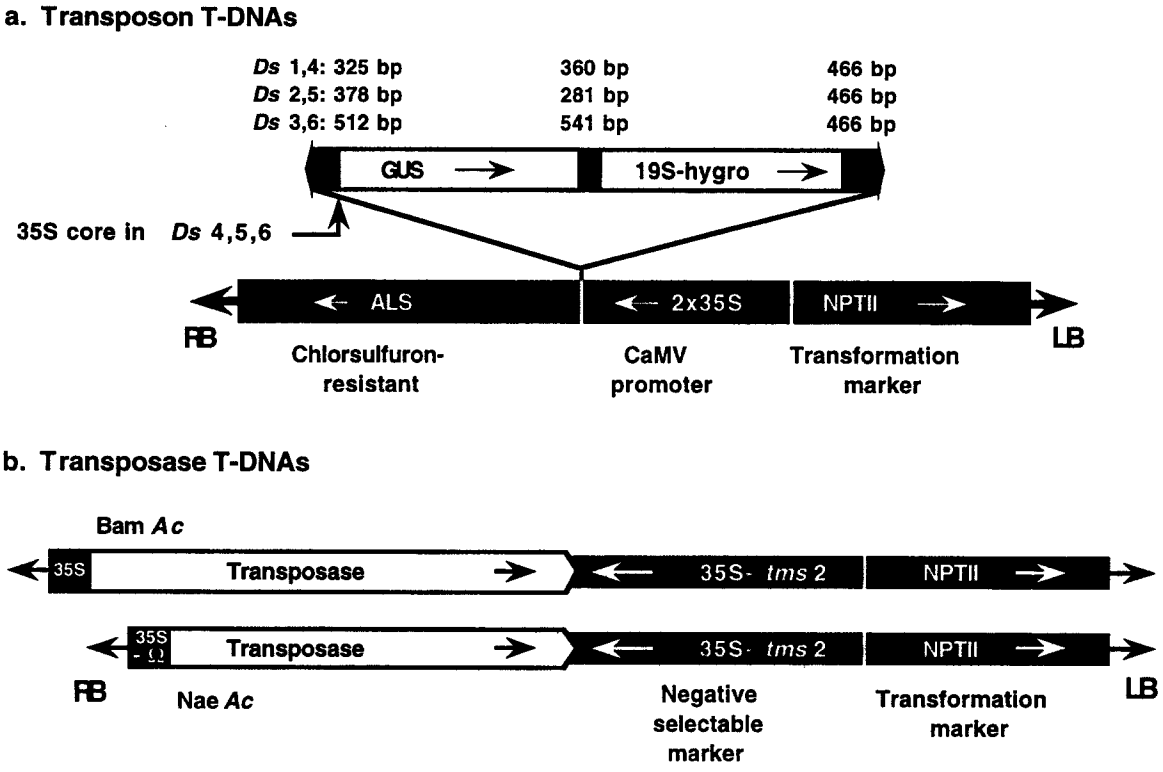


Figure 1. A diagrammatic representation of the T-DNA segments containing the transposon (a) and the transposase gene (b), as well as the selectable markers associated with each.

The constructs used here have been described in detail previously (Fedoroff and Smith, 1993). The amount of the *Ac* element in each *Ds* construct is represented by the filled portions at the beginning, middle and end of the transposon and the exact number of base pairs is indicated above the diagram. *Ds*1 and 4 contain only the first of the *Ac* element's four transcription start sites, while the others contain all of them with (*Ds*3 and 6) or without (*Ds*2 and 4) part of the element's untranslated leader. *Ds* constructs 4–6 contain the 35S core sequence upstream from the GUS gene, while *Ds* constructs 1–3 do not. Abbreviations: RB, T-DNA right border; LB, T-DNA left border; GUS, β-glucuronidase; 35S and 19S, the promoters of the CaMV 35S and 19S transcripts; the Nae*Ac* contains the Ω sequence of the tobacco mosaic virus; ALS, chlorsulfuron-resistant acetolactate synthase gene (Haughn *et al.*, 1988); hygro, the bacterial *aph*4 gene, confers hygromycin resistance; NPTII, bacterial neomycin phosphotransferase II gene; *tms*2, the agrobacterial *tms*2 gene, confers sensitivity to auxin amides; Bam- and Nae-*Ac*, deleted and promoter-substituted *Ac* elements.

which excision was not accompanied by reinsertion of the transposon at a site that co-segregates with the EDS produce C^rH^s progeny. Such plants occasionally produce a few C^rH^r F_2 progeny and these often have long roots. Analysis of the F_3 progeny showed that such exceptional C^rH^r F_2 plants with long roots are usually, but not always, heterozygous for an EDS and an unrearranged *Ds*-GUS-T-DNA. These probably arise because the cell in which the excision event occurred gave rise to a somatic sector whose boundary occasionally separated cells destined to become egg and sperm cells of a single flower. The roots may be long because of the proximity of the *aph4* gene's 19S promoter to the double 35S promoter's strong enhancer in the *Ds*-GUS-T-DNA. Root growth on hygromycin-containing medium of plants with transposed *Ds* elements varies among TrEs at different sites, is characteristic of a given line with a TrE, and is almost always slower than that of plants with the *Ds*-GUS element located in its initial position, which aids in the identification of plants with transposed elements (Figure 2).

Determinants of transposition frequency

Structure and location of the Ac- *and* Ds-*GUS-T-DNAs.* Independent transformants containing a single copy of either the Bam*Ac* or Nae*Ac* (Figure 1b) were each crossed with several independent lines containing one of the *Ds*-GUS-T-DNAs (Figure 1a). Among the four Bam*Ac* T-DNA insertion lines tested, three elicited germinally transmissible transpositions in 20–83% of the F_1 plants whose progeny were tested. There was no evidence of transposition in the 26 F_1 plants arising from crosses to the fourth Bam*Ac* T-DNA line (Table 1). Among the eight Nae*Ac*-T-DNA lines tested, four promoted germinally transmissible transposition in 23–70% of F_1 plants analyzed, while the remaining four promoted transposition in few or none (0–4%). Thus the two different *Ac* constructs behaved similarly in the present experiments, suggesting that expression of the transposase gene was not the rate-limiting factor in transposition frequency. However, there was marked variation in the frequency of *Ds* mobilization between *Ac* lines

