Linked and Unlinked Transposition of a Genetically Marked *Dissociation* Element in Transgenic Tomato

J. Healy,^{*,1} C. Corr,[†] J. DeYoung[†] and B. Baker^{*,†,2}

^{*}Department of Plant Pathology, University of California, Berkeley, California 94720, and [†]USDA-ARS Plant Gene Expression Center, Albany, California 94710

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ABSTRACT

We have introduced a genetically marked *Dissociation* transposable element (*Ds^{neo}*) into tomato. In the presence of *Ac* transposase, *Ds^{neo}* excised from an integrated T-DNA and reinserted at numerous new sites in the tomato genome. The marker genes of *Ds^{neo}* (NPTII) and the T-DNA (HPT) facilitated identification of plants bearing transposon excisions and insertions. To explore the feasibility of gene tagging strategies in tomato using *Ds^{neo}*, we examined the genomic distribution of *Ds^{neo}* receptor sites, relative to the location of the donor T-DNA locus. Restriction fragment length polymorphism mapping of transposed *Ds^{neo}* elements was conducted in two tomato families, derived from independent primary transformants each bearing *Ds^{neo}* within a T-DNA at a unique position in the genome. Transposition of *Ds^{neo}* generated clusters of insertions that were positioned on several different tomato chromosomes. *Ds^{neo}* insertions were often located on the same chromosome as the T-DNA donor site. However, no insertion showed tight linkage to the T-DNA. We consider the frequency and distance of *Ds^{neo}* transposition observed in tomato to be well suited for transposon mutagenesis. Our study made use of a novel, stable allele of *Ac* (*Ac3*) that we discovered in transgenic tomato. We determined that the *Ac3* element bears a deletion of the outermost 5 base pairs of the 5'-terminal inverted repeat. Though incapable of transposition itself, *Ac3* retained the ability to mobilize *Ds^{neo}*. We conclude that a dual element system, composed of the stable *Ac3* *trans*-activator in combination with *Ds^{neo}*, is an effective tool for transposon tagging experiments in tomato.

THE *Activator* and *Dissociation* controlling elements of maize have been studied extensively [reviewed in DORING and STARLINGER (1986) and FEDOROFF (1989)], since their discovery by MCCLINTOCK (1948, 1950). *Activator* (*Ac*) transposes autonomously through the action of an element-encoded transposase. Sequences coding for transposase comprise the bulk of the 4.6-kb transposon (MULLER-NEUMANN, YODER and STARLINGER 1984; POHLMAN, FEDOROFF and MESSING 1984). Deletion studies have delineated regions of only about 200 bp at each terminus of *Ac* that are required in *cis* for transposition (COUPLAND *et al.* 1988, 1989). Transposase binds *in vitro* at subterminal sequences within these regions, while the extreme 5'- and 3'-terminal sequences include short, imperfect inverted repeats (KUNZE and STARLINGER 1989). Nonautonomous *Dissociation* (*Ds*) elements are often derivatives of *Ac* which bear internal deletions that eliminate transposase function. Though a diverse group, *Ds* elements retain sequences that are required in *cis* for transposition. Incapable of transposition alone, *Ds* elements are mobilized in the

presence of transposase supplied in *trans* by an accompanying *Ac*.

Several maize genes have been isolated using *Ac* or *Ds* as insertional mutagens (FEDOROFF, FURTEK and NELSON 1984; CHEN, GREENBLATT and DELLAPORTA 1987; THERES, SCHEELE and STARLINGER 1987; HAKE, VOLLBRECHT and FREELING 1989). Recently, much emphasis has been placed on introducing maize transposable elements into heterologous plant species which lack characterized transposons (BAKER *et al.* 1986; VAN SLUYS, TEMPE and FEDOROFF 1987; YODER *et al.* 1988; JONES *et al.* 1989; HOUBA-HÉRIN *et al.* 1990). The ultimate goal of such studies is to use *Ac* or *Ds* as molecular tags that will permit isolation of plant genes involved in biologically important processes. In transgenic plants, certain salient aspects of *Ac* transposition in maize are retained (HEHL and BAKER 1990; OSBORNE *et al.* 1991; BELZILE and YODER 1992). These include: (i) conservation of *Ac* structural integrity following transposition, (ii) insertion into hypomethylated DNA and generation of an 8-base pair duplication at the site of insertion, and (iii) continued activity over several generations. Unlike the situation in maize, however, increased *Ac* copy number in transgenic plants is associated with an increase, rather than a decrease, in transposition fre-

¹ Present address: Department of Bioengineering, Nagaoka University of Technology, 1603-1 Kamitomioka-Machi, Nagaoka-Shi, Niigata 940-21, Japan.

² To whom correspondence should be addressed.

quency (HEHL and BAKER 1990; JONES *et al.* 1990).

In maize, *Ac* often transposes to sites that are genetically linked to its original location. In genetic studies of transposition from the *P* and *bz-m2* loci, roughly 50% of transposed *Ac* elements showed tight linkage to the donor site of transposition; while the remaining 50% of transposed *Ac* elements showed no linkage to the donor site (GREENBLATT 1984; DOONER and BELACHEW 1989). This predictable tendency toward linked transposition has been incorporated into the design of gene tagging strategies in maize [reviewed in SHEPHERD (1988)]. Similarly, knowledge concerning transposition distance is essential to the design and implementation of transposon tagging schemes in heterologous plants in which *Ac* or *Ds* are deployed. In experiments carried out on *Ac* in tobacco and in tomato, the percentage of linked transposition, as judged by transposition of *Ac* from integrated T-DNA loci, resembled that seen in maize (JONES *et al.* 1990; DOONER *et al.* 1991; OSBORNE *et al.* 1991). Pronounced clustering of *Ac* receptor sites in close proximity to donor loci is a common pattern of *Ac* transposition observed thus far in heterologous plants.

Two component transposon mutagenesis systems, consisting of a stable *Ac* *trans*-activator paired with a *cis*-responsive *Ds* element, have been established in a variety of heterologous species, including tobacco, tomato, and, more recently, *Arabidopsis* (HEHL and BAKER 1989; LASSNER, PALYS and YODER 1989; MASTERSON *et al.* 1989; SCOFIELD *et al.* 1992; SWINBURNE *et al.* 1992). A two-element system holds several advantages over the use of *Ac* alone. Primarily, separation of the transposase and mutator components affords much greater control over the transposition process. Use of a stable *Ac* *trans*-activator permits genetic segregation of *Ac* and *Ds*, and therefore, recovery of stable *Ds* insertions. The stability of *Ds*, in the absence of transposase, makes *Ds* highly desirable as a gene tag. Furthermore, dual element approaches accommodate the use of engineered versions of *Ds* that carry selectable or screenable genetic markers in place of transposase coding sequences. Such marker genes allow for easy identification of plants which harbor *Ds* insertions and facilitate monitoring of cosegregation between a putative insertion allele and a mutant phenotype.

Though the *Ac/Ds* genetic system of maize is apparently applicable to diverse plant species, a thorough understanding of the behavior of *Ds* (or engineered versions of *Ds*) in transgenic situations is a prerequisite to its effective use as an insertional mutagen. Characterization of *Ds* transposition frequency and distance in heterologous plants has received far less attention than corresponding parameters of *Ac* transposition. No studies in transgenic plants, and few in maize, have addressed the issue of *Ds* transposition

distance. Knowledge concerning this parameter is central to the success of gene tagging schemes utilizing *Ds*. For example, if *Ds* transposes with equal probability to all genomic sites, then a *Ds* element positioned at any location in the genome would serve as a suitable donor for insertional mutagenesis of a target gene of interest. Alternatively, if a high percentage of *Ds* transposition events are to linked receptor sites, then it follows that the probability of obtaining an insertion in the target would be increased significantly by first identifying *Ds* at a donor site linked to the gene of interest. Limited data available from maize indicate that *Ds* is capable of transposing to linked sites. One study, of fully colored revertants generated by excision of *Ds* from the *r-sc::m3* allele of the *R* locus, is consistent with linked transposition (KERMICLE, ALLEMAN and DELLAPORTA 1989). When *Ac* was used to *trans*-activate *Ds* in these revertants, five of the nine revertant lines showed new insertions of *Ds* back into the *R* locus. Thus, it was inferred that the five revertants must have contained transposed *Ds* elements linked to *R*. Similarly, two *Ds*s have been identified in maize which transposed to sites closely linked to their starting point in the *bronze* locus (DOWE, ROMAN and KLEIN 1990).

