Germinal Transpositions of the Maize Element *Dissociation* From T-DNA Loci in Tomato

Bernard J. Carroll,[1] Victor I. Klimyuk, Colwyn M. Thomas, Gerard J. Bishop, Kate Harrison, Steven R. Scofield[2] and Jonathan D. G. Jones

The Sainsbury Laboratory, John Innes Centre, Norwich NR4 7UH, United Kingdom

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ABSTRACT

We have analyzed the pattern of germinal transpositions of artificial *Dissociation* (*Ds*) transposons in tomato. T-DNA constructs carrying *Ds* were transformed into tomato, and the elements were *trans*-activated by crossing to lines transformed with a stabilized *Activator* (*sAc*) that expressed the transposase gene. The *sAc* T-DNA carried a *GUS* gene to monitor its segregation. The *Ds* elements were inserted in a marker gene so that excision from the T-DNA could be monitored. The *Ds* elements also carried a genetic marker that was intended to be used for reinsertion selection of the elements after excision. Unfortunately, this gene was irreversibly inactivated on crossing to *sAc*. Germinal excision frequencies of *Ds* averaged 15–40%, but there was large variation between and within plants. Southern hybridization analysis of stable transposed *Ds* elements indicated that although unique transpositions predominate, early transposition events can lead to large clonal sectors in the germline of developing plants and to sibling offspring carrying the same transposition event. Multiple germinal transpositions from three different loci were examined for uniqueness, and 15 different transpositions were identified from each of three T-DNA loci that carried a single independent *Ds*. These were mapped relative to the donor T-DNA loci, and for each locus 70–80% of the transposed elements were closely linked to the donor site.

THE maize transposon *Activator* (*Ac*) and its nonautonomous derivative *Dissociation* (*Ds*) comprise the first transposon system to be discovered by McCLINTOCK (1947, 1948) and to be used for cloning genes from plants (FEDOROFF *et al.* 1984). It would be desirable to deploy transposon tagging to access the wealth of recognized genetic variation in *Arabidopsis thaliana* and tomato, which lack well-characterized endogenous transposons. In principle, a transposon tagging system could be established for these species by introducing elements such as *Ac* or *Ds*, and a number of groups have adopted this objective (YODER *et al.* 1988; SCHMIDT and WILLMITZER 1989; OSBORNE *et al.* 1991; BELZILE and YODER, 1992; DEAN *et al.* 1992).

The behavior of *Ac* in maize has been studied extensively, and excision occurs in both somatic and germline tissue. Studies on *Ac* excision and reinsertion have used the *P* locus, which conditions red pigmentation of the cob and pericarp (GREENBLATT and BRINK 1962; GREENBLATT 1984; CHEN *et al.* 1987, 1992; MORENO *et al.* 1992) and the *bronze* (*bz*) locus required for pigmented aleurone (DOONER and BELACHEW 1989). These studies have highlighted two important features of *Ac/Ds* for tagging: the transposition frequency and the preference of *Ac/Ds* for transposition to linked sites.

Because transposition is linked to DNA replication (GREENBLATT 1984), the fundamental transposition frequency is the probability of transposition per cell division cycle. Conceivably, this is developmentally regulated, and it is certainly affected by *Ac* dosage and expression levels (SCOFIELD *et al.* 1993). The term "germinal excision frequency" has been used to refer to the proportion of gametes that carry an excision event, measured as the relative frequencies of preexcision and excision alleles among sibling offspring. However, this measurement is confounded by various factors. Excision from within a reading frame often leaves nonfunctional alleles; progeny carrying such alleles would not be recorded as carrying excision events, leading to an underestimate of excision frequency. Also, as in the classic experiments of LURIA and DELBRÜCK (1943), a high excision frequency in a particular progeny sample can reflect one early event (in this case, in the development of the flower from which the seed were derived) or many late events. A systematic fluctuation analysis of germinal excision frequencies has never been undertaken to distinguish these interpretations. The molecular characterization of *Ac* (POHLMAN *et al.* 1984) has now made it possible to assess the extent of independent events among sibling progeny by Southern hybridization analysis. With this refinement, the germinal excision frequency can be a useful if crude indicator of

Corresponding author: Jonathan D. G. Jones, The Sainsbury Laboratory, John Innes Centre, Norwich NR4 7UH, United Kingdom.

[1] *Present address:* Department of Agriculture, University of Queensland, Brisbane, 4072, Queensland, Australia.

[2] *Present address:* CEPRAP, University of California-Davis, 1930 Fifth St., Davis, CA 95616.

transposition rates in germline tissue, which is a crucial parameter for transposon tagging experiments.

Germinal excision frequencies of *Ac* are erratic in maize and vary from 0 to 15% between cobs (BRINK and NILAN 1952). Transposition occurs at any time during development, with early events giving rise to large clonal sectors (GREENBLATT 1984; DOONER and BELACHEW 1989). After excision, reinsertion occurs elsewhere in the genome ~50–70% of the time (MCCLINTOCK 1956; GREENBLATT 1968; DOONER and BELACHEW 1989), usually creating an 8-bp duplication of the target sequence (POHLMAN *et al.* 1984).

Ac and *Ds* have been introduced via *Agrobacterium tumefaciens*–mediated transformation into several plant species where they excise and reinsert. The activity of the element varies; in tobacco the element is much more active than in Arabidopsis (JONES *et al.* 1991; SCHMIDT and WILLMITZER 1989). Southern hybridization analysis of transformed lines carrying *Ac* has shown that the element is very active in tomato (YODER 1990), but an accurate assessment of transposition in germline tissue has not been reported.

The strong tendency of *Ac* for transposition to closely linked sites is particularly relevant to tagging strategies (GREENBLATT 1984; DOONER and BELACHEW 1989), indicating that the prospects of tagging a gene at a known location may be enhanced when an *Ac* or *Ds* element is linked (MORENO *et al.* 1992). The tendency of linked transposition is maintained for germinal events in tobacco (JONES *et al.* 1990; DOONER *et al.* 1991) and Arabidopsis (KELLER *et al.* 1993). In tomato, the pattern of somatic transposition of *Ac* has been studied by isolating the sequences flanking transposed *Ac* and *Ds* elements and assigning these to the tomato restriction fragment length polymorphism (RFLP) map. These studies indicated that somatic transposition was often to unlinked sites (OSBORNE *et al.* 1991; BELZILE and YODER 1992; HEALY *et al.* 1993). However, it is difficult to interpret these data when *Ac* is so active in tomato and insertions at unlinked sites may have arisen by multiple transpositions rather than by a single event from the T-DNA . Also, germinal transpositions are generally more relevant to tagging strategies than somatic events, and few data have been generated on linkage relations of germinally transposed elements in tomato.

In this paper, we report the behavior and properties of *Ds* elements in tomato and, in particular, on the locations of a large number of independent germinally transposed *Ds* elements from three T-DNA loci. The *Ds* elements were made by replacing the *Ac* transposase gene with a marker gene, thereby rendering it nonautonomous. Germinal excision and reinsertion frequencies were measured and Southern hybridization analysis was used to assess the extent of clonal transposition events among sibling progeny.

MATERIALS AND METHODS

Plasmid constructions: The *Ds*-carrying binary vector plasmids were designed with five objectives in mind. First, *Ds* elements were cloned into the 5′ untranslated region of an antibiotic resistant gene, so that *Ds* excisions could be easily detected (BAKER *et al.* 1987; JONES *et al.* 1989). We used genes that confer spectinomycin (*SPEC*) and basta (*BAR*) resistance, as previously described (JONES *et al.* 1992, 1993). Second, the *Ds* elements carried another marker gene to select for reinsertion of the *Ds*. Third, they contained a counterselectable gene on the T-DNA, the *Agrobacterium* indoleacetamide hydrolase (*iaaH*) gene, to monitor segregation of the T-DNA. Individuals that carry the *iaaH* gene are sensitive to naphthalene acetamide (NAM) (KLEE *et al.* 1987). Additionally, an oligonucleotide containing recognition sequences for the enzymes *Not*I, *Dpn*I(*Cla*I) and *HO* nuclease (WEIL and MCCLELLAND 1989; NICKOLOFF *et al.* 1986) was incorporated into both the *Ds* and the T-DNA to enable the physical distance of transposition to be determined. These recognition sequences (≥8 bp) occur very rarely in genomic sequences. The *Dpn*I(*Cla*I) site is comprised of a 10-bp recognition sequence, 5′-ATCGATCGAT-3′; *Cla*I methylase can be used to methylate the internal adenine residues, thereby producing a *Dpn*I recognition site in the sequence (WEIL and MCCLELLAND 1989). Finally, the *Ds* element also contained the yeast *lys-2* gene as a selectable marker (BARNES and THORNER 1986) that would complement the *lys-2* mutation in the yeast YAC cloning strain AB1380. YAC libraries made from DNA of plants carrying transpositions could thus be selected for clones containing the *Ds*. The binary plasmids carrying a *Ds* element that were used in this study are shown in Figure 1. A detailed description of the construction of these vectors can be found in the Appendix. SLJ1561 carries a *Ds* with *nos:BAR* as the internal marker, inserted into a *nos:SPEC* excision marker. SLJ1481 has a different *Ds* (*nos:SPEC*) inserted in the 5′ untranslated region of *35S:BAR* excision marker. SLJ1601 is the same, except that the excision marker is *nos:BAR* (Figure 1). The probes and restriction endonuclease recognition sites that were used to characterize transformants and transposed *Ds* elements are also shown in Figure 1, as is the T-DNA of the binary vector that carried the transposase source SLJ10512 *(2′:GUS, 35S:NPT, sAc)* (SCOFIELD *et al.* 1992).