Table 1. Mobilization of *Ds*-GUS by different *Ac* constructs and lines

Ac construct	*Ac* line[a]	*Ac* line as pollen or egg parent	Number of F_2 progenies analyzed[b]	% progenies with EDS or TrE[c]	% progenies with TrEs only[d]
Bam*Ac*	364-22	Egg	83	ND[e]	83
	364-23	Egg	26	ND	0
	364-29	Egg	36	ND	20
	374-23	Egg	49	ND	61
Nae*Ac*	380-1	Egg	95	27	23
	380-6	Egg	205	33	26
		Pollen	29	69	55
	380-10	Egg	247	51	36
			62	ND	60
		Pollen	15	87	70
	380-13	Egg	46	4	4
	380-16	Egg	67	79	61
	380-18	Egg	46	4	4
	380-21	Egg	110	7	4
	380-23	Egg	30	0	0

[a]The number designates an independently derived transformant containing the construct indicated in the previous column.
[b]Seeds obtained from crosses between the indicated *Ac* line and different *Ds* lines were grown and selfed; the F_2 progeny seeds of each were plated independently on selective media.
[c]Seedlings were scored for chlorsulfuron resistance (C^r) and hygromycin resistance (H^r). Progenies yielding primarily or exclusively C^rH^s plants were scored as having an empty donor site (EDS) only, while those that showed a majority or all C^rH^r plants were scored as having both an EDS and a transposed element (TrE).
[d]Only those progenies that had a transposed element are reported in this column.
[e]ND, not determined.

containing identical constructs. *Ac* lines were compared for their ability to mobilize each of the six different *Ds*–GUS transposons (Figure 1). The *Ds*–GUS transposon in most of the tested lines responded similarly to each of the Nae*Ac* elements, although there were occasional exceptions (Table 2). Thus, for example, lines *Ds*2 389-14 and *Ds*6 398-5 showed low transposition frequencies in crosses to highly active *Ac* lines.

The Ac*-T-DNA insertion site is a dominant determinant of excision/transposition frequency.* To determine whether either the *Ac* or the *Ds*–GUS insertion site was the predominant determinant of transposition frequency, we compared the results obtained with a set of five different *Ds*6 lines, each containing the same *Ds*–GUS construct, crossed by a set of seven different Nae*Ac* lines, each containing the same transposase construct. A log-linear model was fitted to the data using the SAS procedure CATMOD (Bishop *et al.*, 1975). The independent variables were the seven different *Ac* lines and the five different *Ds* lines and the dichotomous response was given by the counts of crosses yielding and failing to yield transpositions. The null hypothesis is that the different *Ac* and *Ds* lines have no effect on the rate of transposition and a binomial sampling of progeny with transposed and untransposed elements was assumed. The model was found to fit the data adequately and the *Ds* element line did not have a statistically significant effect on the transposition frequency in the tested sample. By contrast, differences between *Ac* lines in the ability to promote excision and transposition were highly significant. There was no evidence of interaction between particular *Ac* and *Ds* pairs, indicating that no particular combinations of transposon and transposase were either particularly strong or particularly weak in causing transposition in the tested sample of 448 progenies obtained from 35 crosses. The results are displayed in a simple graphic form in Figure 3. Clustering of high and low frequencies of transposition is not evident in Figure 3(a), in which the data are grouped by *Ds* line, but are strikingly apparent in Figure 3(b), where the same data are grouped by *Ac* line. Thus the primary determinant of transposition frequency is the *Ac* line. Because all of the *Ac* lines used in this experiment had a single copy of an identical *Ac*-T-DNA with an intact transposase gene, it appears likely that the genomic location of the transposase gene influences its expression.

The gametic source of transposase affects excision and transposition frequencies. The *Ds* line was used as the pollen parent in a majority of the crosses because the presence of the H^r marker made emasculation unnecessary by providing a selectable marker for F_1s receiving the transposon. However, some reciprocal crosses were also carried out with emasculated flowers to determine whether transmission of the transposon and transposase through different parents influences transposition frequency. Quite

Table 2. Mobilization of different *Ds*–GUS transposons from several insertion sites

		% progenies: EDS only or EDS+TrE	Number of progenies analyzed[c]	Percent of progenies: EDS only or EDS+TrE	Number of progenies analyzed[c]	Percent of progenies: EDS only or EDS+TrE	Number of progenies analyzed[c]
	Ds–GUS[a]	Nae*Ac*-6[b]		Nae*Ac*-21		Nae*Ac*-10	
	*Ds*1 388-4	–	–	10	10	–	–
*	*Ds*1 388-5	–	–	10	10	97[d]	62
*	*Ds*1 388-30	–	–	10	10	–	–
*	*Ds*2 389-2	–	–	10	10	–	–
	*Ds*2 389-7	–	–	10	10	–	–
	*Ds*2 389-12	20	10	–	–	61	38
*	*Ds*2 389-13	13	8	–	–	97	35
*	*Ds*2 389-14	0	5	–	–	0	9
	*Ds*2 389-23	51	35	–	–	27	26
*	*Ds*2 389-25	30	10	–	–	30	10
*	*Ds*3 390-1	–	–	10	10	–	–
*	*Ds*4 391-20	36	14	–	–	53	17
	*Ds*5 392-9	20	10	–	–	48	21
*	*Ds*5 392-12	20	10	–	–	61	38
*	*Ds*5 392-13	20	10	–	–	20	10
	*Ds*5 392-24	33	3	–	–	60	10
	*Ds*6 393-3	44	61	0	10	40	10
	*Ds*6 393-9	20	10	0	10	30	10
	*Ds*6 393-19	10	10	20	10	50	10
	*Ds*6 393-21	33	9	0	10	0	4
	*Ds*6 398-5	0	10	0	10	10	10
	Average	23	Σ=215	7	Σ=100	43	Σ=320

[a]The number of the parental *Ds* line (the number after the hyphen designates the transformant).
[b]The number of the parental *Ac* line (the number after the hyphen designates the transformant).
[c]F_1 plants from the indicated crosses were grown and selfed; the F_2 seeds produced by each F_1 plant were plated separately on medium containing both chlorsulfuron and hygromycin.
[d]These progenies were scored for the presence of both an EDS and a TrE, while the remainder were assessed for the presence of either only an EDS or both an EDS and a TrE.
*Mapped *Ds*–GUS transposons (see Figure 5).

unexpectedly, excision and transposition frequencies were about twice as high when the transposase was transmitted through the pollen parent and the transposon through the egg parent than in the reciprocal crosses (Table 1). Despite the difference in sample size, these results are highly statistically significant (P = 0.007 for Nae*Ac* 380-10 and P = 0.021 for Nae*Ac*380-6). The results obtained from the analysis of 100 F_2 progenies resulting from a subset of crosses that was reciprocal with respect to transposon and transposase lines is shown in Table 3.