In tomato, *trans*-activation of *Ds* has been investigated thus far using both a naturally occurring element from maize (*Ds1*), and an *in vitro* modified element marked with a bacterial *lacZ* gene (LASSNER, PALYS and YODER 1989). Though excision of *Ds1* occurred in the F₁ progeny of crosses between plants containing *Ds1* and plants expressing transposase, no reintegration was detected. However, subsequent examination of F₂ progeny derived by selfing of an F₁ individual revealed several *Ds1* insertions at new locations. Transposition of the marked *Ds* element was found to be much less efficient than that of *Ds1*. Only one transposition event was detected in twenty F₂ plants.

Here we report on several aspects of the behavior of a genetically marked *Ds* element, *Ds^{neo}*, in transgenic tomato. Our *Ds* element carries a selectable kanamycin-resistance gene (NPTII), which facilitates identification of plants bearing transposition events. In two independent tomato families, we demonstrate that *Ds^{neo}* excises from a T-DNA locus, in the presence of transposase supplied by an *Ac* *trans*-activator, and reintegrates frequently at new genomic sites. As an extension of our assessment of *Ds^{neo}* activity, we also investigated the genomic distribution of *Ds^{neo}* receptor sites in each of our tomato families. Our strategy was to map the locations of the donor site of transposition in each family, that is the T-DNA, as well as the locations of transposed copies of *Ds^{neo}*. This approach exploits the existence of a detailed RFLP map of tomato (BERNATZKY and TANKSLEY 1986; TANKSLEY

et al. 1989, 1992), as well as established procedures for the isolation of genomic regions flanking transposable element insertions and assignment of these flanking DNAs to the restriction fragment length polymorphism (RFLP) map (EARP, LOWE and BAKER 1990; OSBORNE *et al.* 1991). Our results demonstrate both linked and unlinked transposition of *Ds^{neo}*.

Finally, we describe the isolation and characterization of a stable derivative of *Ac* (*Ac3*), that was identified in a tomato line harboring wild-type *Ac*. The *Ac3* allele, which bears a mutation in the 5'-terminal inverted repeat sequence, serves as an effective *trans*-activator of *Ds^{neo}* transposition. Taken together, our studies lay the ground work for future transposon mutagenesis experiments in tomato.

MATERIALS AND METHODS

Tomato transformation and selection conditions: Agrobacterium-mediated transformation of *Lycopersicon esculentum* cv. VF36 with plasmid pBH2 was carried out according to a published procedure (McCORMICK *et al.* 1986). pBH2 was built by insertion of *Ds^{neo}* into the binary Ti-plasmid vector, pDW-Hph, at a unique *Sma*I site in the untranslated leader region of a hybrid hygromycin phosphotransferase gene, *P_{Nos}-HPT*. In construction of *Ds^{neo}*, the *Hind*III interval of *Ac* was deleted and a chimeric neomycin phosphotransferase II gene, *P_{Nos}-NPTII* was inserted at the *Xho*I site. Figure 1 shows *Ds^{neo}* positioned within the *P_{Nos}-HPT* gene fusion, as well as the uninterrupted *P_{Nos}-HPT* gene generated by *Ds^{neo}* excision. Transformants A90 and A106 were selected with kanamycin at 100 µg/ml. Excision of *Ds^{neo}* from the *P_{Nos}-HPT* gene construct was selected with hygromycin at 10 µg/ml. Generation of *Ac* transformant A61 by transformation of VF36 with plasmid pGV3850HPT::pKU3 has been described previously (OSBORNE *et al.* 1991).

Activation of *Ds^{neo}* transposition: *Ds^{neo}* transposition frequency and distance were assessed in two independent tomato families: family 90, *Ac* (A61) × *Ds^{neo}* (A90); and family 106, *Ac3* (D42-1) × *Ds^{neo}* (A106) (Figure 2, A and B). To generate family 90, *Ac* transformant A61 was used as the female parent in crosses to *Ds^{neo}* transformant, A90. To enrich for transposition events, *F₁* seedlings resistant to hygromycin and kanamycin were selected. One such seedling, B60-2, was the *F₁* progenitor of family 90. B60-2 carried, in addition to *Ds^{neo}*, transposed *Ac*s, but did not inherit the T-DNA of the *Ac* (A61) parent. *F₂* progeny of B60-2, C205-2 to C205-30, were derived by selfing. Similarly, selfing of C205-6 to C205-30 yielded *F₃* progeny. In the *F₃*, selections for kanamycin resistance identified individuals containing transposed *Ds^{neo}*. *F₂* plants C205-14 and C205-18 and their *F₃* progeny contained *Ac* elements whereas C205-6, C205-21, C205-29, C205-30 and their *F₃* progeny were void of *Ac* (data not shown). To generate family 106, *R₃* D42-1, an individual bearing stable *Ac3* (but no other *Ac*-hybridizing sequences detectable by Southern blotting) was used as the female parent in crosses to *R₂* C217-1 of *Ds*-line 106. The stable *Ac3* allele arose in self progeny of *Ac* line 61. *F₁* seedlings were subjected to selection with hygromycin and kanamycin. Similarly, *R₄* E11-2, another member of *Ac* line 61 bearing only stable *Ac2*, served as the female parent in separate crosses to *R₂* C218-1 of *Ds^{neo}* line 106.

Southern blot analysis: Plant genomic DNA was prepared from leaf tissue as previously described (BERNATZKY and TANKSLEY 1986). Restriction enzyme-digested DNA (approximately 10 µg per lane) was electrophoresed on agarose gels (0.8%) and blotted to nitrocellulose filters. Inverse polymerase chain reaction (IPCR) products or restriction fragments were gel-purified using NA45 paper (Schleicher and Schuell), and radioactively labeled by random priming according to the supplier's specifications (Amersham). Hybridizations were carried out as reported previously (HEHL and BAKER 1990). The T-DNA right border probe was a 650-bp *Pst*I-*Bam*HI fragment of the HPT gene, while *Ds^{neo}* excision and reintegration was assessed with a 1.3-kb *Eco*RI-*Bam*HI fragment containing the *Nos* promoter.

Cloning of T-DNA flanking regions by IPCR: IPCR reactions were performed using 2.5 µg of tomato genomic DNA. IPCR product #90 was obtained from DNA of *R₃* C205-2 (line 90), while #106 was obtained from DNA of *R₂* B91-1 (line 106). Templates were prepared by digestion of genomic DNAs with *Taq*I, followed by phenol extraction and ligation as described previously (OSBORNE *et al.* 1991). Ligated DNAs were heated briefly to 65°, then cleaved with *Asp*718 and precipitated with 0.1 volume of 3 M NaAc and two volumes of ethanol. Precipitated DNAs were resuspended in 100 µl of PCR buffer (Promega). Reactions were carried out using 2 units of *Taq* DNA polymerase (Promega) in a Cetus Thermal Cycler according to the following parameters: 1 min/94°, 2 min/55°, 3 min/72°, 35 cycles. Reactions included 1.0 µg each of oligonucleotide primers that were complementary to sequences in the T-DNA right border: 5'-CAACAAGTCACGGATATTTCG-3' (positions 459-478) and 5'-AACACGCACACTTACGATAG-3' (positions 330-311). IPCR products were cloned into either *Sma*I-cut pUC19, or *Eco*RV-cut pKR (provided by J. JONES).