Plasmids were mobilized into *Agrobacterium tumefaciens* strain LBA4404 (HOEKEMA *et al.* 1983) using the *Escherichia coli* helper strain HB101/pRK2013.

Plant transformation: Transformation of tomato cotyledons (variety Moneymaker) and plant regeneration were performed essentially as described by FILLATTI *et al.* (1987). Transgenic lines were named after the appropriate DNA construct, and each transformant was given a unique letter designation.

Axenic seedling culture and *GUS* enzyme assays: Seeds were sterilized and plated on hormone-free Murashige and Skoog salts (MS) medium (1% glucose, 0.8% agarose) with or without the selection agent for each of the marker genes. When screening for the transformation marker (*NPT*), the excision marker or marker gene inside the *Ds* (*BAR* or *SPEC*), ~50 seeds were placed on 140-mm Petri plates and sealed with Nescofilm (Nippon Shoji Kaishi, Osaka, Japan) but with a small slit made in the seal at the top of the plate to allow gas exchange. The plates were incubated vertically such that the roots did not grow into the agarose, thereby allowing efficient screening for the *GUS* reporter gene (see below). Unless otherwise stated, the concentration of antibiotic incorporated into the medium to screen for the *NPT*, *BAR* and

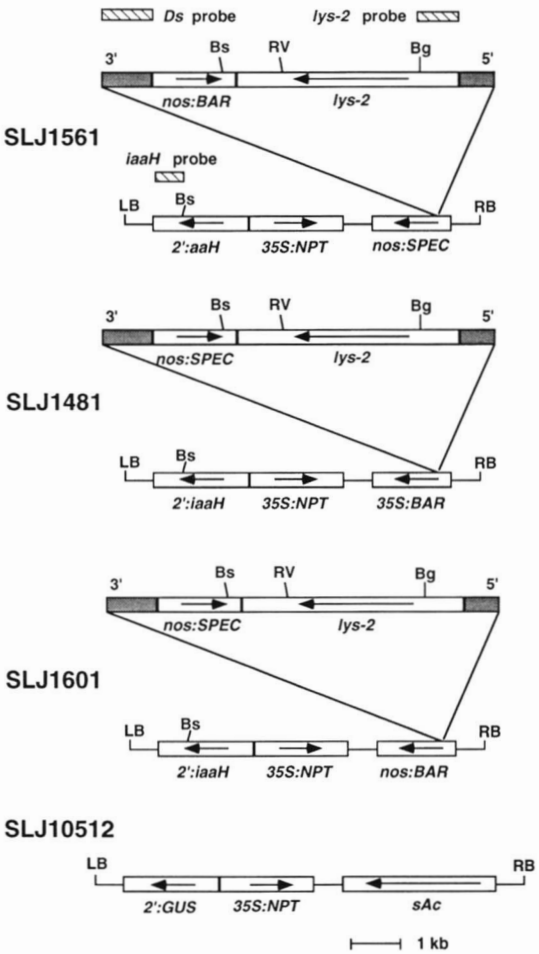

FIGURE 1.—T-DNA regions of binary vectors carrying a *Ds* element or the transposase gene (*sAc*). The three *Ds*-containing constructs were SLJ1561, SLJ1481 and SLJ1601, and the *sAc* construct was SLJ10512. The designation of the ends of the element, 5′ and 3′, refers to the orientation of the transposase gene within the analogous wild-type *Ac* element. Also shown in the figure are marker genes, restriction enzyme recognition sites and probes used in Southern hybridization analysis to characterize the T-DNA insertions and transposed *Ds* elements. Restriction sites: Bs, *Bst*YI; RV, *Eco*RV; Bg, *Bgl*II. Probes: *Ds* , 3′ 1100 bp of *Ac*; *lys-2*, 5′ 800-bp *Bgl*II fragment of *lys-2* gene; *iaaH*, 3′ 800-bp *Bst*YI fragment of *iaaH* gene.

SPEC genes was 300 μg/ml kanamycin, 100 μg/ml phosphinothricin and 100 μg/ml spectinomycin, respectively.

At 10–12 days after planting and culture at 25°, the seedlings were screened for resistance to the selection agent and then, depending on the experiment, whole plates of seedlings were screened for the *GUS* reporter gene. Excess water from condensation was drained from the plate, and then 15 ml of 0.5 mg/ml 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) was dispensed into the plate to cover the roots of the seedlings. The plate was then incubated at 37° for 2–3 hr, after which time only seedlings carrying the *GUS* gene showed strong blue coloration of the roots. This procedure was confirmed to be reliable by Southern hybridization analysis on selected plants. Individual seedlings could be recovered after this treatment and sent to the glasshouse for propagation, isolation of DNA and crossing.

Screening for the *iaaH* gene was conducted in sterile plastic 100-mm food pots [item numbers: 14693–309–8 (pot) and 13591–109–1 (lid), Borden Ltd., Bridgeport, Somerset, UK] containing 90 ml of MS medium with 1 μg/ml NAM. In this case, the roots of the seedlings grew into the agarose medium. Scoring was based largely on the phenotype of the root. To detect the presence or absence of the *2′.iaaH* gene reliably, it was necessary to ensure that the seeds were well spaced and not plated in contact with each other (20 seeds were distributed per pot) and to score the phenotypes before the seedlings grew too large (≤12 days after planting). *GUS* assays were performed on some of these seedlings. Root pieces (3–5 mm long) were incubated in 20 μl of 0.5 mg/ml X-gluc in 0.5-ml Eppendorf tubes at 37° for 1 hr before scoring.

Crossing strategy and progeny testing: To characterize the behavior of *Ds* elements in tomato, the following crossing strategy was used. *Ds* and *sAc* transformants carrrying a single copy of the T-DNA were crossed and F_1 seedlings were identified that were heterozygous for both T-DNAs. These plants were used as either the female or male parent (sometimes pooling pollen from several plants) in test crosses to nontransformed plants. The resulting TC_1F_1 progenies were assessed for frequency and characteristics of germinal transposition events inherited from the double heterozygous parents. Germinal excision frequencies of the *Ds* elements and frequencies of reinsertion after excision were estimated (see below), and individuals carrying stable transposed *Ds* elements were identified by selecting for expression of the excision marker, absence of transposase (absence of *GUS* gene activity) and for reinsertion of a *Ds* element elsewhere in the genome. Unfortunately, the plant selectable marker (*BAR* or *SPEC*) inside the *Ds* element was ineffective once exposed to *Ac* transposase (see RESULTS) and therefore PCR was used to monitor reinsertion (see below).

It was important to test cross the double heterozygotes to produce TC_1F_1 progeny, rather than selfing to produce F_2 progeny, because this strategy greatly simplified the analysis of germinal transposition events by ensuring that progeny inherited a single copy of the *Ds*-containing T-DNA. Positive detection of a *Ds* element by using polymerase chain reaction (PCR) on TC_1F_1 progeny expressing the excision marker (and lacking transposase) ensures that the detected element has excised from the excision marker. The test-cross strategy also facilitated segregation of the transposase gene away from stable transposed *Ds* elements. DNA samples of sibling progeny carrying stable transposed *Ds* elements were analyzed by Southern hybridization analysis to assess the extent of clonal transposition events among sibling progeny. TC_1F_1 plants were identified in this analysis that carried single unique transposed *Ds* elements, and these were selfed to produce TC_1F_2 progeny that were screened to estimate the genetic distance of transposition.