The reason for the asymmetry described above is not at all obvious in view of the fact that most transposition events, as judged by the number of C^r F_2 progeny, occur well after fertilization. When the transposase gene is transmitted through female and male gametes, 67% and 97%, respectively, of F_1 plants in which transposition was detected produce less than 25% C^r F_2 progeny. The subset of data in Table 3 show, not surprisingly, that the fraction of C^r F_2 progeny is usually larger when the transposase gene is transmitted through the female than through the male (overall averages were 17 vs. 7%, respectively, among reciprocal crosses). This difference reflects a higher frequency of early transposition events when the element is inherited through the female than through the male germline. Thus among F_1 plants in which transposition events have occurred, 33% produce more than 25% C^r F_2 progeny when the transposase is transmitted through the female, while only 3% do when it is transmitted through the male. This is consistent with the earlier expression of maternal genes or maternal transmission of some active transposase in female germ cells.

Despite the tendency toward earlier transposition in those lines that receive the transposase through the female parent, the probability that transposition will occur at some time during development is about twice as high if the transposase gene is transmitted through the male and the transposon through the female (Tables 1 and 3). In view of the straightforward correlation between the early transposition and transmission of the transposase gene through the female, it appears unlikely that the explanation lies in

transposase overproduction. Rather, we suggest that there may be an underlying persistent difference between the paternal and maternal genomes such that a transposon is more likely to be accessible to transposase if it is transmitted through the female germline, regardless of when in development the transposase accumulates to a sufficient concentration to promote transposition. Thus we suggest that perhaps both transposase expression and transposon accessibility are maximized upon transmission through the female.

Short-range transposition of the Ds*-GUS element*

Facile detection of short-range transposition events. The ability to detect nearby transpositions on the same sister chromatid depends on the ease with which the frequency of recombination between the donor site and the reinserted element can be monitored. The structure of the present *Ds*-GUS-T-DNAs permits easy assessment of the linkage between the transposed element and the EDS by the association of the donor site (C^r) and element (H^r) markers. Among the C^r progeny of a self-pollinated plant, an unlinked element will yield 25% C^rH^s progeny, while a linked element will yield less than 25% C^rH^s recombinants. If a single early transposition event dominates the progeny of the *Ac*/+ *Ds*/+ doubly hemizygous F_1 plant, as is generally the case, linkage of the TrE to the donor site can be assessed immediately in the progeny of the plant in which the transposition event occurred. Table 4 shows a sample of such primary segregation data for several F_2 progenies and the linkage deduced from analysis of F_3 plants. In many cases, the fraction of C^r progeny is sufficiently large so that closely linked elements can be identified immediately in the F_2 progeny.

Frequency of short-range transposition events. Short-range transpositions of elements belonging to the *Ac* family are frequent in transgenic plants, as they are in maize (Bancroft and Dean, 1993; Dooner and Belachew, 1989; Greenblatt, 1984; Greenblatt and Brink, 1963; Jones *et al.*, 1990; Keller *et al.*, 1993; Osborne *et al.*, 1991). The results of linkage analysis for 92 elements that transposed from a previously described donor site containing both an *Ac*- and *Ds*-GUS transposon at the same insertion site (Fedoroff and Smith, 1993) are given in Table 5 and Figure 4. As in the *Ac* × *Ds* crosses, 1/4 of the original group of 121 lines with an EDS lacked a TrE. Among those with TrEs, transposition to linked sites was about twice as frequent as to unlinked sites (Table 5a). It should be noted that if the EDS lines arise predominantly by transposition to a sister chromatid (see below), then the bias toward linked transpositions is closer to 3:1. Among linked TrEs, almost half are within