Cloning of *Ds^{neo}* flanking regions by IPCR: Plant DNAs of families 90 and 106 from which IPCR products were obtained, are listed in Table 1. Templates for IPCR were prepared as described above, except that genomic DNAs were digested with *Pst*I prior to the ligation step, and *Acc*I was used for linearization. Primers were designed to incorporate *Eco*RI or *Bam*HI sites. 5' flanking DNAs were amplified with primer, 5'-CACGGATCCATACGATAACGGTCCGTACGGGA-3' (position 48 to position 26 of *Ds^{neo}*), and a *P_{Nos}-NPTII* gene primer, 5'-CAGGAATTCGTGATCTCGATCGACCAAG-3' (position 1943 to position 1962 of *Ds^{neo}*). Similarly, 3'-flanking DNAs were amplified with primer, 5'-CAGGAATTCGGTTTTCGTTTCCGTC CCGCAAG-3' (position 5238 to position 5260 of *Ds^{neo}*), and *P_{Nos}-NPTII* gene primer, 5'-CACGGATCCCCAACAGACAATCGGCTGCTC-3' (position 3722 to position 3703 of *Ds^{neo}*). IPCR products were digested with *Eco*RI and *Bam*HI, and cloned into *Eco*RI- and *Bam*HI-cleaved pUC19.

Construction of a λ EMBL4 library: Pooled genomic DNAs from 18 *F₃* progeny of family 90 (Table 1) were partially digested with *Mbo*I as described (MANIATIS, FRITSCH and SAMBROOK 1982). Restriction fragments in a 10-20 kb size range were isolated from agarose gels and ligated into purified λ EMBL4 arms that had been cleaved with *Bam*HI and *Sal*I (FRISCHAUF *et al.* 1983). Ligations were packaged according to the manufacturer's protocol (Promega) and bacteriophage were plated on *Escherichia coli* host Q358 (MANIATIS, FRITSCH and SAMBROOK 1982). Duplicate filters from a library of approximately 5 × 10⁵ recombinants were screened differentially using a *Nos* promoter fragment, or an HPT gene fragment as hybridization probes. HPT-hybridization was used as a basis to eliminate from our study *Nos*-hybridizing clones that consisted of *Ds^{neo}*

"in place" in the T-DNA. Three positive clones were identified which hybridized to the P_{Nos} probe, but not to the HPT probe. Purified λ DNAs were digested with *Eco*RI and *Hind*III, and analyzed by Southern blotting using the 5' end of *Ac* as a probe. Hybridizing fragments, containing the junction between genomic DNA and *Ds*^{neo}, were subcloned into pUC19.

RFLP mapping of flanking DNAs: T-DNA and *Ds*^{neo} flanking regions (Table 1) were assigned to the tomato RFLP map essentially as described (OSBORNE *et al.* 1991). Flanking DNAs were hybridized to enzyme survey filters bearing *L. esculentum* cv. VF36 and *Lycopersicon pennellii* genomic DNAs digested with each of six different restriction enzymes: *Bst*NI, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hind*III. Enzymes that produced polymorphic restriction fragments between the two species were used to digest genomic DNAs of 56 F₂ progeny of an *L. esculentum* cv. VFNT $\times$ *L. pennellii* hybrid (BERNATZKY and TANKSLEY 1986). Southern blots of digested F₂ DNAs were hybridized with flanking probes. Hybridization data were analyzed, as described, using the computer program, MapMaker (LANDER *et al.* 1987), and a database consisting of 76 RFLP markers distributed over the 12 tomato chromosomes (provided by S. TANKSLEY).

Statistical analysis of insertion site distribution: Statistical analysis of data from family 90 was performed using a multinomial distribution (FELLER 1957). We began by assuming that each *Ds*^{neo} insertion is made randomly and has an equal likelihood of being found at any given location. By this definition, the insertions are uniformly distributed independent random variables. First, we determined the probability of finding sets of insertions arranged on chromosomes as shown in Figure 7: three insertions on chromosome 11 (101 cM), two insertions on chromosome 9 (109 cM), two insertions on chromosome 6 (118 cM), and two solo insertions on different chromosomes. The total length between the most distal markers in our RFLP database, 1260 cM, represented the combined length of all tomato chromosomes. Next, we calculated the probability with which insertions on each chromosome were found in clusters. A cluster was defined as a set of insertions in which each insertion was positioned within 20 cM of at least one other insertion in the set. Thus, for the array of insertions of Figure 7, $P = 0.0009\%$.

For statistical analysis of data from family 106 (Figure 8), we again assumed that *Ds*^{neo} is equally likely to insert anywhere in the tomato genome. Using a binomial distribution (FELLER 1957), we then computed the probability of finding three out of five insertions on chromosome 8 (the chromosome on which T-DNA 106 also mapped). We used 114/1260, the ratio of the length of chromosome 8 (114 cM) to the summed length of all 12 tomato chromosomes (1260 cM), and $n = 5$ for the total number of *Ds*^{neo} insertions. Thus, for the data of Figure 8, $P = 0.7\%$.

Cloning and characterization of stable *Ac* termini: Sequences flanking *Ac*2 and *Ac*3 were isolated by IPCR from *Eco*RI-digested genomic DNAs of R₂ C201-17 and R₂ C203-29, respectively, each a member of *Ac* line 61 containing a single stable *Ac* element. Following linearization of the template DNA with *Hind*III, amplification of *Ac* 5' and 3' ends was performed using primers designed to include *Pst*I and *Bgl*II sites. Primer pairs for the 5' end of stable *Ac* were as follows: 5'-CACCTGCAGAGATCTTACGATAACGGTCGGTACGG-3' (position 47 to position 28 of *Ac*), and 5'-CACCTGCAGAGATCTGATCTCATATGATGTCTCAA-3' (position 2456 to position 2475 of *Ac*). Primer pairs for the 3' end were as follows: 5'-CACCTGCAGAGATCTTTACCGACCGTTACCGACCG-3' (position 4531 to position 4550 of *Ac*), and 5'-CACCTGCAGAGATCTGC

FIGURE 1.—Restriction map of *Ds*^{neo} at its donor location in the T-DNA, and map of the predicted empty donor fragment following excision. Panel A depicts *Ds*^{neo} that is "in place" in the T-DNA, while panel B depicts the uninterrupted P_{Nos}-HPT gene created by excision of *Ds*^{neo}. Open boxes showing positions of the NPTII and HPT marker genes are indicated. Speckled regions represent *Ds* sequences. 5' and 3' termini of *Ds*^{neo} are indicated by filled triangles. Hatched boxes represent the Nos promoter. The thin line depicts T-DNA left (LB) and right (RB) borders. Filled rectangles delimit junctions between T-DNA borders and flanking genomic DNA. The hatched box below each map represents the P_{Nos} probe, while the open box below the map of panel A represents the HPT probe. Numbers refer to sizes of *Eco*RI-*Pst*I restriction fragments, in kilobase pairs (kb), detected by P_{Nos} or HPT probes. E, *Eco*RI; P, *Pst*I, H, *Hind*III.

AGGCTTATAATATAAGGC-3' (position 2531 to position 2511 of *Ac*). IPCR products were cloned into *Pst*I-digested pUC19. Dideoxynucleotide sequencing was carried out using a commercially available kit (U.S. Biochemical Corp.).

As means to verify sequencing results obtained with IPCR-generated DNAs, we sequenced portions of PCR products consisting of about 350 bp at each terminus of *Ac*3 plus 50 bp of flanking genomic DNA. PCR products were generated using a primer directed against genomic sequence (determined by sequencing of IPCR products) in combination with an internal *Ac* primer. These primer pairs incorporated *Eco*RI and *Bam*HI sites to facilitate cloning. The 5' end of *Ac*3 was amplified with primer, 5'-CATGGATCCC GTGTGAATGTGTGATGCT-3' (position 376 to position 358 of *Ac*), and flanking primer, 5'-CACGAATTCCTGTTAATTGTTGAAC-3'. The 3' end of *Ac*3 was amplified with primer, 5'-CATGGATCCGCTAGTAGAC TTATATGGC-3' (position 4186 to position 4204 of *Ac*), and flanking primer 5'-CACGAATTCCTTTGCTGGCC ATG-3'. PCR products were digested with *Eco*RI and *Bam*HI, and cloned into *Eco*RI- and *Bam*HI-cleaved pUC19 prior to nucleotide sequencing.