Estimation of germinal excision frequency: Germinal excision frequency was measured through the gametes of F_1 plants heterozygous for both the *Ds*-containing and *sAc*-containing T-DNAs. These double heterozygotes were used either as the female or male parent in crosses to untransformed tomato, and the resulting progeny were screened for expression of the excision marker and the *GUS* reporter gene linked to the *Ac* transposase source. Both *SPEC* or *BAR* genes confer non-cell autonomous phenotypes in tomato (JONES *et al.* 1993), and in plants carrying the transposase gene, somatic and germinal transposition events cannot be distinguished; therefore, only progeny lacking the *sAc* were used in calculating the germinal excision frequency. The formula and rationale for calculating this parameter is given below:

germinal excision frequency

$$= \frac{\text{(no. of } \textit{GUS} \text{ negative plants expressing the excision marker)}}{(1 - a) \times \text{(total no. of } \textit{GUS} \text{ negative plants}} \times 100,$$

where a is the recombination frequency between the *Ds*- and *sAc* T-DNAs (equals 0.5 for independent segregation).

The estimate relies on scoring the progeny for expression of the excision marker and the *GUS* gene only and does not rely on determining the number of individuals with the *Ds*-carrying T-DNA within a population. The expected number of individuals carrying this T-DNA (based on Mendelian segregation) is used in the calculation and can therefore give an estimate in small populations that exceeds the maximum excision frequency of 100%. The expected number of *GUS*-negative plants carrying the *Ds*-related T-DNA is increased when the T-DNAs are linked in repulsion, and therefore the calculation must take into account the genetic distance between the *Ds*- and *sAc*-containing T-DNAs. All of the T-DNA loci described here segregate independently (Table 1), except for 1561E and 10512I, which were shown to be linked in repulsion (see RESULTS).

Genetic distance of germinal transposition events: TC_1F_1 individuals were identified that expressed the excision marker, lacked transposase and had a single transposed *Ds* element as determined by Southern hybridization analysis with at least two restriction endonucleases. Self (TC_1F_2) progeny derived from these plants were analyzed to determine the genetic distance of transposition. The *NPT* gene was used as the genetic marker for the T-DNA. The progeny were germinated on kanamycin (300 μg/ml), and ≥10 kanamycin-sensitive seedlings in each family (representing a unique transposition event) were tested for the presence of the *Ds* by using PCR (see below). Kanamycin-sensitive plants were homozygous for the absence of the T-DNA, and recombinants for either or both chromosomes could be detected by the PCR assay. The proportion of nonrecombinant individuals (kanamycin sensitive and *Ds* negative) in a minimum sample size of 10 kanamycin-sensitive plants (representing 20 gametes) was used to estimate the genetic distance of transposition. The following formula accounts for the possibility of recombination occuring for both chromosomes:

$$\text{genetic distance (cM)} = [1 - \sqrt{(\text{proportion of kanamycin-sensitive plants lacking } \textit{Ds})}] \times 100.$$

PCR analysis for detection of *Ds* elements: Template preparation from leaf or cotyledon tissue and PCR analysis was performed as described by KLIMYUK *et al.* (1993). This procedure involves using intact tissue as the PCR template after it has been boiled for 30 sec in 0.25 M NaOH and neutralized with 0.25 M HCl and 0.5 M Tris-HCl, pH 8.0. For leaf tissue it was often necessary to include β-mercaptoethanol in the 0.25 M NaOH (10 μl/ml). Nontransformed and transformed controls were always included in the analyses, and four oligonucleotide primers were added to each PCR. Two primers (GGT AAA CGG AAA CGG AAA CGG TAG and TGC CTG GCC GCC TGG GAG AGA) recognize sequences at the 5′ end of *Ac* and the *Ds* elements described here to amplify a 208-bp fragment, and two (TAT AAC CAA ATG CAA CTC CGT CTT and CGA GAG AGA TTC AAG AAT AGA CCC) recognize sequences of tomato DNA on chromosome *11*, which amplify a 141-bp fragment. The latter primers were an essential positive control because template from all tomato genotypes contain the sequences recognized by these oligonucleotides.

The PCR reaction was performed on pieces of tissue in a volume of 50 μl in the presence of 0.25 μM of each of the four primers, 250 μM dNTPs (Pharmacia) in a buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.05% Nonidet P-40 and 1.0–2.5 units of "AmpliTaq" thermostable DNA polymerase (Perkin-Elmer Cetus). Cycling conditions were as follows: 94° for 15 sec; 55° for 15 sec; 72° for 1 min; 35 cycles, followed by 10 min extension at 72°C. A total reaction volume (15 μl) was loaded onto a 3% agarose gel (3:1 Nusieve GTG Agarose:Seakem LE agarose).

DNA isolation for Southern hybridization analysis: The procedure for DNA isolation was a modification of a published method (LASSNER *et al.* 1989). One gram of leaf tissue was harvested and stored on ice. The rollers of a sap extractor (Erich Pollarne, Hannover, Germany) were rinsed with water before feeding in the tissue and then washed with 3 ml nuclear extraction buffer (0.2 M Tris pH 7.5, 0.05 M EDTA, 2 M NaCl, 2% w/v hexadecyltrimethyl ammonium bromide) followed by 0.6 ml 5% w/v sarkosyl. The eluate was collected, inverted several times and incubated at 65° for 20 min. The samples were then extracted once with phenol:chloroform, and nucleic acids were precipitated from the supernatant by adding an equal volume of isopropanol and leaving at room temperature for 15 min before centrifugation. The pellet was washed on ice in 70% ethanol for 1 hr, centrifuged and resuspended in 2 ml sterile distilled water incubated with 10 μg RNase A for 15 min at 37° then extracted again with phenol:chloroform. DNA was precipitated in 70% ethanol with 1 M ammonium acetate and hooked out with a Pasteur pipette into 70% ethanol, centrifuged and resuspended in 200 μl of sterile distilled water. The yield of DNA from 1 g of starting tissue was ~50 μg. For Southern hybridization analysis, 10 μg of DNA was digested to completion with one of several restriction endonucleases, electrophoresed in 0.8–1.2% agarose gels containing Tris-borate/EDTA buffer (SAMBROOK *et al.* 1989) and blotted onto Genescreen Plus membranes. Thereafter, the hybridization of the probe to the membranes was as described by THOMAS *et al.* (1994).

Cloning and characterization of sequences flanking transposed *Ds* elements: Plants were identified that lacked transposase and carried a single unique transposed *Ds*. Inverse PCR (IPCR) was used to amplify the tomato sequences flanking these elements; DNA isolation for IPCR, IPCR reactions, cloning of IPCR products and DNA sequence analysis was as described by THOMAS *et al.* (1994).

RESULTS

Expression of genetic markers in transformants carrying a *Ds* element: Eleven transformants were obtained that carried a functional *NPT* gene: 8 from SLJ1561, 1 from SLJ1481 and 2 from SLJ1601. Only three, designated 1561E, 1481J and 1601D, were diploid, expressed the full complement of plant selectable makers and resulted from the insertion of a single complete copy of the T-DNA. Figure 2 shows Southern hybridization analysis of the right border for these transformants; when genomic DNA was digested with *Bgl*II, electrophoresed, blotted and probed with the 5′ 800-bp *Bgl*II fragment of *lys-2*, only a single strong band was detected. The single copy nature of these insertions was confirmed by similar analysis of the left border, after digestion with *Bst*YI and probing with the 3′ 800-bp *Bst*YI fragment of the *iaaH* gene (data not shown).

TABLE 1

Germinal excision frequencies of *Ds* elements in progeny derived from individual fruits and individual plants

Ds line	*sAc* line	Plant number	Fruit number	Segregation ratio in TC_1				Germinal excision frequency (%)	
				GUS⁻ resistant	*GUS*⁻ sensitive	*GUS*⁺ resistant	*GUS*⁺ sensitive	For individual fruit	For whole plant
1561E	10512I	G289	1	29	3	0	27	95	
			2	25	0	1	30	105[b]	
			Total	54	3	1	57		99
1561E	10512A	G248	1	1	40	16	14	5	
			2	3	32	18	21	17	
			3	6	27	20	18	36	
			4	4	14	9	5	44	
			5	2	25	16	24	15	
			6	7	22	11	16	48	
			7	2	18	9	19	20	
			8	7	22	8	22	48	
			9	13	36	22	30	53	
			Total	45	236	129	169		32
1561E	10512A	G249	1	2	36	8	22	10	
			2	1	35	10	17	6	
			3	1	3	10	6	50	
			4	3	23	13	19	23	
			Total	7	97	41	64		13
1561E	10512A	G254	1	12	6	10	9	133[b]	
			2	12	15	9	20	89	
			3	20	25	29	26	89	
			4	10	10	4	10	100[b]	
			5	4	24	21	17	29	
			6	15	12	11	11	111[b]	
			7	1	29	11	19	7	
			8	2	17	10	10	21	
			9	0	17	9	11	0	
			10	6	20	8	14	46	
			Total	82	175	122	147		64
1561E	10512I	G282	1	4	11	0	18	28	28
1561E	10512A	G281	1	0	12	7	6	0	
			2	1	35	9	18	6	
			Total	1	47	16	24		4
1481J	10512I	G287	1	3	23	7	23	23	
			2	11	17	10	24	79	
			3	9	16	5	13	72	
			Total	23	56	22	60		58
1481J	10512I	G280	1	4	41	23	19	18	18
1481J	10512I	G279	1	33	37	27	33	94	
			2	15	23	22	20	79	
			Total	48	60	49	53		89
1481J	10512I	K4	1	0	37	0	31	0	
			2	3	26	24	26	21	
			3	5	46	21	21	20	
			4[a]	4	21	16	21	32	
			5[a]	8	65	24	26	27	
			6[a]	0	15	0	10	0	
			Total	20	210	85	135		17
1481J	10512I	K3	1	8	38	33	29	35	
			2	2	58	31	30	7	
			3	3	17	2	16	30	
			4[a]	3	29	16	21	19	
			5[a]	9	44	17	26	34	
			Total	25	186	99	122		24