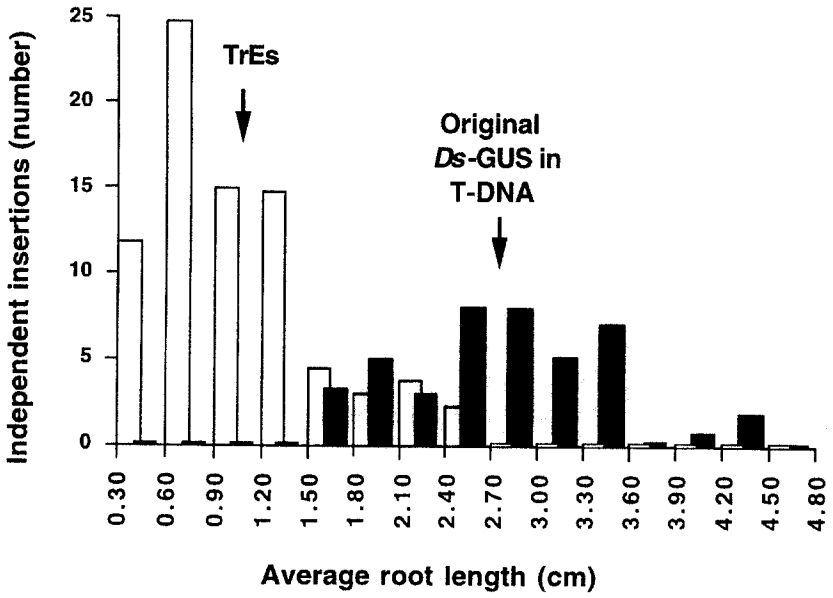
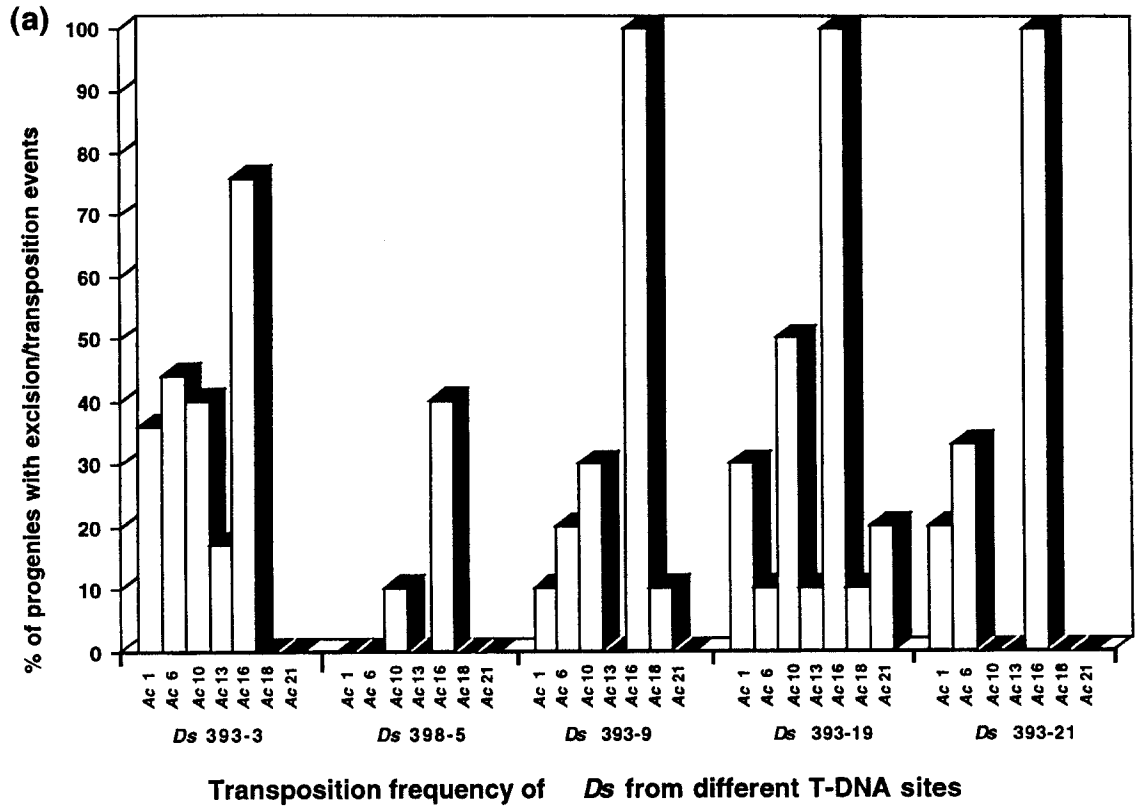
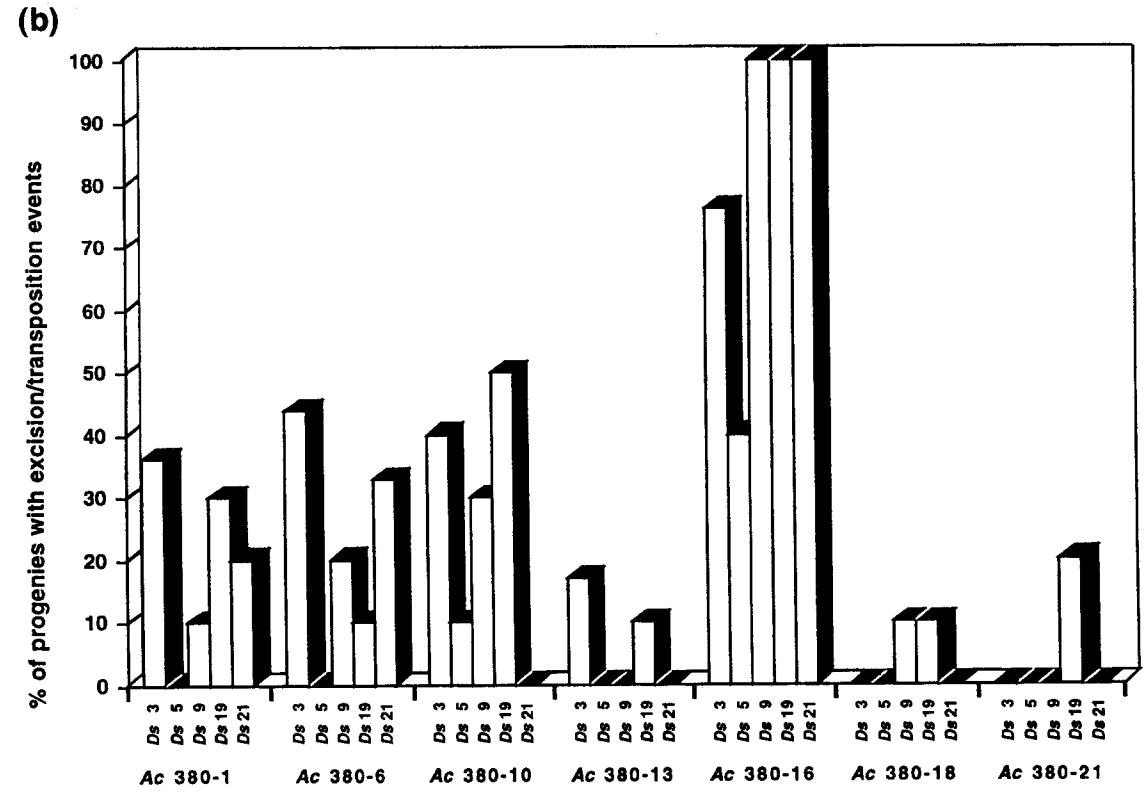


Figure 2. The average root length at 7 days after germination of siblings homozygous for different transposed elements (white bars) or the *Ds*-GUS transposon at its original position in the T-DNA (black bars).
Root measurements were made as described in Experimental procedures.

Figure 3. The transposition frequency of *Ds* from different T-DNA insertion sites in the crosses with different *Ac* lines.
The figure is based on the analysis of a total of 448 progenies from the indicated 35 pairwise crosses.
(a) The percentage of F_1 plants with either an excision or transposition event (inferred from the presence of C^r F_2 progeny) is plotted for different *Ds*-GUS-T-DNA insertion lines (pollen parent) in crosses to seven different Nae*Ac* insertion lines (egg parent).
(b) Data from the same crosses as shown in (a), but grouped by *Ac* insertion line.



Transposition frequency of *Ds* from different T-DNA sites



***Ac* activity at different T-DNA insertion sites**

Table 3. Mobilization of *Ds*-GUS by *Ac* transmitted through pollen and egg

Egg parent	Pollen parent	Number of F_2 progenies analyzed[a]	Percent with TrE + EDS	Percent with TrE + EDS or EDS only	Average % C^r siblings per progeny[d]
Nae*Ac* 380-6[b]	*Ds*4 391-20[c]	14	29	36	23
	Ds 5 392-9	10	10	20	16
Total		24			
Average			20	28	20
*Ds*4 391-20	Nae*Ac* 380-6	10	60	70	8
*Ds*5 392-9		9	56	67	13
Total		19			
Average			58	69	11
Nae*Ac* 380-10	*Ds* 4 391-20	17	35	53	22
	Ds 5 392-12	27	48	70	18
Total		44			
Average			42	62	20
*Ds*4 391-20	Nae*Ac* 380-10	10	80	100	3
*Ds*5 392-12		3	100	100	7
Total		13			
Average			90	100	5

[a]F_1 plants were grown and selfed; all F_2 progenies were tested for the presence of the *Ds*- and *Ac*-linked markers.
[b]The number of the parental *Ac* line (the number after the hyphen designates the transformant).
[c]The number of the parental *Ds* line (the number after the hyphen designates the transformant).
[d]The percentages of C^r F_2 progeny were averaged over the number of F_1 plants in which transposition/excision was detected.