RESULTS

Characterization of tomato transformants bearing a marked *Ds* element within a T-DNA: A *Ds* element carrying a chimeric, P_{Nos}-NPTII, kanamycin resistance (Kn^R) gene was introduced into tomato cultivar VF36 by *Agrobacterium*-mediated transformation using plasmid pBH2 (McCORMICK *et al.* 1986). The marked *Ds* (*Ds*^{neo}) of pBH2 is positioned within the untranslated leader of a gene fusion (P_{Nos}-HPT), that confers hygromycin resistance (Hyg^R) only upon excision of the element (Figure 1).

FIGURE 2.—Derivation of plants used for RFLP mapping of *Ds^{neo}* insertion loci. Crossing schemes by which *Ac/Ds^{neo}* family 90 (panel A) and *Ac3/Ds^{neo}* family 106 (panel B) were generated are shown. Seedlings were selected for Hyg^R/Kn^R or Kn^R as described in MATERIALS AND METHODS. Plants from which genomic DNAs flanking *Ds^{neo}* and T-DNA were isolated by IPCR are indicated in bold type.

Kanamycin selection yielded several independent primary (R_0) transformants, two of which, A90 and A106, were judged to carry single T-DNA loci. Kn^R segregated roughly 3:1 (102 Kn^R :35 Kn^S) in self-progeny of A106. Furthermore, Southern blot analysis of A106 DNA, using a fragment of the HPT gene as a T-DNA right border probe, detected a unique 5.8-kb *Hind*III fragment containing the junction between the T-DNA and tomato genomic DNA (not shown). A90 was shown to bear a single T-DNA locus by similar criteria. Hybridization of A90 DNA with the T-DNA right border probe revealed a single 8.0-kb *Eco*RI-*Pst*II junction fragment (not shown). Southern blot analysis of restriction enzyme-digested DNA isolated from R_0 lines using *Ds*, NPTII, HPT, and P_{Nos} fragments as hybridization probes, indicated that no gross structural rearrangements of *Ds* nor of its NPTII marker had accompanied transformation (not shown).

We examined the frequency and distance of *Ds^{neo}* transposition in two independent tomato families, 90 and 106, derived from A90 and A106. These families were produced by crossing each *Ds^{neo}*-bearing line, to a plant containing either an autonomous or a stable *Ac trans*-activator (Figure 2, A and B).

FIGURE 3.—Southern blot analysis of *Ds^{neo}* transposition from T-DNA 90 and from T-DNA 106. *Eco*RI-*Pst*II digests of genomic DNAs were hybridized to a P_{Nos} probe. P_{Nos} -hybridizing fragments of 2.7 and 2.0 kb are diagnostic of *Ds^{neo}* that is “in place” within the T-DNA. Excision of *Ds^{neo}* yields a 1.1-kb empty donor fragment. P_{Nos} -hybridizing fragments of other sizes represent transposed copies of *Ds^{neo}*. (A) *Ds^{neo}* transposition in F_1 , F_2 , and F_3 progeny of *Ac/Ds^{neo}* family 90. DNAs from R_0 (lane a, A90); F_1 (lane b, B60-2); F_2 (lane c, C205-14); and four F_3 (lane d, D32-1; lane e, D32-2; lane f, D32-4; lane g, D32-7) individuals are shown. (B) *Ds^{neo}* transposition in F_1 progeny of *Ac/Ds^{neo}* family 106. DNAs from line 106 (lane a, R_2 C217-1) and from four *Ac3* $\times$ 106 F_1 progeny (lane d, D48-1; lane e, D48-2; lane f, D48-3; lane g, D48-7) are shown. Lanes b and c show *Ds^{neo}* transposition in two F_1 offspring of an *Ac2* $\times$ 106 cross (lane b, D47-2; lane c, D47-10). Restriction fragment sizes in kb are indicated.

***Ds^{neo}* transposition from T-DNA 90 is trans-activated by autonomous *Ac*:** In the presence of *Ac*, *Ds^{neo}* excised from its original location in the T-DNA of line 90 and reintegrated at numerous new sites in the tomato genome. Moreover, *Ds^{neo}* continued to transpose, in plants containing *Ac*, for at least three generations.

Figure 3A depicts a Southern blot analysis of *Ds^{neo}* transposition in F_1 , F_2 and F_3 progeny generated from a cross between line 90 and a plant harboring an autonomous *Ac* element (see Figure 2A). Selection for Hyg^R and Kn^R allowed us to identify F_1 seedlings in which transposition of *Ds^{neo}* to new genomic sites had occurred. F_2 and F_3 offspring were generated by selfing of F_1 and F_2 individuals, respectively, followed by selection for Kn^R in the F_3 . The P_{Nos} fragment used as a probe (Figure 1) detects two fragments (2.0 and 2.7 kb) in *Eco*RI- and *Pst*II-digested genomic DNA that are diagnostic of *Ds^{neo}* that is “in place” within the T-DNA of line 90 (Figure 3A, lane a). In an F_1 individual bearing an *Ac trans*-activator (lane b), P_{Nos} also detects a 1.1-kb empty donor fragment created by *Ds^{neo}* excision from the T-DNA. The empty donor fragment is absent in the self F_2 and F_3 offspring shown in lanes c–g, as these plants did not inherit the T-DNA from their F_1 parent. Additional P_{Nos} -hybridizing fragments

detected in F₁, F₂, and F₃ progeny denote transposed *Ds^{neo}* elements (Figure 3A, lanes b–g).

Interestingly, little or no “in place” *Ds^{neo}* was seen in the F₁ progenitor of family 90 (Figure 3A, lane b), indicating that excision of *Ds^{neo}* must have occurred at an early stage in the development of this plant. Furthermore, new P_{Nos}-hybridizing bands, representing reintegrated copies of *Ds^{neo}* were visible in the F₁ parent (lane b). *Ds^{neo}* continued to transpose in the F₂ generation (Figure 3A, lane c), and novel *Ds^{neo}* insertions, absent in the F₂, were observed in F₃ progeny (lanes d–g). The copy number of transposed *Ds^{neo}* in members of family 90 ranged from one to about four insertions per plant.

***Ds^{neo}* transposition from T-DNA 106 is trans-activated by stable *Ac* derivatives:** Two stable *Ac* elements, *Ac2* and *Ac3*, each effectively activated transposition of *Ds^{neo}* from the T-DNA of line 106 to new locations in the tomato genome. Isolation of these mutant *Ac* alleles and further characterization of the *Ac3* trans-activator, which bears a small 5′ terminal deletion, are described below.

The Southern blot analysis of Figure 3B, lanes b–g, shows several Hyg^R and Kn^R F₁ progeny of crosses between line 106 (lane a) and plants bearing either *Ac2* or *Ac3* (see Figure 2B). As in Figure 3A, P_{Nos} detects fragments specifying *Ds^{neo}* that is “in place” in the T-DNA of line 106, transposed *Ds^{neo}*, as well as the empty donor site. In most individuals, some *Ds^{neo}* remained “in place” (2.0- and 2.7-kb P_{Nos}-hybridizing fragments). However, in every F₁ individual examined, somatic excision of *Ds^{neo}* was signaled by the presence of the 1.1-kb P_{Nos}-hybridizing empty donor fragment. The 1.1-kb excision band was detected in Figure 3B, lane c with longer exposures of the autoradiogram. Furthermore, three of the four F₁ progeny of *Ac3* and 106 parents contained new integrations of *Ds^{neo}* (Figure 3B, lanes d–f). Approximately one to two new *Ds^{neo}* insertions were detected per plant. In one F₁ plant, substantial excision of *Ds^{neo}* from the T-DNA occurred (lane g), though no reintegrated copies of *Ds^{neo}* were detected with the P_{Nos} probe. Insertion of *Ds^{neo}* at new locations was also evident in two F₁ offspring of *Ac2* and 106 parents (Figure 3B, lanes b and c).