Plants were selected for heterozygosity of both the *Ds* T-DNA and the *sAc* T-DNA and used in test crosses (as the female parent, unless otherwise stated) to produce individual fruit. Progeny from these individual fruit was screened for resistance to the selection agent for the excision marker and expression of the *GUS* gene (*i.e.*, presence of the transposase gene), and the germinal excision frequency was estimated as described in MATERIALS AND METHODS. The estimate takes into account the recombination frequency between the *Ds* and *sAc* T-DNA loci, which was 0.044 in the case of 1561E (*Ds*) and 10512I (*sAc*) (see text); all other combinations of *Ds* and *sAc* segregated independently.

[a] For these fruit the double heterozygote was used as a male parent in the test cross; otherwise it was used as a female.

[b] The expected (not the actual) number of individuals carrying *Ds*-related T-DNA was used in the calculation and therefore some estimates exceed the maximum possible frequency of 100%.

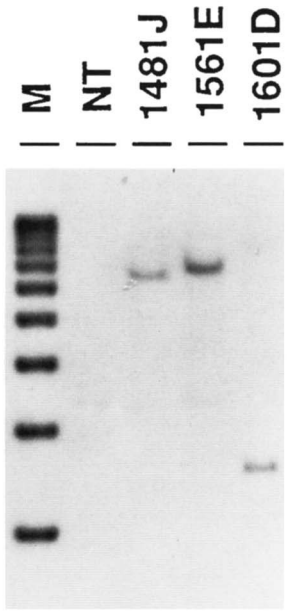

FIGURE 2.—Southern hybridization analysis of the right border of T-DNA insertions in *Ds* transformants 1561E, 1481J and 1601D. Genomic DNA was digested with *Bgl*II, electrophoresed, blotted and probed with the 5′ 800-bp *Bgl*II fragment of *lys-2* (see Figure 1). M, marker DNA (1-kb ladder, BRL, smallest marker fragment shown is ~3 kb); NT, nontransformed. Each hybridizing fragment represents a single copy insertion of the T-DNA.

Selfed progeny derived from 1561E, 1481J and 1601D expressed the *NPT* gene, the *iaaH* gene and the marker gene (*BAR* or *SPEC*) inside the *Ds* element at the expected Mendelian segregation ratio for single copy T-DNA insertions (data not shown).

As for other plant species (KLEE *et al.* 1987), tomato seedlings carrying the *iaaH* gene can be distinguished from nontransformed seedlings in the presence of NAM. Tomato plants expressing the *2′:iaaH* gene could be distinguished from nontransformed plants because they developed callus-like roots on NAM (data not shown). This marker gene was used to estimate the genetic distance between *Ds* and *sAc* T-DNAs (see below).

Phenotypes resulting from crossing *Ds* lines to *sAc* lines: These *Ds*-containing lines (1561E, 1481J and 1601D) were crossed to other transformants, 10512A or 10512I, that expressed the *Ac* transposase gene from a single copy insertion of the T-DNA . When the F_1 progeny from a cross between a *Ds* line and a transposase line were plated on the selection agent for the excision marker, all individuals that inherited both T-DNAs were resistant due to somatic excision of the *Ds* element. No variegation was observed in these resistant individuals because the *BAR* and *SPEC* genes confer noncell autonomous phenotypes in tomato (JONES *et al.* 1993).

Surprisingly, F_1 progeny that inherited both T-DNAs (determined by *GUS* assays and PCR) showed greatly decreased expression of the plant-selectable marker inside the *Ds* element compared with individuals inheriting the *Ds* but not the *sAc* T-DNA. This is shown in Figure 3 for 1601D × 10512I double heterozygotes, germinated on 100 μg/ml spectinomycin. A similar phenomenon was observed when either 1481J or 1561E × 10512 double heterozygotes were plated on the selection agent for the *Ds* marker gene. The decreased expression of the marker gene was shown to be heritable, even after the transposase source had segregated away (data not shown). A detailed analysis of this phenomenon will be reported elsewhere. An adverse consequence of this interesting result was that PCR or Southern hybridization analysis was required to detect reliably the presence of *Ds* elements once transposase had been introduced into the same genome. Expression of all other marker genes described above was not adversely affected by *Ac* transposase, and gene silencing was confined to the marker gene inside the *Ds* element.

Germinal excision and frequency of reinsertion of the *Ds* elements: Germinal excision frequencies were measured through the germline of F_1 plants heterozygous for both the *Ds*-containing and *sAc*-containing T-DNAs. These double heterozygous plants were used either as the female or male parent in crosses to untransformed tomato, and the resulting progeny were screened for expression of the excision marker and *GUS* genes. Only progeny lacking *sAc(GUS)* were used in calculating the germinal excision frequency. The calculation takes into account the genetic distance between the *Ds*- and *sAc*-containing T-DNAs (see MATERIALS AND METHODS). All of the T-DNA loci described here segregate independently, except for 1561E and 10512I, which are linked in repulsion in F_1 individuals, and TC_1F_1 individuals derived from these plants usually inherit either the *Ds*-carrying T-DNA (1561E) or the T-DNA carrying *sAc* (10512I).

To estimate the distance between these loci, TC_1F_1 progeny was plated on 1 μg/ml NAM to detect the presence or absence of the *iaaH* gene (1561E) and subsequently assayed for *GUS* activity (10512I). The segregation ratio among the progeny was 77:75:4:3 (*iaaH* positive, *GUS* negative:*iaaH* negative, *GUS* positive:*iaaH* positive, *GUS* positive:*iaaH* negative, *GUS* negative). Thus, the recombination frequency between these two T-DNAs was estimated to be 0.044 (=7/159), and this value was used to calculate the germinal excision frequency in progeny derived from this cross. Linkage between these loci was confirmed by assigning sequences flanking the T-DNAs to the tomato RFLP map (THOMAS *et al.* 1994).

Both *sAc* lines (10512A and 10512I) gave rise to variable germinal excision frequencies, often high but sometimes low. Large variation was observed between individual plants and between individual flowers on the

FIGURE 3.—Inactivation of the plant selectable marker gene on the 1601D *Ds* in the presence of *Ac* transposase. F_1 progeny obtained by crossing 1601D and 10512I were germinated on 100 μg/ml spectinomycin. (Left) Seedlings with *Ds* T-DNA only (high level of resistance, equal to the resistance of self progeny of 1601D). (Center) Seedlings with both the *Ds* and *sAc* T-DNAs (very low level of resistance). (Right) Seedlings without *Ds* T-DNA (sensitive, completely bleached).

same plant. This important feature of *Ds* excision in tomato is emphasized by the data from 11 double heterozygotes presented in Table 1 and indicates that the first excision event in the generation when transposase is introduced may occur at any point during the development of the plant. Among progeny derived from individual fruit on the same plant, excision frequencies ranged from 0 to 100% and from 4 to 99% between progeny from different plants. Although most of the data presented in Table 1 were from progeny derived from the female cell lineage of double heterozygotes, the excision frequency through the female and male cell lineages of some individuals was compared and shown to be similar. The data in Table 1 were derived from a total of 2803 TC_1F_1 individuals, and as expected, about half of these (1413) inherited the transposase (*GUS*) gene. The average germinal excision frequency over the 11 double heterozygotes was ~40%.

In other experiments, pollen was collected continually throughout development and pooled from several double heterozygotes for each of the *Ds* lines before crossing to a nontransformed tester. At least 1000 TC_1F_1 individuals were screened for each *Ds* line, and the average germinal excision frequency ranged from 16 to 35% (Table 2). In populations 2 and 3 listed in Table 2, the number of progeny expressing the *GUS* gene was slightly less than expected (561/1329 and 424/1034, respectively). None of 65 *GUS*-negative individuals from these populations analyzed by Southern analysis inherited the transposase gene (data not shown), thereby indicating that the *GUS* assay was reliable and that in some individuals the transgenes may be inherited at a lower frequency than expected. Individuals expressing the excision marker and lacking transposase (*GUS*) were screened by PCR analysis for the presence of a transposed *Ds*. The percentage of these individuals carrying a *Ds* defined the frequency of reinsertion after excision, which ranged from 27 to 61% in the pooled populations (Table 2). In this way, individuals carrying stable transposed *Ds* elements were identified for further analysis.