Table 4. Segregation of transposed elements in F_2 progeny

Nae*Ac*[a]	*Ds*-GUS[b]	F_1[c]	No. F_2 seed tested	Percent C^r F_2 progeny	Percent H^r/C^r F_2 progeny	Genotype[d]
380-21	*Ds*1 388-5	16-2	300	20	75	Unlinked TrE
380-21	*Ds*2 389-2	32-6	300	5	86	Linked TrE
380-10	*Ds*2 389-12	44-9	300	15	82	Unlinked TrE
380-6	*Ds*2 389-25	59-5	400	6	95	Linked TrE
380-10	*Ds*2 389-25	60-5	400	8	79	Unlinked TrE
380-10	*Ds*2 389-25	60-6	400	9	70	Unlinked TrE
380-10	*Ds*2 389-25	60-9	400	23	72	Unlinked TrE
380-21	*Ds*3 391-1	68-4	400	6	100	Linked TrE

[a]The number of the parental *Ac* line (the number after the hyphen designates the transformant).
[b]The number of the parental *Ds* line (the number after the hyphen designates the transformant).
[c]The number of the F_1 plant resulting from the indicated cross yielding the results shown.
[d]The genotype was determined from the linkage between the donor site, marked by chlorsulfuron-resistance, and the TrE, marked by hygromycin-resistance, among progeny of hemizygous F_2 plants.

30 cM of the donor site and, of those within 10 cM, 73% are within 5 cM and 35% within 1 cM of the donor site, respectively (Table 5b and Figure 4). Assuming a total map length of 500 cM and a genome size of 100 Mb, roughly one out of four transpositions is to a site no more than a megabase from the transposon's initial position and one out of 10 is to a site no more than 200 kb from the donor site in the sample analyzed. If there is no insertion site bias, the probability per transposition event examined of inactivating a gene located within 1 cM of a transposon is 28 times greater, and that for a gene located within 5 cM is 16 times greater, than for an unlinked gene. Thus using a transposon that is located close to the target gene is likely to reduce the requisite time and labor of tagging the gene between one and two orders of magnitude.

Plants with empty donor sites only

It has been reported that in tobacco and *Arabidopsis*, *Ac*-based transposon tagging systems give high percentages of progeny with an EDS that lack a transposed element, suggesting that excised *Ds* elements frequently fail to reinsert (Bancroft and Dean, 1993; Jones *et al.*, 1990; Long *et al.*, 1993b). With the present lines, we have found the

Table 5. Yield and linkage of TrEs among lines with excision events
(a) Summary

	Linkage of TrEs and EDS frequency[a]	
EDS or TrE	Number of progenies with C^r plants[b]	Percent of progenies with C^r plants
Linked TrE (0–45 cM)	58	48
Unlinked TrE (>45 cM)	34	28
EDS	29	24
Total analyzed	121	100

(b) Data for Linked TrEs

	All TrEs			Very closely linked TrEs	
Linkage of TrE to donor site	Number of progenies with C^r plants	Percent of progenies with C^r plants	Linkage of TrE to donor site	Number of progenies with C^r plants	Percent of progenies with C^r plants
0–30 cM	38	41	<1 cM	9	35
30–45 cM	20	22	1–5 cM	10	38
>45 cM	34	37	5–10 cM	7	27
Total	92	100	Total	26	100

[a]The parental plants contained both an *Ac*-T-DNA and a *Ds*-T-DNA inserted adjacent to each other (Fedoroff and Smith, 1993). Linkage relationships were determined in progeny of plants hemizygous for both the EDS and TrE.
[b]Each progeny is that of a different plant in which a transposition event occurred, as judged by the appearance of C^r plants.

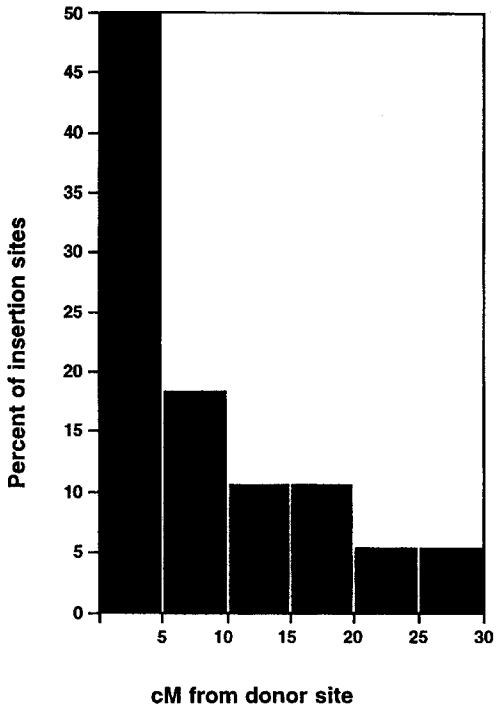


Figure 4. The percent of insertion sites is plotted as a function of the distance from the empty donor site (EDS) expressed in centimorgans (cM). Data are given only for insertions within 30 cM of the EDS.

frequency of such plants to be low, relatively constant and independent of the excision/transposition frequency. Among the 846 F_2 progenies from *Ac* × *Ds* crosses that had an EDS, 2/3 to 3/4 also contained a TrE, regardless of transposition frequency. Similarly, in the sample of 121 lines for which data are given in Table 5, about 3/4 contained a TrE.

The origin of progeny with empty donor sites and no TrEs. Progeny in which an excision event has occurred, but which lack a TrE can arise by several mechanisms. In maize, *Ac* elements often excise from one sister chromatid and re-insert nearby either on the other sister chromatid or in an as yet unreplicated region of the same chromosome (Dooner and Belachew, 1989; Greenblatt, 1984; Greenblatt and Brink, 1963). Transposition during plant development from one sister chromatid to the other in the present transposon tagging system would result in the recovery of progeny containing an EDS, but no TrE. This is because reinsertion of an element into a sister chromatid would be followed by the mitotic segregation of a sister chromatid containing an unrearranged T-DNA and the TrE (C^sH^r) from a sister chromatid containing only an EDS (C^rH^s). These, in turn, would give rise to twinned plant sectors producing gametes of the corresponding genetic constitutions. Progeny lacking the transposon-borne hygromycin-resistance marker can also arise in other ways, either by failure of

the transposon to reinsert or as the result of a transposition event that causes breakage and rearrangement of the transposon. However, it appears likely that such events are relatively infrequent in the present lines. Indeed, the observation that roughly equal fractions of the lines in Table 5 had either only an EDS (24%) or had a TrE located within 10 cM of the donor site (21%) suggests that among the plants analyzed here, most of those with an EDS but no TrE arose from interchromatid transpositions.