Isolation and characterization of stable *Ac* elements: The trans-activator, *Ac3*, that we employed to mobilize *Ds^{neo}* in line 106, is a stable derivative of *Ac*. Both *Ac3*, and a second stable, yet trans-active element, *Ac2*, were identified in tomato transformants bearing otherwise active *Acs*.

The *Ac3* and *Ac2* elements were detected initially by Southern blot analysis of progeny derived from *Ac* transformant A61. Using an internal fragment of *Ac* as a probe, discrete pairs of *Ac*-hybridizing restriction fragments, each corresponding to a single transposed

FIGURE 4.—Southern blot analysis of tomato transformants harboring *Ac2* and *Ac3*. The internal 1.6-kb *HindIII* fragment of *Ac* was used as a probe on *EcoRI* digested genomic DNAs of *Ac2* (lane a, C201-17) and *Ac3* (lane b, C203-29) bearing plants. Each stable element is represented by a different pair of *Ac*-hybridizing fragments. No other *Ac*-hybridizing sequences were detected in these individuals or in their selfed progeny (not shown). Restriction fragment sizes in kb are indicated.

FIGURE 5.—Nucleotide sequence of 5′ and 3′ ends of *Ac3* and adjacent genomic DNA. The termini of *Ac3* are underlined. The upper underlined sequence shows the five base pairs (CAGGG, positions 1–5) which are deleted from the 5′-inverted repeat of *Ac3*.

element, were observed in *EcoRI*-digested genomic DNAs from certain individuals of this line (Figure 4). Each *Ac* had apparently transposed successfully from the T-DNA donor locus of line 61 to a new genomic site and, strikingly, had come to represent the only *Ac*-hybridizing sequence detectable by Southern blotting. In an examination spanning three generations of tomato, of progeny derived from plants in which each putative stable *Ac* was originally identified, we detected no further transposition of either *Ac* element by Southern hybridization. That is, no new *Ac*-hybridizing bands appeared in 97 progeny containing only the *Ac3* allele, or 25 progeny containing only the *Ac2* allele (not shown). As illustrated in Figure 3B, *Ac3* and *Ac2* each activate transposition of *Ds^{neo}*. Thus, each stable *Ac* element encodes a functional transposase.

To identify the lesion responsible for the stability of the *Ac3* trans-activator, we isolated its 5′ and 3′ termini using an IPCR-based strategy (see MATERIALS AND METHODS). DNA sequencing of IPCR products revealed a deletion of 5 bp (CAGGG, positions 1–5) in the 5′-terminal inverted repeat of *Ac3* (Figure 5). Presence of this 5-bp deletion was confirmed by sequencing of PCR products, containing both ends of

Ac3, which were amplified from genomic DNA using genomic and internal *Ac* primers. Inspection of 124 bp at the 5' end, and 164 bp at the 3' end of *Ac3* revealed no other departure from the published *Ac* sequence. Notably however, sequences flanking the 5' and 3' termini of *Ac3* did not include the 8-bp duplication of target DNA characteristic of *Ac* integration (Figure 5). Interestingly, the *Ac3* allele segregated independently of the T-DNA 61 locus from which it originated (not shown). This observation suggests that the element transposed to an unlinked receptor site prior to the deletion event. Using genomic flanking DNA as a molecular probe, we determined that the stable *Ac3* element maps to chromosome 2 of tomato, between RFLP markers TG48 and TG140 (not shown).

The lesion rendering *Ac2* immobile has not yet been identified. However, examination of an IPCR product generated from the 5' end of the *Ac2* allele revealed no alterations in its 5'-terminal inverted repeat sequence.

Experimental strategy for investigation of *Ds^{neo}* transposition distance: We investigated patterns of *Ds^{neo}* transposition in each of two independent tomato families described above: family 90, *Ac* × *Ds^{neo}* (A90), and family 106, *Ac3* × *Ds^{neo}* (A106) (Figure 2). Our strategy was to determine both the location of the starting point of transposition, that is, the single T-DNA locus of each family, as well as the locations of transposed copies of *Ds^{neo}*. Toward this end, we isolated genomic sequences flanking the T-DNA and transposed copies of *Ds^{neo}*, and assigned flanking DNAs to positions on the tomato RFLP map. In family 90 we mapped transposed *Ds^{neo}* elements isolated from self F₂ and F₃ progeny descended from a single F₁ individual. This approach allowed for continued transposition over several generations, and for recovery of insertions present in different progeny that shared common lineages. In contrast, in family 106 we mapped transposed *Ds^{neo}* elements isolated from several F₁ individuals, reasoning that insertions present in different F₁ progeny could bear no relationship to one another.

Isolation of genomic sequences flanking transposed *Ds^{neo}* elements: Southern blot analyses, similar to those displayed in Figure 3, established that our *Ac* trans-activators caused *Ds^{neo}* to transpose from its "in place" position in the T-DNAs of lines 90 and 106, to new locations in the tomato genome. To identify these *Ds^{neo}* receptor sites, we isolated tomato genomic DNA flanking transposed elements. Cloning of flanking regions was accomplished using IPCR and by construction of a bacteriophage λ library. Table 1 lists pertinent characteristics of IPCR and λ clones. Sequences flanking the 5' or 3' termini of *Ds^{neo}* insertions were recovered by IPCR, as described in MATERIALS AND

TABLE 1
List of flanking probes

Marker	T-DNA or <i>Ds</i>	Plant lineage	Chromo- some	Flanking region (kb)	Isolation method
Family 90					
#90	T-DNA	R ₃ C205-2	9	1.8	IPCR
#5	<i>Ds</i>	F ₂ C205-5	11	0.35	IPCR
#15	<i>Ds</i>	F ₂ C205-6	1	0.75	IPCR
#301	<i>Ds</i>	F ₃ D30-1	1	0.75	IPCR
#321	<i>Ds</i>	F ₃ D32-1	9	1.4	IPCR
#322	<i>Ds</i>	F ₃ D32-2	11	1.0	IPCR
#324	<i>Ds</i>	F ₃ D32-4	11	2.5	IPCR
#327	<i>Ds</i>	F ₃ D32-7	9	0.6	IPCR
#361	<i>Ds</i>	F ₃ D36-1	11	2.5	IPCR
#1	<i>Ds</i>	— ^a	6	2.4	λ
#10	<i>Ds</i>	— ^a	6	5.0	λ
#11	<i>Ds</i>	— ^a	2	6.8	λ
Family 106					
#106	T-DNA	R ₂ B91-1	8	0.5	IPCR
#31	<i>Ds</i>	F ₁ D48-3	8	1.2	IPCR
#32	<i>Ds</i>	F ₁ D48-3	2	0.25	IPCR
#71	<i>Ds</i>	F ₁ D48-7	8	0.8	IPCR
#72	<i>Ds</i>	F ₁ D48-7	1	0.3	IPCR
#91	<i>Ds</i>	F ₁ D48-9	8	0.4	IPCR

^a The bacteriophage λ library was constructed from pooled genomic DNAs of 18 F₃ progeny of family 90: D30-1, D30-4, D30-5, D32-1, D32-2, D32-3, D32-4, D32-5, D32-7, D32-9, D32-11, D34-1, D34-2, D34-3, D35-1, D36-1, D36-5, D37-1. As a result, plant lineages of flanking DNAs isolated by λ cloning cannot be reconstructed.

METHODS. Oligonucleotide primer pairs were chosen to amplify only regions flanking *Ds^{neo}* termini. *Ac* flanking regions were not amplified, since one primer of each pair anneals to sites present in the P_{Nos}-NPTII marker gene of *Ds^{neo}*.

In family 90, regions flanking the 3' end of *Ds^{neo}* were isolated from two F₂ and six F₃ plants, all derived from the same F₁ parent (Figure 2 and Table 1). IPCR products were expected to include an interval of tomato genomic DNA bounded at each end by a short stretch of *Ds* sequence. Nucleotide sequencing allowed us to verify that IPCR-generated DNAs conformed to this expected structure (not shown). In addition, sequencing of genomic flanking regions enabled us to determine whether IPCR products that were obtained in separate reactions, especially products of similar size, actually comprised different *Ds^{neo}* insertions. For example, similar size products (#301 and #15) were recovered from an F₃ individual (D30-1), as well as from its F₂ parent (C205-6, Table 1). Indeed, nucleotide sequencing of the flanking DNAs proved that they were identical to one another. Therefore, we inferred that this insertion arose in or prior to the F₂ generation and was germinally transmitted. Identical products (#324 and #361) were also obtained from two F₃ individuals (D32-4 and D36-1). Thus, these products comprise a single, inherited transposition event rather than two different events.