Large and small sectors in the germline of double heterozygotes: A high germinal excision frequency can conceivably result from an early excision event in the plant development to produce progeny carrying a clonal transposed *Ds*. To assess the extent of this possibility in tomato, samples of individuals carrying a stable transposed *Ds* element(s) were subjected to Southern hybridization analysis to determine *Ds* copy number and the uniqueness of transposition events among sibling progeny. *Ds* copy number was usually one but was sometimes more, indicating multiple transposition events in the single generation that transposase was present (Figures 4 and 5). Large clonal sectors occurred in some double heterozygotes and gave rise to many sibling progeny carrying the same transposed *Ds*.

Figure 4A shows Southern hybridization analysis of a sample of progeny derived from two test-cross fruit of double heterozygote G289 (F_1 of 1561E × 10512I). Two other plants derived from other double heterozygotes and carrying transposed *Ds* elements are also represented in Figure 4A. Genomic DNA was digested with *Bst*YI and probed with the 3′ 1100 bp of *Ds* (see Figure 1). Each band in the figure represents a single *Ds* element, and the preexcision allele gave a band of 2.6 kb. All of the individuals derived from G289 and represented in this figure carried a clonal transposed *Ds* element defined by a 5.8-kb *Bst*YI band, and two of these plants had an additional transposed *Ds* element. The clonal identity of the 5.8-kb band was confirmed using separate restriction enzymes.

A total of 24 individuals derived from these two fruits of G289 were analyzed by DNA gel blots; 19 carried only the major clonal transposition event described above, 2 (1 from each fruit) carried the major clonal event plus another less common clonal event, 2 had the major

TABLE 2

The frequency of germination excision and reinsertion of *Ds* elements estimated in progeny pooled from several double heterozygous plants

Population	*Ds* line	*sAc* line	Number of double heterozygotes[a]	Segregation ratio in TC_1				Germinal excision frequency (%)	Frequency of reinsertion[b]	Frequency of reinsertion as a percentage
				GUS$^-$ resistant	*GUS*$^-$ sensitive	*GUS*$^+$ resistant	*GUS*$^+$ sensitive			
1	1561E	10512I	5	264	491	16	786	35	30/110	27
2	1481J	10512I	4	60	708	205	356	16	25/59	42
3	1601D	10512I	4	80	530	118	306	26	42/69	61
4	1561E	10512A	2	92	631	230	402	25	ND[c]	ND[c]
5	1561E	10512I	4	195	428	20	642	31	ND[c]	ND[c]

Pollen was collected throughout development from plants heterozygous for both the *Ds* and *sAc* containing T-DNAs. Pollen from groups of these plants was pooled and test crossed to untransformed female plants. The resulting progeny were screened for resistance to the selection agent for the excision marker and expression of the *GUS* gene, linked to and reporting for the *Ac* transposase source. Germinal excision frequency was estimated as described in MATERIALS AND METHODS and the legend of Table 1.

[a] Number of double heterozygotes that contributed to the test-cross progeny.

[b] Denominator is total number of *GUS*$^-$ plants expressing excision marker that were tested for the presence of a transposed *Ds* by PCR analysis; numerator is number of *GUS*$^-$ plants expressing excision marker and carrying a transposed *Ds*.

[c] Not determined.

clonal event plus another unique one and the remaining individual possessed the less common clonal event plus two other unique events (data not shown). Thus, among the 24 individuals, only five independent transposition events were detected. The germinal excision frequency estimated from the progeny of these two fruits of G289 was ~100% (see Table 1), thereby indicating that excision events early in the development of double heterozygotes results in both a high transmission of excision alleles to the subsequent generation and many sibling progeny containing the same *Ds* insertion event.

Sibling progeny derived from the fruit of other double heterozygotes were more heterogeneous for stable transposed *Ds* elements, as demonstrated in Figure 4B. These progeny were derived from male and female test-cross fruit of K4 (F_1 of 1481J × 10512). In this case, all of the individuals carried at least one unique transposed element. The germinal excision frequency measured in the progeny of K4 was 17% (see Table 1) and among the 11 individuals analysed, 13 unique transposed elements were detected.

Clonal events were also evident in pooled progeny from several double heterozygous plants. Figure 5 represents 34 plants in the pooled test-cross progeny from four 1601D × 10512I F_1 plants (population 3 in Table 2). Two clonal events were detected in this population: plants 5 and 7–9 carried a clonal transposed element,as did plants 18 and 24. About 50 unique elements were detected in this sample of plants (Figure 5). Twenty-five plants carrying a stable *Ds* element(s) were sampled from another pooled population derived from four 1481J × 10512I F_1 plants (population 2 in Table 2) and analyzed by Southern hybridization analysis with at least two restriction enzymes (data not shown). Twenty-four unique events were detected in this second pooled population, but over a quarter of these (7 of 25) carried the same transposition event, indicating that one of the parents contributed pollen throughout its entire development that contained a clonal event.

Genetic distance of germinal transposition events: Fifteen individuals containing a single unique transposed *Ds* were identified for each *Ds* line, selfed and analyzed in the next generation to estimate the genetic distance of transposition. The T-DNA was monitored using the *NPT* gene, and the presence of the *Ds* was detected using PCR. The progeny were plated on kanamycin and segregated as expected at a frequency that approximated three resistant to one sensitive (data not shown). At least 10 kanamycin-sensitive seedlings in each family, representing ≥20 gametes, were tested for the presence of the *Ds* by using PCR analysis (Figure 6). The proportion of kanamycin-sensitive plants devoid of the transposed element was used to estimate the genetic distance of transposition (see MATERIALS AND METHODS). If the *Ds* element transposed to a closely linked site, individuals that did not inherit the T-DNA (*i.e.*, were kanamycin sensitive) would also fail to inherit the *Ds*.

An example of such a family is shown in Figure 6 (top), where none of the 10 kanamycin-sensitive plants inherited the *Ds* element. In contrast, three quarters of kanamycin-sensitive progeny would be expected to inherit the *Ds* if the T-DNA and the transposed element segregated independently, as was the case for the family represented in the lower panel of Figure 6, where 7 of 10 kanamycin-sensitive plants carried the transposed element.

The genetic pattern of germinal transposition from each of the T-DNAs was very similiar, and the majority