Mapped Ds*–GUS-T-DNA insertion sites*

Selection of strains with elements located near a gene to be tagged by insertional mutagenesis can markedly reduce the labor involved in identifying desired insertion mutations. We have therefore embarked on mapping *Ds*–GUS-T-DNA insertion sites and here report the map positions of 10 sites (Figure 5). Mapping was done by hybridization of either fragments flanking the T-DNA insertion site amplified by thermal asymmetric PCR or by isolating a P1 clone containing the sequence flanking the insertion site (Liu and Whittier, 1995). The sites are located on three of the five *Arabidopsis* chromosomes, with chromosome 5 containing the largest number of sites (5). Crosses have been carried out with all of the mapped transposons and the results are incorporated into Table 2. While some of the *Ds*-GUS lines were crossed only to relatively inactive *Ac* lines, data presented in Figure 3 as well as in Table 2 suggest that these lines will respond well to more active transposase lines. The mapped lines are being made available through the Ohio State University's *Arabidopsis* stock center. We believe that the increasing availability of mapped transposon insertion sites, together with easier and more rapid means of identifying nearby transpositions and insertions into desired sequences, will substantially increase the utility of insertional mutagenesis for gene inactivation, as well as gene cloning.

Experimental procedures

Genetic analyses

Previously characterized plant lines carrying single insertions of *Ds*–GUS- and *Ac*-T-DNAs were grown and crosses were carried out, generally using a plant either homo- or hemizygous for the *Ds*–GUS-T-DNA as the pollen parent and a homozygous *Ac*-T-DNA plant as the female parent (Fedoroff and Smith, 1993). Seeds collected from the crosses were sterilized and germinated on agar-solidified MS medium containing 20 μg ml^{-1} hygromycin, as previously described (Fedoroff and Smith, 1993). Hygromycin-resistant plants were grown and allowed to self-pollinate. The progeny seeds were surface sterilized and germinated on agar-solidified MS medium containing both hygromycin (20 μg ml^{-1}) and chlorsulfuron (Chem Service, West Chester, PA) at 6 p.p.m. The genetic constitution of plants was determined as described previously (Fedoroff and Smith, 1993). Recombination frequencies

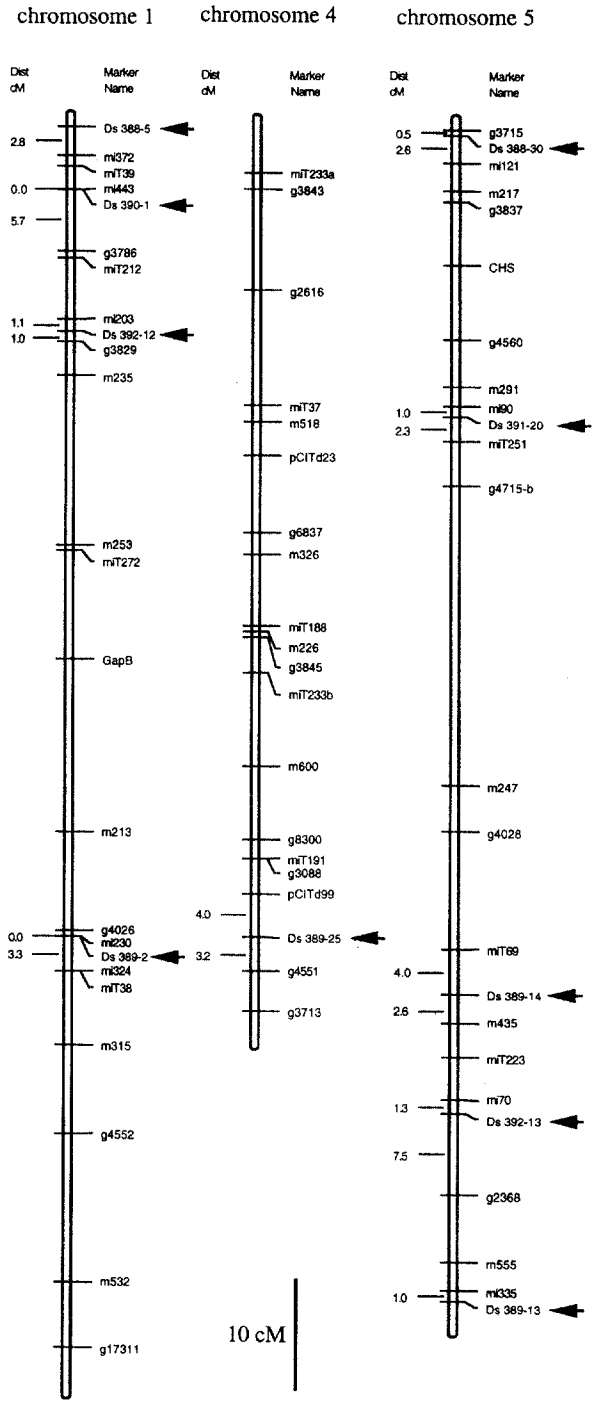


Figure 5. Location of *Ds*–GUS-T-DNAs on *Arabidopsis* chromosomes 1, 4 and 5.

between the EDS and the TrE were assessed in plants derived from a previously described strain carrying adjacent *Ds*–GUS- and *Ac*-T-DNA insertions (Fedoroff and Smith, 1993). Several (two to six) sibling plants hemizygous for both the EDS and the TrE resulting from outcrosses to wild-type plants were selfed and recombination frequencies were determined from the fraction of plants resistant to both napthalene acetamide (absence of *tms2* gene of the EDS) and hygromycin (presence of TrE). Sibling

progenies yielding markedly different values, probably originating from secondary transposition events, were excluded from the sample.

Root analysis

Root length measurements were made on 20–30 sibling seedlings of F_3 or F_4 plants with transposed elements and of identical genetic constitution grown on upright hygromycin-containing MS agar plates under identical conditions and at the same interval after plating (3 day cold treatment, 7 days at 25° C, 16 h light/8 h dark).