In total, six unique IPCR products, each representing a distinct *Ds^{neo}* insertion site, were obtained from *Ac/Ds^{neo}* family 90 (Table 1). Nucleotide sequences of genomic regions flanking the 3' ends of several of these *Ds^{neo}* insertions are displayed in APPENDIX 1.

Genomic regions flanking three additional transposed *Ds^{neo}* elements in family 90 were isolated from a bacteriophage λ library constructed from pooled F₃ progeny DNAs (see Table 1 and MATERIALS AND METHODS). These λ clones (#1, #10, and #11) hybridized to a P_{Nos} probe, but not to an HPT gene probe (which detects *Ds^{neo}* that is "in place" within the T-DNA). Southern analysis of purified λ DNAs using a 5' *Ac* probe identified fragments containing the junction between genomic DNA and transposed *Ds^{neo}* (Table 1).

In family 106, regions flanking the 5' end of *Ds^{neo}* were isolated from three F₁ siblings (Figure 2 and Table 1). Two different IPCR products (#31 and #32) were recovered from a single F₁ plant (D48-3). A second F₁ individual (D48-7), also yielded two IPCR products (#71 and #72). In total, five IPCR products, each representing a distinct *Ds^{neo}* insertion, were obtained from *Ac3/Ds^{neo}* family 106 (Table 1). APPENDIX 1 shows *Ds^{neo}* 5' ends and genomic sequences flanking several of these insertions. No sequence alterations in the 5' or 3' termini of transposed *Ds^{neo}* elements were detected in flanking DNAs isolated from either family 90 or family 106 (APPENDIX 1).

IPCR products or λ clones served as hybridization probes with which to assign *Ds^{neo}* insertions in each family to positions on the tomato RFLP map (see MATERIALS AND METHODS and later in RESULTS).

Isolation of genomic sequences flanking T-DNA loci: The integrated T-DNAs of transformants A90 and A106 each served as starting points for *Ds^{neo}* transposition in our studies. Genomic regions flanking the T-DNA locus of line 90 and of line 106 were isolated by IPCR using oligonucleotide primers specific for the T-DNA right border (see MATERIALS AND METHODS). Flanking regions were expected to contain T-DNA right border sequence, as well as adjacent genomic DNA. We confirmed the identities of IPCR products #90 and #106 by Southern blotting (Figure 6). Products generated by IPCR hybridized both to the T-DNA right borders of A90 and A106 and to unique genomic restriction fragments.

When used as a hybridization probe on *Hind*III-digested genomic DNA from progeny of transformant A90, IPCR product #90 detected a 16-kb T-DNA border fragment and an additional 12-kb fragment (corresponding to the wild-type copy of the locus into which the T-DNA integrated) in an individual that was hemizygous for the T-DNA (Figure 6A, lane a). In contrast, an individual that was homozygous for the T-DNA (lane b) contained only the 16-kb border

FIGURE 6.—IPCR products #90 and #106 hybridize to the T-DNA right border fragments of A90 and 106, and flanking genomic DNA. (A) IPCR product #90 was used as a hybridization probe on *Hind*III digested genomic DNAs from three selfed R₃ progeny (D25-1, D25-2 and D25-3) descended from R₀ A90 (lanes a–c, respectively). (B) IPCR product #106 was used as a hybridization probe on *Hind*III digested genomic DNAs of tomato cultivar VF36 (lane a) or R₀ A106 (lane b). Restriction fragment sizes in kb are indicated.

fragment, while an individual in which the T-DNA was absent contained only the 12-kb genomic fragment (lane c). The IPCR #90 probe also detected the same 8.0-kb T-DNA border fragment in an *Eco*RI-*Pst*I digest of A90 DNA, as a T-DNA right border probe (not shown). Similarly, when used as a hybridization probe on *Hind*III-digested genomic DNA from untransformed tomato VF36 and from transformant A106, IPCR product #106 detected the expected 5.8-kb fragment corresponding to the T-DNA border fragment (Figure 6B, lane b). In addition, the probe detected a 2.8-kb genomic fragment also present in VF36 DNA (lane a).

IPCR products #90 and #106 were utilized as hybridization probes to place the T-DNA loci of A90 and A106 on the RFLP map of tomato (described below and in MATERIALS AND METHODS).

RFLP map positions of flanking regions: We assigned IPCR or λ clones from each family, corresponding to regions flanking the T-DNA and transposed copies of *Ds^{neo}*, to positions on the RFLP map. Our method involved the use of a tomato F₂ mapping population, and a database of RFLP markers (both gifts of S. TANKSLEY) in combination with the MapMaker computer program (LANDER *et al.* 1987) (see MATERIALS AND METHODS). As reported previously for *Ac* (OSBORNE *et al.* 1991; BELZILE and YODER 1992), nearly all *Ds^{neo}* target sites in tomato were single copy genomic DNA sequences (not shown).

Figure 7 illustrates the distribution of *Ds^{neo}* receptor sites and the location of the integrated T-DNA of *Ac/Ds^{neo}* family 90. Nine sequences flanking *Ds^{neo}* insertions were assigned to the RFLP map. These insertions were recovered from self F₂ and F₃ progeny derived from a single F₁ individual. Since *Ds^{neo}* remained active in tomato for several generations, we

FIGURE 7.—RFLP map positions of the T-DNA and transposed *Ds^{neo}* elements in *Ac/Ds^{neo}* family 90. The open arrow indicates the position of the single T-DNA locus in family 90, while filled arrows indicate positions of transposed copies of *Ds^{neo}* on a partial RFLP map of tomato. Brackets denote the maximum separation between loci in centimorgans (cM). RFLP markers are indicated beside hatch marks on numbered tomato chromosomes. The figure shows 5 of the 12 tomato chromosomes. For clarity, the 3-cM cluster of three insertions on chromosome 11 is not drawn to scale.

anticipated that each transposed copy of *Ds^{neo}* would serve as a new donor site for further transposition events. Here, we observed both linked and unlinked transposition of *Ds^{neo}*. Receptor sites were distributed over 5 of the 12 tomato chromosomes. However, most insertions were grouped in small clusters (less than 20 cM), composed of two to three elements each, on just three chromosomes. One cluster was loosely linked to T-DNA 90 on chromosome 9.

To determine whether the clustered pattern of *Ds^{neo}* receptor sites seen in family 90 could arise by random transposition, we calculated the probability of finding the array of insertions depicted in Figure 7 using a multinomial distribution coupled with cluster analysis (see MATERIALS AND METHODS). Each *Ds^{neo}* did not insert at all genomic sites with equal probability. If each *Ds^{neo}* insertion site were determined according to a uniform distribution, the probability that the arrangement of insertions seen in family 90 would arise by chance is only 0.000009, or less than 1 in 10^5 trials.

Figure 8 depicts the distribution of *Ds^{neo}* receptor sites and the location of the integrated T-DNA of

Ac3/Ds^{neo} family 106. Five flanking DNAs, obtained from three different F_1 siblings, were placed on the RFLP map. Transposed copies of *Ds^{neo}* were located on 3 of the 12 tomato chromosomes. No *Ds^{neo}* element within linkage distance, that is, less than 40 cM, of the T-DNA was identified. However, three of the five insertions mapped to the same chromosome as T-DNA 106 (chromosome 8). Strikingly, each of the three transposed *Ds^{neo}* elements located on chromosome 8 (#31, #71 and #91), originated in a different F_1 individual. Thus, these insertions must have arisen independently of one another.