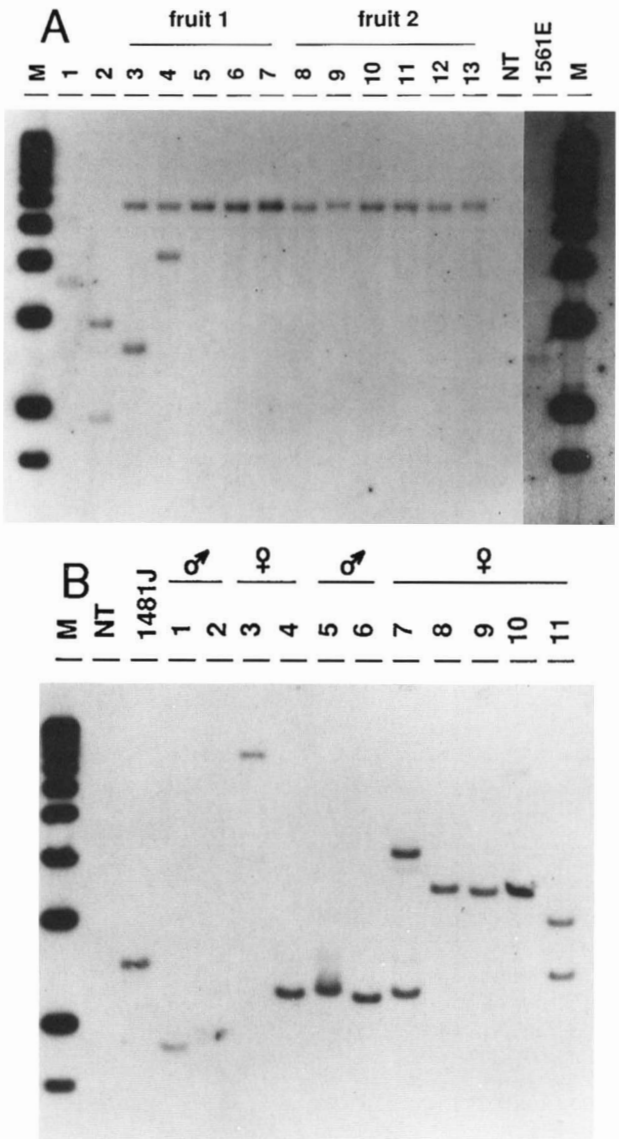

FIGURE 4.—Southern hybridization analysis of stable transposition events in sibling progeny. Genomic DNA of nontransformed, the progenitor 1561E or 1481J and TC_1F_1 individuals carrying stable transposed *Ds* elments was digested with *Bst*YI, electrophoresed, blotted and probed with the 3′ 1100 bp of *Ds* (see Figure 1). (A) Lane M, marker DNA (1-kb ladder, BRL, smallest marker fragment shown is ~1.6 kb); lanes NT and 1561E, nontransformed and progenitor 1561E, respectively; lanes 1 and 2, TC_1F_1 individuals from separate double heterozygotes derived from 1561E; and lanes 3–13, TC_1F_1 individuals from two test-cross fruit of G289 (heterozygous for both the 1561E and 10512I T-DNAs). All of the individuals derived from G289 and represented in the figure carried a clonal transposed *Ds*, identified as a 5.8-kb *Bst*YI band. The germinal excision frequency inherited through the female gametes of these fruit are listed in Table 1 (rows 1 and 2) and approximate 100%. (B) Lane M, marker DNA (1-kb ladder, BRL, smallest marker fragment shown is ~1.6 kb); lanes NT and 1481J, nontransformed and progenitor 1481J, respectively; lanes 1 and 2, TC_1F_1 individuals from a test cross with K4 (heterozygous for the 1481J and 10512I T-DNAs) as the male; lanes 3 and 4, TC_1F_1 individuals from a test cross with K4 as the female male; lanes 5 and 6, TC_1F_1 individuals from a separate test cross with K4 as the male; lanes 7–11, TC_1F_1 individuals from a separate test cross with K4 as the female. Some of the bands in the figure are a similar size, but analysis with another enzyme (*Eco*RV) demonstrated that they all represented separate events (data not shown). The germinal excision frequency estimated for K4 was ~20% (Table 1).

of transpositions (29 out of the 45 independent events studied) were to closely linked sites, with no recombinants in the 20 gametes that were analyzed (Figure 7). The data presented in Figure 7 are not precise estimations of the genetic distance of transposition, and the cluster of events at 0 cM is probably a reflection of the small sample size that was tested for recombination (≥10 kanamycin-sensitive TC_1F_2 plants representing 20 gametes from meiosis). It is likely, however, that many of these reinsertions occurred very close to the T-DNA. This conclusion is supported by the following consideration of the probability of registering no recombinants in a sample of 10 (P_0). An estimation of the genetic distance for a particular transposed *Ds* represents a binomial experiment (BYRKIT 1987) and P_0 can be calculated as follows:

$$P_0 = q^0 \times p^n$$

where q is the probability of detecting recombinants based on the real genetic distance, $p = 1 - q$ and n is the sample size of kanamycin-sensitive plants tested for recombination (≥10).

P_0 decreases exponentially as the genetic distance increases, as illustrated in Table 3. Thus, most of the transposed elements represented as being 0 cM from the T-DNA in Figure 7 are likely to be close to the T-DNA. Sequence analysis of the flanking DNA of one these events showed that the element had actually transposed from the untranslated leader region of the excision marker to another region of the T-DNA, just downstream from the NPT gene, a distance of ~2.5 kb (data not shown). Because the 45 TC_1F_1 seedlings that gave rise to the progeny analyzed for the genetic distance of transposition were originally selected for expression of the excision marker, the screen was biased toward recovering linked transpositions and half of the unlinked events would not have been recovered. Even when this bias is taken into account, it can be predicted that two thirds of germinal transpositions in these lines were to sites close to the T-DNA.

Sequence analysis of inverse PCR clones derived from transposed *Ds* elements: Inverse PCR was used to amplify and clone the genomic sequences flanking transposed *Ds* elements. Clones were obtained for both sides of three germinal transposed *Ds* elements, for the 5′ side only of four others and for the 3′ side of another four stable transposed elements. The termini of these elements and the flanking tomato DNA were sequenced

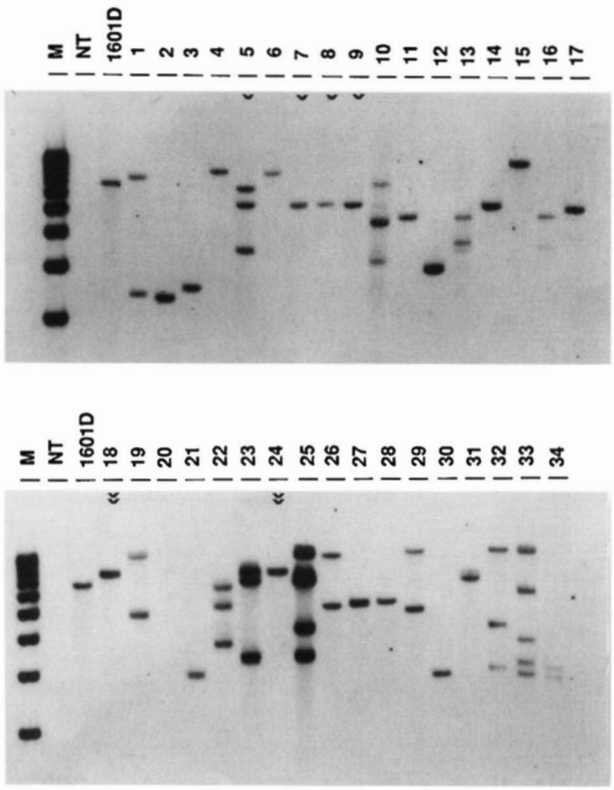

FIGURE 5.—Southern hybridization analysis of stable transposition events in pooled sibling progeny. TC_1F_1 pooled progeny was derived from the pollen of four plants, each heterozygous for both the 1601D and 10512 I T-DNAs. Genomic DNA of nontransformed, the progenitor 1601D and TC_1F_1 individuals carrying stable transposed *Ds* elements was digested with *Eco*RV, electrophoresed, blotted and probed with the 3′ 1100 bp of *Ac* (see Figure 1). Lane M, marker DNA (1-kb ladder, BRL, smallest marker fragments shown in the top and bottom panels are ~4 and 3 kb, respectively); lanes NT and 1601D, nontransformed and progenitor 1601D, respectively; lanes 1–34, TC_1F_1 individuals. Analysis with separate enzymes confirmed that the plants represented in lanes 5 and 7–9 (highlighted with arrow heads) carried a clonal transposed element, and another two individuals, corresponding to lanes 18 and 24 (highlighted with double arrow heads), possessed a separate clonal event.

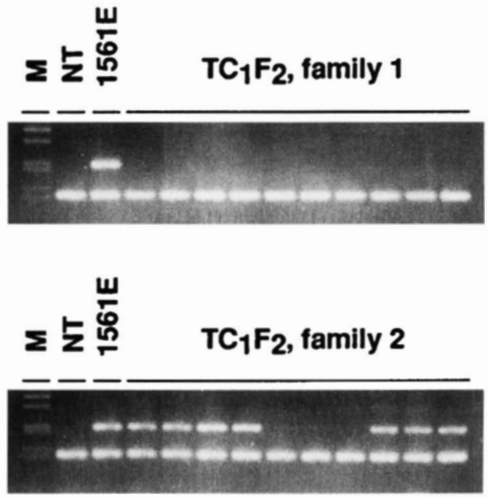

FIGURE 6.—PCR analysis for the detection of *Ds* elements and estimation of the genetic distance of transposition. TC_1F_1 individuals carrying the T-DNA and a single transposed *Ds* were selfed to produce TC_1F_2 progenies that were plated on kanamycin to identify individuals that lacked the T-DNA (~25% of the progeny). For each family, 10 kanamycin-sensitive individuals (representing 20 gametes) were subjected to PCR analysis for detection of the transposed *Ds*. Lane M, marker DNA (1-kb ladder, BRL); lanes NT and 1561E, nontransformed and progenitor 1561E, respectively. Upper and lower panels, markers, controls and PCR analysis on 10 kanamycin-sensitive individuals from TC_1F_2 families 1 and 2, respectively, both derived from 1561E. The smaller fragment is an internal control and represents a sequence on chromosome 11 of tomato and the larger fragment is only amplified when the *Ds* elements is present in the genome. For further details see text. None of the 10 kanamycin-sensitive individuals from family 1 analyzed by PCR inherited the transposed *Ds*, thereby indicating close linkage of the element and the T-DNA. In contrast, about three quarters of kanamycin-sensitive individuals carried the *Ds* in family 2, demonstrating that in this case transposition was to an unlinked site.