Amplification of genomic sequences flanking Ds–*GUS-T-DNA insertions*

Genomic sequences flanking the Ds–GUS-T-DNA insertions were amplified using Thermal Asymmetric Interlaced (TAIL-) PCR (Liu and Whittier, 1995). The reaction conditions and thermal cycling settings were as described (Liu *et al.*, 1995a). TAIL-PCR utilizes a set of three nested, target-specific primers along with a shorter arbitrary degenerate primer. The sequences of the target-specific primers were: L1, 5′-TATAATAACGCTGCGGACATCTACAT; L2, 5′-CTCCAT-ATTGACCATCATACTCA; and L3, 5′-GATTTCCCGGACAT-GAAGCCA for the outer, middle and inner nested primers, respectively. Altogether five different arbitrary primers were used. Their sequences were: AD2, 5′-TCTTICGNACITNGGA; AD3, 5′-(AT)GTG-NAG(AT)ANCANAGA; AD10, 5′-TTGIAG-NACIANAGG; AD20, 5′-TCTTICGNACITNGGA; and W4, 5′-AG(AT)GNAG(AT)ANCANAGA. These arbitrary primers were utilized in successful amplification of sequences flanking Ds/T-DNA insertions as follows: AD2, line 389-2; AD3, lines 390-1 and 391-20; AD10, lines 388-5, 388-30, 389-14, 389-25 and 392-12; AD20, line 392-13; and W4, line 389-13.

Mapping of Ds–*GUS-T-DNA insertions*

The amplified flanking sequences were used as probes to detect RFLPs between the ecotypes Columbia (Col) and Landsberg *erecta* (Le). RFLP segregation was scored in 100 recombinant inbred lines (Lister and Dean, 1993). In cases when no RFLP was detected by the TAIL-PCR product directly, an indirect mapping procedure was employed (Shibata *et al.*, manuscript in preparation). Briefly, P1 clones were isolated overlapping the TAIL-PCR products (Liu *et al.*, 1995b) and total P1 clone DNA was then used to probe for Col-Le RFLPs. Segregation was scored as above. To preclude mapping artifacts which might arise through low-level repetitive sequences, Southern blots were carried out to confirm the presence of an identically sized band in the P1 clone as in the Col RFLP allele. Linkage analysis was carried out using the MapMaker program (Lander *et al.*, 1987). The segregation data used for mapping included scoring for 'mi' markers (Liu *et al.*, unpublished), 'miT' T-DNA insertion loci (Liu *et al.*, 1995a) and other markers mapped or remapped by Lister and Dean (Lister and Dean, 1993).

Acknowledgments

This work was supported by NSF grant no. DIR-9123775 to N.V.F., a National Institutes of Health National Research Service Award to D.L.S. and by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Sports and Culture of Japan to K.O. and S.I., and from Special Coordination Funds of the Science and Technology Agency of Japan to K.O. We thank Dr A. Clark for assistance with statistical analyses, N. Mitsukawa for sharing filters of RI lines, T. Haas, K. Menchey, Y. Shirano, and Yuko Takahashi for technical assistance, and L. Savage and S. Wright for their participation as undergraduate students.

References

Altmann, T., Schmidt, R. and Willmitzer, L. (1992) Establishment of a gene tagging system in *Arabidopsis thaliana* based on the maize transposable element *Ac*. *Theor. Appl. Genet.* **84**, 371–383.

Altmann, T., Felix, G., Jessop, A., Kauschmann, A., Uwer, U., Pena-Cortes, H. and Willmitzer, L. (1995) *Ac*/*Ds* transposon mutagenesis in *Arabidopsis thaliana*: mutant spectrum and frequency of *Ds* insertion mutants. *Mol. Gen. Genet.* **247**, 646–652.

Baker, B., Schell, J., Lorz, H. and Fedoroff, N.V. (1986) Transposition of the maize controlling element *Activator* in tobacco. *Proc. Natl Acad. Sci. USA*, **83**, 4844–4848.

Bancroft, I. and Dean, C. (1993) Transposition pattern of the maize element *Ds* in *Arabidopsis thaliana*. *Genetics*, **134**, 1221–1229.

Bancroft, I., Bhatt, A. M., Sjodin, C., Scofield, S., Jones, J. D. and Dean, C. (1992) Development of an efficient two-element transposon tagging system in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **233**, 449–461.

Bancroft, I., Jones, J. D. and Dean, C. (1993) Heterologous transposon tagging of the *DRL1* locus in *Arabidopsis*. *Plant Cell*, **5**, 631–638.

Bishop, Y.M.M., Fienberg, S.E. and Holland, P.W. (1975) *Discrete Multivariate Analysis: Theory and Practice*. Cambridge: MIT Press.

Chuck, G., Robbins, T., Nijjar, C., Ralston, E., Courtney-Gutterson, N. and Dooner, H. K. (1993) Tagging and cloning of a *Petunia* flower color gene with the maize transposable element *Activator*. *Plant Cell*, **5**, 371–378.

Dooner, H.K. and Belachew, A. (1989) Transposition pattern of the maize element *Ac* from the *bz-m2*(*Ac*) allele. *Genetics*, **122**, 447–457.

Fedoroff, N. (1989) Maize transposable elements. In *Mobile DNA* (Howe, M. and Berg, D., eds). Washington, DC: American Society for Microbiology, pp. 375–411.

Fedoroff, N.V. and Smith, D.L. (1993) A versatile system for detecting transposition in *Arabidopsis*. *Plant. J.* **3**, 273–289.

Fedoroff, N., Wessler, S. and Shure, M. (1983) Isolation of the transposable maize controlling elements *Ac* and *Ds*. *Cell*, **35**, 243–251.

Feldmar, S. and Kunze, R. (1991) The ORFa protein, the putative transposase of maize transposable element *Ac*, has a basic DNA binding domain. *EMBO J.* **10**, 4003–4010.

Greenblatt, I.M. (1984) A chromosome replication pattern deduced from pericarp phenotypes resulting from movements of the transposable element, *Modulator*, in maize. *Genetics*, **108**, 471–485.

Greenblatt, I. M. and Brink, R.A. (1963) Transpositions of *Modulator* in maize into divided and undivided chromosome segments. *Nature*, **197**, 412–413.

Haughn, G.W., Smith, M., Mazur, B. and Somerville, C. (1988) Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. *Mol. Gen. Genet.* **211**, 266–271.

Hehl, R. (1994) Transposon tagging in heterologous host plants. *Trends Genet.* **10**, 385–386.