Though no insertion tightly linked to T-DNA 106 was identified, we thought it significant that so large a fraction of the insertions isolated in this family mapped to the same chromosome as the transposition donor site. We calculated the probability of finding three out of five *Ds^{neo}* insertion sites on chromosome 8 (Figure 8) using a binomial distribution (see MATERIALS AND METHODS). If *Ds^{neo}* had an equal likelihood of inserting anywhere in the genome, the probability that this arrangement would occur by chance is only 0.007, or less than 1 in 100 trials.

FIGURE 8.—RFLP map positions of the T-DNA and transposed Ds^{neo} elements in $Ac3/Ds^{neo}$ family 106. The open arrow indicates the position of the single T-DNA locus in family 106, while filled arrows indicate positions of transposed copies of Ds^{neo} on a partial RFLP map of tomato. RFLP markers are indicated beside hatch marks on numbered tomato chromosomes. The figure shows 3 of the 12 tomato chromosomes.

DISCUSSION

We have examined transposition in tomato of a genetically marked Ds element, Ds^{neo} , in response to transposase supplied by either an autonomous Ac element, or by a novel, stable derivative, $Ac3$. In the presence of each Ac *trans*-activator, Ds^{neo} excised from an integrated T-DNA and reinserted at new locations in the tomato genome.

Ds^{neo} transposes frequently in tomato: We studied the behavior of Ds^{neo} for several generations in progeny of Ac and Ds^{neo} parents (family 90). Ds^{neo} remained active and continued to transpose, in the presence of Ac , for at least three generations of tomato. The excision marker of the T-DNA (HPT) and the marker gene of Ds^{neo} (NPTII) allowed us to enrich for F_1 progeny carrying transposition events by selecting simultaneously for excision (Hyg^R) and reintegration (Kn^R). Of 32 F_2 plants of family 90 examined by Southern blotting (a sample of this analysis is shown in Figure 3A), 29 (91%) contained transposed copies of Ds^{neo} . Typically, between one and four Ds^{neo} transposition events were scored per plant. Ds^{neo} insertions visualized by Southern hybridization in Figure 3A were indicative of somatic transposition since Ac was present in these individuals. However, using genomic DNAs flanking Ds^{neo} , isolated from four Ac containing F_3 plants (D32-1, D32-2, D32-4, D32-7; Table 1), as

hybridization probes, we demonstrated that three of these Ds^{neo} insertions (D32-1, D32-2, D32-4) were germinally transmitted to F_4 progeny (not shown). Thus, a sizeable proportion of transposition events must have occurred in cell lineages which eventually differentiated into either male or female gametophytes. In other work, not presented here, we have examined inheritance of Ds^{neo} in self F_3 progeny which did not inherit Ac from their F_2 parent. By definition, transposed Ds^{neo} elements present in progeny lacking Ac must have arisen in the parent and been germinally transmitted. In such self F_3 offspring, we generally detect between one and four germinally transmitted transposed copies of Ds^{neo} . Increases in Ds^{neo} copy number, over the single copy present in the T-DNA of the parent, may be attributed to Ds^{neo} excision from one recently replicated daughter chromatid and insertion into an as yet unreplicated chromosomal region (GREENBLATT and BRINK 1963).

We confined our analysis of Ds^{neo} transposition to a single generation in progeny of $Ac3$ and Ds^{neo} parents (family 106). The immobile $Ac3$ element, which we discovered in an Ac line of tomato, functioned as an effective *trans*-activator of Ds^{neo} . As in family 90, we subjected F_1 progeny of family 106 to selection for Hyg^R and Kn^R . Somatic excision of Ds^{neo} from the T-DNA was detected in seven out of nine F_1 offspring (an example of this analysis is shown in Figure 3B). Here, we observed between one and two new integrations of Ds^{neo} per plant. Apparently, transposed copies of Ds^{neo} were often represented in relatively large sectors of leaf tissue in these F_1 plants, facilitating their detection by Southern analysis. However, in two individuals (one of which is shown in Figure 3B, lane g), we detected excision of Ds^{neo} from the T-DNA, but saw no evidence of reintegration. The failure to detect transposed Ds^{neo} elements in these instances can likely be explained by transposition very late in development, resulting in a low representation of any given insertion. We favor this explanation, since DNA of the plant shown in Figure 3B, lane g contained transposed copies of Ds^{neo} that were detectable by the IPCR method (Table 1). Alternatively, we may have sampled mitotic sectors of plant tissue in which Ds^{neo} had segregated away from the T-DNA, following transposition to a sister chromatid.

RFLP mapping of Ds^{neo} insertion loci in two tomato families: We extended our characterization of Ds^{neo} transposition in tomato to include an analysis of the genomic distribution of Ds^{neo} insertion sites. Regions flanking transposed copies of Ds^{neo} were used as DNA markers to assign insertions to the tomato RFLP map. We mapped the locations of 14 transposed Ds^{neo} elements in two independently derived families, each bearing an integrated T-DNA at a unique position in the genome (Table 1). Thus, transposed copies of

Ds^{neo} originated from a different donor site in each family, which we defined by assignment of T-DNA flanking regions to the RFLP map. By examining the pattern of *Ds^{neo}* transposition in two different families, we hoped to account for position effects on donor loci that could potentially distort the activity or transposition distance of *Ds^{neo}*.

Preferential insertion of *Ds^{neo}* on the same chromosome as the T-DNA donor locus: In family 106, we isolated transposed *Ds^{neo}* elements from three F₁ offspring. Insertions that arose in different F₁ siblings necessarily represented independent, unrelated transposition events. This approach is analogous, though smaller in scope, to studies of *Ac* transposition distance carried out in maize. Maize experiments determined the fraction of transposed *Ac* elements, each the product of a distinct excision event, that showed linkage to single donor loci (GREENBLATT 1984; DOONER and BELACHEW 1989). In these maize studies, however, positions of unlinked *Ac*s were not determined precisely. We, on the other hand, determined precise RFLP map positions for all *Ds^{neo}* insertion loci regardless of their linkage status with respect to the T-DNA.

In family 106, transposed copies of *Ds^{neo}* were distributed over 3 of the 12 tomato chromosomes (Figure 8). Strikingly, three out of five insertions were located on the same chromosome as the T-DNA 106 locus. Moreover, each of the three insertions on chromosome 8 was obtained from a different F₁ individual. Statistical tests showed that this arrangement was unlikely to occur by chance ($P = 0.007$), if *Ds^{neo}* transposed randomly in the tomato genome. Therefore, we consider that the probability of recovering *Ds^{neo}* insertions on a given chromosome is increased significantly by positioning of the T-DNA donor locus on the same chromosome. Though the distribution of *Ds^{neo}* elements depicted in Figure 8 is unlikely to have arisen by random transposition, the small number of insertions mapped in family 106 precludes a meaningful statistical estimate of the frequency with which a transposed element will fall on the same chromosome as the donor locus.

Surprisingly however, the transposition distance of *Ds^{neo}* in family 106 was greater than that expected from earlier studies of linked *Ac* transposition. In maize, approximately 50% of *Ac* receptor sites displayed tight linkage to the donor loci, *P*, and *bz-m2* (GREENBLATT 1984; DOONER and BELACHEW 1989). In contrast, all of the *Ds^{neo}* receptor sites on chromosome eight were positioned greater than 40 cM from the T-DNA donor locus of line 106. Additionally, in cases where two insertions were obtained from the same F₁ plant (#31 and #32, or #71 and #72), no linkage between receptor sites was observed.

Linked and unlinked transposition of *Ds^{neo}*: In family 90, we isolated transposed *Ds^{neo}* elements from

self F₂ and F₃ progeny, all descended from the same F₁ parent. This approach provided an opportunity to isolate insertions that arose over several generations, some of which could share common lineages. For example, we predicted that linked transposition of *Ds^{neo}* at a frequency comparable to that reported previously for *Ac* (roughly 50%) in tomato, would yield clustered *Ds^{neo}* insertions at sites that are both linked and unlinked to the T-DNA (OSBORNE *et al.* 1991; BELZILE and YODER 1992). Clusters of insertions in proximity to the T-DNA could presumably arise by linked primary transposition, while clusters at locations that are unlinked to the T-DNA could arise by primary transposition from the T-DNA to unlinked loci, followed by secondary linked transposition in the vicinity of new insertion sites. As a corollary of this model, insertions that belong to a cluster are likely to be related to one another.