(Figure 8) and showed that in all cases transposition did not result in deletion of the transposon termini, which is consistent with previous reports on *Ac* transposition in heterologous species (HEHL and BAKER 1990; OSBORNE *et al.* 1991). Of the three events where the tomato sequences flanking both sides of the element were cloned and sequenced, two showed the typical 8-bp duplication of the target sequence. The other characterized event had a 7-bp duplication, but this sequence was separated from the 5′ end of the element by 6 bp and by 2 bp at the 3′ end (Figure 8).

DISCUSSION

An assessment of the germinal transposition frequency of *Ac* in tomato has not been reported for tomato, mainly because a suitable cell-autonomous excision marker has not been identified to distinguish somatic from germinal excision events (JONES *et al.* 1993). We have now addressed this problem by using a two-component system of *Ds* and *sAc*, because the transposase gene can be introduced for a single generation and germinal transposition events can be analyzed in progeny lacking *sAc*.

There are differences and similarities between *Ds* germinal transposition in tomato and maize. Germinal excision frequencies are on average very high in tomato (15–40%; Tables 1 and 2), compared with 10% or less in maize (BRINK and NILAN 1952; DOONER and BELACHEW 1989). The reinsertion frequency after excision was similar in maize (MCCLINTOCK 1956; GREENBLATT 1968; DOONER and BELACHEW 1989) and tomato (30–70%; Table 2), and sequence analysis of the termini of transposed *Ds* elements and the flanking DNA indicated that the transposition mechanism was not discernibly different in tomato. The typical 8-bp duplication of the

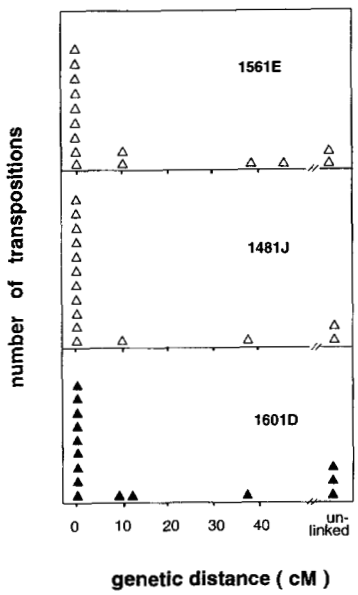

FIGURE 7.—The genetic distance of germinal transposition from three T-DNA loci. Fifteen individuals for each *Ds* line were identified that were heterozygous for the T-DNA and a single stable transposed *Ds* element. These were selfed and the progeny analyzed for recombination between the T-DNA (monitored by screening for kanamycin sensitivity) and the transposed *Ds* (monitored by using PCR). The location of each event is not precise because generally only 10 individuals (20 gametes) were assessed for recombination. Those events depicted as 0 cM from the T-DNA showed no recombinants in the 20 gametes that were analyzed. For further details see MATERIALS AND METHODS, RESULTS and Figure 6.

target sequence (POHLMAN *et al.* 1984) was observed in two of the three germinally transposed *Ds* elements characterized here (Figure 8), and in four out of six transpositions of *Ac* reported elsewhere (OSBORNE et al. 1991). We sequenced the 5′ end of seven transposed *Ds* elements and the 3′ end of eight transposed *Ds* elements and in all cases the integrity of the terminal sequences was maintained after transposition (Figure 8), as was usually the case for *Ac* transpositions (OSBORNE et al. 1991).

The tendency of *Ac* to transpose to genetically linked sites in maize (GREENBLATT 1984; DOONER and BELACHEW 1989), tobacco (JONES *et al.* 1990) and Arabidopsis (KELLER *et al.* 1993) is also strongly conserved in tomato (Figure 7). This pattern of germinal transposition of nonautonomous *Ds* elements from three T-DNA loci was in contrast to the pattern of dispersed clusters of insertion sites observed for the autonomous *Ac* element in somatic shoot tissue (OSBORNE *et al.* 1991; BELZILE and YODER 1992). There may be an inherent difference in transposition pattern between somatic and germline tissue, but an alternative and more likely explanation is that *Ac* is more active and secondary or even higher order transpositions are more common in somatic than in germline tissue. The pattern of transposition studied here was based on lines that inherited a single transposed *Ds* element and as such we may have enriched for primary or lower order transposition events.

TABLE 3

Probability of recovering no recombinants between two loci in a sample size of 10

Real genetic distance (cM)	Expected proportion of recombinants	P_0 (n = 10)
0	0	1
5	0.10	0.35
10	0.20	0.11
17	0.30	0.03

The probability of registering no recombinants (P_0) and the expected proportions of recombinants are listed for several genetic distances between the loci. P_0 was calculated as described in the text.

Although the average germinal excision frequency is much higher in tomato than in maize, the timing of germinal transposition in both species can vary considerably between plants and between different parts of the same plant (Table 1) (GREENBLATT 1984; DOONER and BELACHEW 1989). In some cases, high germinal excision frequencies resulted from early transposition events and gave rise to many sibling progeny, each carrying the same transposed *Ds* element (Table 1, Figure 4). However, our data show that provided sufficient gametes from sufficient plants are deployed, independent transpositions can constitute the majority of events (Figure 5). The fact that clonal transposition events can be detected among sibling progeny (Figures 4 and 5) is important for the prospects of tagging genes because it indicates that the element often discontinues transposition after the initial transposition. In some cases, however, transposition continues and gives rise to multiple transposed elements in individual plants, consistent with transposition of *Ac* from replicated to unreplicated DNA (GREENBLATT 1984; DOONER and BELACHEW 1989; CHEN *et al.* 1992).

In conclusion, we can assess the merits and limitations of this two-component s*Ac*/*Ds* system for tagging genes in tomato. *Ds* elements excise and reinsert frequently and efficiently, predominantly to linked sites. The prospects of tagging a gene at a known location should be enhanced if the transposon is linked to the target locus. For tagging experiments, it will be important to maximize the number of parent plants contributing to a population to be screened for tagged genes, to maximize the proportion of progeny screened that carry unique events. The actual number of plants required to generate a tagging population with the s*Ac*/*Ds*-based system described here is difficult to predict and will depend on the distance between the starting position of the transposon and the target locus. Despite some clonal events, unique transposition events were