Honma, M.A., Baker, B.J. and Waddell, C.S. (1993) High frequency germinal transposition of *Ds*ALS in *Arabidopsis*. *Proc. Natl Acad. Sci. USA*, **90**, 6242–6246.

Houba-Herin, N., Becker, D., Post, A., Larondelle, Y. and Starlinger, P. (1990) Excision of a *Ds*-like maize transposable element (*Ac* delta) in a transient assay in Petunia is enhanced by a truncated coding region of the transposable element *Ac*. *Mol. Gen. Genet.* **224**, 17–23.

Izawa, T., Miyazaki, C., Yamamoto, M., Terada, R., Iida, S. and Shimamoto, K. (1991) Introduction and transposition of the maize transposable element *Ac* in rice (*Oryza sativa* L.). *Mol. Gen. Genet.* **227**, 391–396.

James, Jr D., Lim, E., Keller, J., Plooy, I., Ralston, E. and Dooner, H.K. (1995) Directed tagging of the *Arabidopsis* FATTY ACID ELONGATION1 (FAE1) gene with the maize transposon *Activator*. *Plant Cell*, **7**, 309–319.

Jones, J.D., Carland, F., Lim, E., Ralston, E. and Dooner, H.K. (1990) Preferential transposition of the maize element *Activator* to linked chromosomal locations in tobacco. *Plant Cell*, **2**, 701–707.

Keller, J., Lim, E. and Dooner, H. (1993) Preferential transposition of *Ac* to linked sites in *Arabidopsis*. *Theor. Appl. Genet.* **86**, 585–588.

Kunze, R. and Starlinger, P. (1989) The putative transposase of transposable element *Ac* from *Zea mays* L. interacts with subterminal sequences of *Ac*. *EMBO J.* **8**, 3177–3185.

Kunze, R., Stochaj, U., Laufs, J. and Starlinger, P. (1987) Transcription of transposable element *Activator* (*Ac*) of *Zea mays*. *EMBO J.* **8**, 3177–3185.

Lander, E.S., Green, P., Abrahamson, J., Barlow, A., Daly, M.J., Lincoln, E.S. and Newberg, L. (1987) Mapmaker: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics*, **1**, 174–181.

Laufs, J., Wirtz, U., Kammann, M., Matzeit, V., Schaefer, S., Schell, J., Czernilofsky, A.P., Baker, B. and Gronenborn, B. (1990) Wheat dwarf virus *Ac/Ds* vectors: expression and excision of transposable elements introduced into various cereals by a viral replicon. *Proc. Natl Acad. Sci. USA*, **87**, 7752–7756.

Lister, C. and Dean, C. (1993) Recombinant inbred lines for mapping RFLP and phenotypic markers in *Arabidopsis thaliana*. *Plant J.* **4**, 457–463.

Liu, Y.-G. and Whittier, R.F. (1995) Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. *Genomics*, **25**, 674–681.

Liu, Y.-G., Mitsukawa, N., Oosumi, T. and Whittier, R.F. (1995a) Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. *Plant J.* **8**, 457–463.

Liu, Y.-G., Mitsukawa, N., Vazquez-Tello, A. and Whittier, R.F. (1995b) Generation of a high-quality P1 library of *Arabidopsis* suitable for chromosome walking. *Plant J.* **8**, 351–358.

Long, D., Martin, M., Sundberg, E., Swinburne, J., Puangsomlee, P. and Coupland, G. (1993a) The maize transposable element system *Ac/Ds* as a mutagen in *Arabidopsis*: identification of an albino mutation induced by *Ds* insertion. *Proc. Natl Acad. Sci. USA*, **90**, 10 370–10 374.

Long, D., Swinburne, J., Martin, M., Wilson, K., Sundberg, E., Lee, K. and Coupland, G. (1993b) Analysis of the frequency of inheritance of transposed *Ds* elements in *Arabidopsis* after activation by a CaMV 35S promoter fusion to the *Ac* transposase gene. *Mol. Gen. Genet.* **241**, 627–636.

McClintock, B. (1951) Chromosome organization and genic expression. *Cold Spring Harbor Symp. Quant. Biol.* **16**, 13–47.

Osborne, B.I., Corr, C.A., Prince, J.P., Hehl, R., Tanksley, S.D., McCormick, S. and Baker, B. (1991) *Ac* transposition from a T-DNA can generate linked and unlinked clusters of insertions in the tomato genome. *Genetics*, **129**, 833–844.

Schmidt, R. and Willmitzer, L. (1989) The maize autonomous element *Activator* (*Ac*) shows a minimal germinal excision frequency of 0.2%–0.5% in transgenic *Arabidopsis thaliana* plants. *Mol. Gen. Genet.* **220**, 17–24.

Shimamoto, K., Miyazaki, C., Hashimoto, H., Izawa, T., Itoh, K., Terada, R., Inagaki, Y. and Iida, S. (1993) Trans-activation and stable integration of the maize transposable element *Ds* cotransfected with the *Ac* transposase gene in transgenic rice plants. *Mol. Gen. Genet.* **239**, 354–360.

Smith, D.L. and Fedoroff, N.V. (1995) *LRP1*, a gene expressed in lateral and adventitious root primordia of *Arabidopsis*. *Plant Cell*, **7**, 735–745.

Springer, P.S., McCombie, W.R., Sundaresan, V. and Martienssen, R.A. (1995) Gene trap tagging of *PROLIFERA*, an essential *MCM*2-3-5-like gene in *Arabidopsis*. *Science*, **268**, 877–880.

Sundaresan, V., Springer, P., Volpe, T., Haward, S., Jones, J.D., Dean, C., Ma, H. and Martienssen, R. (1995) Patterns of gene action in plant development revealed by enhancer trap and gene trap transposable elements. *Genes Devel.* **9**, 1797–1810.

Van Sluys, M.A., Tempe, J. and Fedoroff, N. (1987) Transposition of the maize *Activator* element in *Arabidopsis thaliana* and *Daucus carota*. *EMBO J.* **13**, 3881–3889.

Walbot, V. (1992) Strategies for mutagenesis and gene cloning using transposon tagging and T-DNA insertional mutagenesis. *Ann. Rev. Plant Physiol.* **43**, 49–82.

Wessler, S.R., Baran, G., Varagona, M. and Dellaporta, S.L. (1986) Excision of *Ds* produces waxy proteins with a range of enzymatic activities. *EMBO J.* **5**, 2427–2432.

Yoder, J.I., Palys, J., Alpert, K. and Lassner, M. (1988) *Ac* transposition in transgenic tomato plants. *Mol. Gen. Genet.* **213**, 291–296.