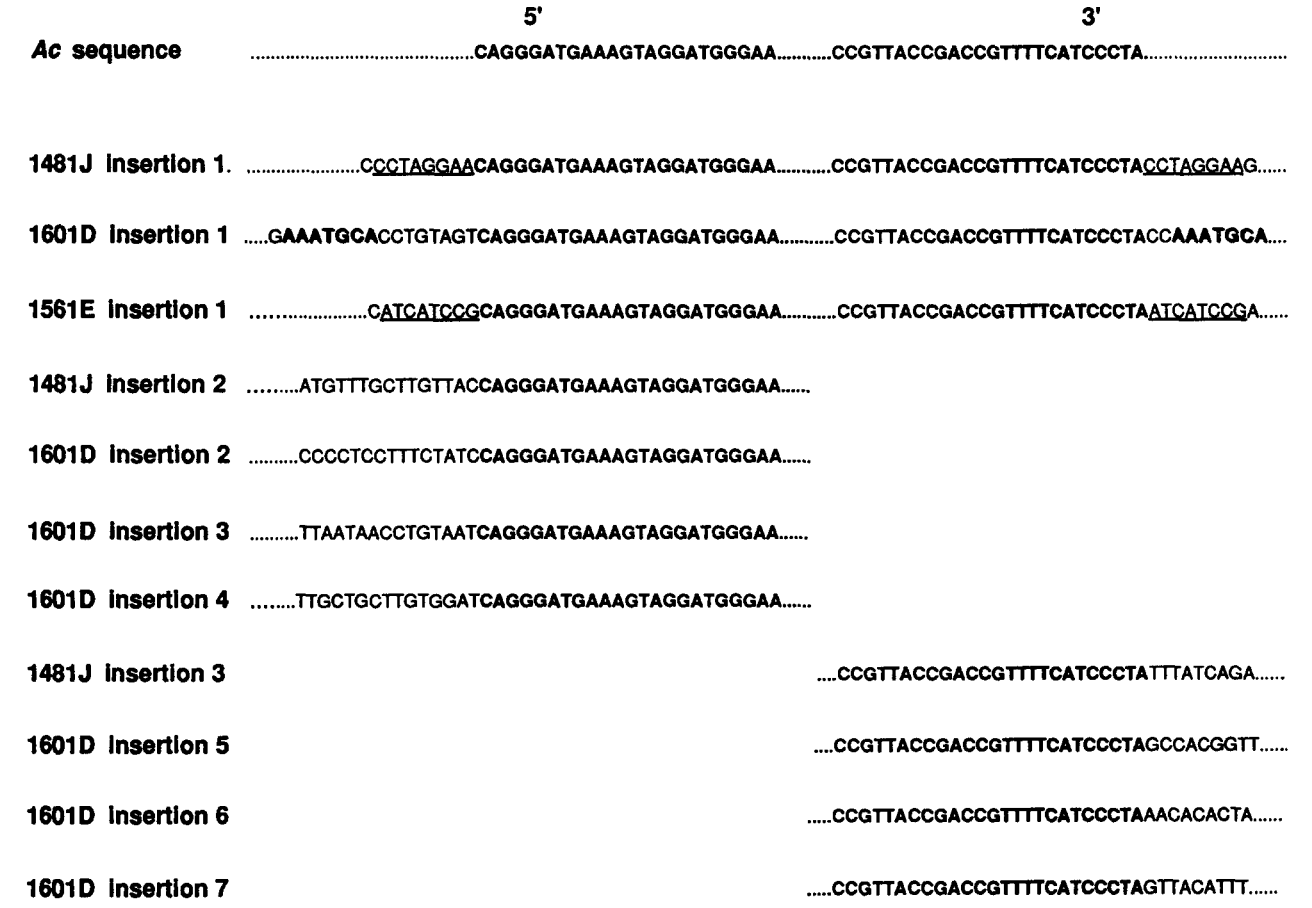

FIGURE 8.—Sequence analysis of IPCR clones derived from transposed *Ds* elements. The *Ac* sequence at the 5′ and 3′ ends of the element is shown and the 8-bp duplication of the target sequence for 1481J-insertion 1 and 1561E-insertion 1 are underlined. In 1601D-insertion 1 there was a 7-bp duplication of sequence in the vicinity of the insertion site (which is shadowed in the figure).

the majority of events in the sample sizes reported here (Figures 4 and 5) and for closely linked tagging (within 5 cM) it is very likely that a large enough population could be generated from 30 parent plants.

As a future refinement, alternative promoters could be fused to the *Ac* transposase gene to allow expression just before or concurrent with gametogenesis, thus increasing the frequency of unique transposition events. The heritable inactivation of the plant selectable marker inside the *Ds*, although interesting, precludes tagging strategies that require selection for reinsertion of the transposon. The inactivated marker genes described here were either *nos:BAR* or *nos:SPEC*. Gene fusions with a stronger promoter, such as *35S*, might not be silenced and could permit selection for the transposed *Ds*. There are now several examples of tagging genes in heterologous species by using the maize *Ac/Ds* transposon system (AARTS *et al.* 1993; BANCROFT *et al.* 1993; CHUCK *et al.* 1993). With the improvements outlined above, there are good prospects for efficient tagging of genes in tomato with a two-component *Ds/sAc* system.

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APPENDIX: PLASMID CONSTRUCTIONS

The plasmid SLJ1471 carrying *Ds(SPEC)* was made as follows. The *nos* promoter was fused to the *SPEC* coding sequence by recombining a plasmid carrying an *Xho*I site in the *nos* 5′ leader, with plasmid SLJ6B1 (carrying a *35S:SPEC* fusion) to make SLJ411 carrying a *nos:SPEC* fusion (JONES *et al.* 1992). This plasmid was digested with *Xho*I and *Sst*I, treated with T4 DNA polymerase and dNTPs and recircularized to remove these sites in the 5′ leader to facilitate future manipulations. The *ocs*

3′ end was replaced with a *nos* 3′ end by exchanging fragments with SLJ4K1 (JONES *et al.* 1992) between the *Bam*HI and *Hin*dIII sites. The resulting plasmid was partially digested with *Bcl*I (avoiding a *Bcl*I site within the *SPEC* gene), treated with DNA polymerase I (Klenow fragment) in the presence of dGTP and dATP and fully digested with *Hin*dIII. The resulting fragment was ligated to DNA of the *Ac* carrying plasmid SLJ7C3 (JONES *et al.* 1992) that had been cut with *Xho*I, treated with DNA polymerase I (Klenow fragment) in the presence of dTTP and dCTP and then cut with *Hin*dIII to make plasmid SLJ1452 that carried a *nos:SPEC* gene in a *Ds*. The yeast *lys-2* gene was excised from plasmid YEP620 (BARNES and THORNER 1986) as a *Hin*dIII / *Eco*RI fragment in which the *Eco*RI site had been blunt ended using Klenow and dNTPs. This fragment was ligated to SLJ1452 DNA that had been cut with *Nru*I and *Hin*dIII. The *Nru*I/*Eco*RI (blunt) ligation restored the *Eco*RI site. The oligonucleotide carrying *Not*I, *Dpn*I(*Cla*I) and *HO* sites carried *Hin*dIII compatible ends and was ligated to *Hin*dIII cut DNA of the resulting plasmid to make SLJ1471.

A plasmid SLJ155J carrying *Ds(BAR)* was made as follows. A *nos:BAR* fusion was made in a manner analagous to the *nos:SPEC* fusion, using SLJ2011 (JONES *et al.* 1992) rather than SLJ6B1. Then the *Sst*I and *Xho*I sites were removed, and using *Bcl*I partial digestion followed by treatment with DNA polymerase I (Klenow fragment) in the presence of dNTPs and complete digestion with *Hin*dIII, the *nos:BAR* fusion was ligated into SLJ7C3 that had been cut with *Xho*I and half filled in and subsequently cut with *Hin*dIII. Again, the yeast *lys-2* gene was inserted as a *Hin*dIII/ *Eco*RI (blunt) fragment to *Ds(nos:BAR)* that had been cut with *Hin*dIII and *Nru*I. At this stage, the *ocs* 3′ end of the *BAR* gene was replaced (after partial *Bam*HI digestion then *Hin*dIII digestion) with a *nos* 3′ end confined to a digested *Bam*HI/*Hin*dIII fragment. The resulting plasmid was cut with *Hin*dIII and ligated to an annealed oligonucleotide carrying the *Not*I, *HO* and *Dpn*I(*Cla*I) sites, giving rise to SLJ155J.

The T-DNAs of the binary vectors, SLJ1561, SLJ1481 and SLJ1601, used in these studies are shown in Figure 1. All of these were derived from plasmid SLJ491 carrying a *35S:NPT* gene divergently transcribed from the *A. tumefaciens 2′* promoter (JONES *et al.* 1992). Plasmid SLJ561 (JONES *et al.* 1993) carries a *nos:SPEC* gene convergently transcribed with the *35S:NPT* gene. A unique *Cla*I site in the 2′ promoter 5′ untranslated region provided a location to clone in the *iaaH* gene, engineered to carry *Cla*I sites at the translational initiation site and also at a location 3′ to the *iaaH* poly adenylation sequence (BANCROFT *et al.* 1992). This *Cla*I site was used to insert the *iaaH* ClaI fragment, making plasmid SLJ954. The resulting plasmid carries a unique *Hin*dIII site, into which was cloned the *Not*I, *HO, Dpn*I(*Cla*I) oligonucleotide, making plasmid SLJ99C4. This plasmid *(2′:iaaH, 35S :NPT, nos :SPEC)* carries in the 5′ untranslated leader of the *nos:SPEC* gene, unique *Sst*I and *Xho*I sites, into which was cloned a *Sst*I /*Sal*I fragment derived from SLJ155J, to make SLJ1561.

A plasmid SLJ543, containing convergently transcribed *35S:NPT* and *35S:BAR* genes, was made by ligating SLJ2011 (cut with *Bgl*II and *Hin*dIII) to SLJ491 (cut with *Bam*HI and *Hin*dIII) (JONES *et al.* 1992). This also carries a *Cla*I site that enabled a *Cla*I fragment carrying *iaaH* to be inserted, bringing the *iaaH* gene under the control of the *2′* promoter. The resulting plasmid was digested at a unique *Hin*dIII site and ligated to the *Not*I, *HO, Dpn*I(*Cla*I) oligonucleotide. This plasmid carries unique *Sst*I and *Xho*I sites in the 5′ untranslated leader of the *35S:BAR* gene, and these were used to insert a *Sst*I/*Sal*I fragment from SLJ1471 to make SLJ1481.

SLJ99C4 carries unique *Bam*HI and *Xho*I sites flanking the *SPEC* gene. SLJ99C4 DNA was digested with these enzymes, and the *SPEC* reading frame was replaced with the *BAR* reading frame, brought in from plasmid SLJ2011 on a *Bam*HI/*Xho*I fragment. The resulting plasmid carries a T-DNA of the form *(2′:iaaH, 35S: NPT, nos :BAR)*, and again, the *nos :BAR* gene carries unique *Sst*I and *Xho*I sites, into which the *Ds (SPEC)* of SLJ1471 was introduced as a *Sst*I/*Sal*I fragment to make SLJ1601.