Like patterns of *Ac* transposition seen previously in tomato (OSBORNE *et al.* 1991; BELZILE and YODER 1992), the RFLP map of insertion sites in family 90 (Figure 7) illustrated both linked and unlinked transposition of *Ds^{neo}*. The most striking feature of this array was the arrangement of insertions in small clusters on three of the 12 tomato chromosomes. Significantly, seven of the nine mapped insertions showed linkage to at least one other insertion, though, only one insertion site was genetically linked to the donor locus, T-DNA 90. Our statistical analysis showed that the clustered pattern of receptor sites seen in family 90 was highly unlikely to arise by chance if *Ds^{neo}* transposed randomly in the tomato genome ($P = 0.000009$). Nor can the observed distributions of clusters be explained adequately by a mechanism in which *Ds^{neo}* transposed exclusively to sites linked to its starting point. A 100% linked mode of transposition would be expected to produce a single cluster of *Ds^{neo}* receptor sites in close proximity to the T-DNA. The data of Figure 7, in which *Ds^{neo}* transposition generated clusters of insertions at different positions in the tomato genome, can best be explained by a model involving an intermediate percentage of linked transposition.

A common feature of *Ac* transposition seen thus far in maize, tobacco, and tomato is a pronounced clustering of receptor sites that are tightly linked to the transposition donor locus (GREENBLATT 1984; DOONER and BELACHEW 1989; JONES *et al.* 1990; DOONER *et al.* 1991; OSBORNE *et al.* 1991). In this respect, the distributions of *Ds^{neo}* insertions seen here differed from those reported previously for *Ac* in maize and heterologous plants. We saw little evidence of primary linked transposition of *Ds^{neo}* in close proximity to the T-DNA donor site. In family 90, two *Ds^{neo}* insertions (#321 and #327) were clustered together (within 17 cM of one another) on the same chromo-

some as the T-DNA. However, only one member of that cluster (#321) showed genetic linkage to the T-DNA. This linkage was loose: insertion #321 was positioned just under 40 cM from the donor site. Furthermore, although we recovered several insertions located on the same chromosome as the T-DNA in family 106, none was within linkage distance of the donor site. The clustered pattern of *Ds^{neo}* insertions depicted in Figure 7 is consistent with a model of predominantly unlinked primary transposition from the T-DNA, followed by linked secondary transposition around new receptor sites.

Recovery of so few *Ds^{neo}* insertions linked to the T-DNA may reflect a substantive difference between modes of transposition employed by *Ac* and our marked *Ds^{neo}* element. It is formally possible that *Ac* sequences removed in construction of *Ds^{neo}* may somehow facilitate transposition to sites that are linked to the T-DNA donor locus. We consider this possibility unlikely, however, since we readily detected transposed *Ds^{neo}* elements that were linked to one another. Moreover, a recent study of somatic *Ac* transposition in tomato showed a distribution of *Ac* receptor sites similar to that reported here for *Ds^{neo}* (BELZILE and YODER 1992). Instead, we consider the more likely possibility that the observed distributions of transposed *Ds^{neo}* elements reflect some property of the genomic insertion sites of T-DNA loci 90 and 106. For example, in tobacco, percentages of linked *Ac* transposition are known to vary considerably depending upon the particular chromosomal location of the donor site. In a comparison of patterns of *Ac* transposition from six different T-DNA loci in tobacco, frequencies of tightly linked transposition displayed a significant degree of locus-to-locus variation (DOONER *et al.* 1991). However, unlike distributions of *Ds^{neo}* reported here, the majority of those *Ac* receptor sites in tobacco still tended to cluster well within 20 cM of the T-DNA donor loci. Alternatively, it is conceivable that the discrepancy reflects a skewing of our data in favor of insertion sites that were unlinked to the T-DNA. Though we used complementary molecular approaches, IPCR and λ cloning, to isolate flanking DNAs, we may not have recovered all classes of insertions with equal efficiency.

We favor the interpretation that the clustered arrays of *Ds^{neo}* receptor sites in family 90 result from rounds of unlinked and linked transposition. However, another plausible explanation for the observed clustering of *Ds^{neo}* is the existence of insertion hot-spots. No obvious target site specificity was revealed by inspection of nucleotide sequences of genomic regions flanking the 5' or 3' ends of several transposed *Ds^{neo}* elements (APPENDIX 1). However, our data do not eliminate the possibility that *Ds^{neo}* may insert preferentially at certain loci, or in regions with a

special chromosomal architecture. The most compelling evidence against the idea that clustered receptor sites represent insertion hot-spots, is the fact that different distributions of transposed *Ds^{neo}* elements were seen in families 90 and 106. Perhaps the most obvious candidate for an insertion hot-spot is the tight cluster, in family 90, of three transposed *Ds^{neo}* elements on chromosome eleven, delimited by markers TG46 and TG26 (Figure 7). Insertions of *Ac* have also been identified in this region (OSBORNE *et al.* 1991; BELZILE and YODER 1992). However, we detected no insertion within this chromosomal interval in family 106. Conversely, while several insertions on chromosome eight were identified in family 106, not one of the nine insertions mapped in family 90 was positioned on this chromosome. The only clear region of overlap between the insertion site distributions of families 90 and 106, is an interval of about 40 cM on chromosome one, which bears a single transposed *Ds^{neo}* in each family. We appreciate, however, that the total number of insertions analyzed in our study is relatively small, and that a thorough analysis of hot-spots for *Ds^{neo}* insertion requires genetic mapping of a large number of genomic receptor sites.

Stable *Ac3* bears a small 5' terminal deletion: The *Ac3* element, used to *trans*-activate *Ds^{neo}* in family 106, was first detected as a single, transposed copy of *Ac* present in certain individuals of an *Ac* tomato line. *Ac3* appeared as a distinctive pair of *Ac*-hybridizing restriction fragments that were stably inherited (Figure 3). In maize, a reversible inactivation of *Ac* transposition correlates with methylation of subterminal sequences (DENNIS and BRETTELL 1990). However, we consider it unlikely that the apparent stability of *Ac3* results from such methylation. We monitored inheritance of the *Ac3* allele for several generations in tomato and saw no evidence of instability.

Through introduction of internal deletions at each end of *Ac*, *cis*-acting regions of about 200 bp have been defined at each terminus, as both necessary and sufficient for excision [COUPLAND *et al.* (1989); reviewed in KUNZE *et al.* (1990)]. These regions include short (11 bp) imperfect inverted repeat sequences that are found at the extreme 5' and 3' termini. Identification of a mutant *Ac* allele, *Ac18*, bearing a deletion at its 3' terminus, established that the 3'-inverted repeat sequence is essential for transposition. This deletion, which removed just 4 bp of the 3'-inverted repeat, generated a stable *Ac* element (HEHL and BAKER 1989). Like the *Ac3* element of tomato described here, *Ac18* arose spontaneously in a transgenic plant, in that instance tobacco, which harbored *Ac*. Moreover, like the *Ac3* allele, *Ac18* retained the capacity to induce *Ds* transposition.

Nucleotide sequencing of *Ac3* termini revealed only one deviation from the published *Ac* sequence: a dele-

tion removing just the first five nucleotides (CAGGG, positions 1–5) of the 5' terminal inverted repeat (Figure 4). We infer that this small 5'-terminal deletion is responsible for the immobility of *Ac3*, and speculate that the element may have sustained the deletion during the process of transposition itself. Discovery of the *Ac3* allele affords a novel example of a mutation, disrupting only the structural integrity of the 5'-inverted repeat, that abolishes transposition. We note, however, that since the entire nucleotide sequence of *Ac3* has not been determined, we cannot rule out the possibility that a mutation elsewhere in the element might also contribute to its stability.

Future transposon tagging strategies using *Ds^{neo}*: In summary, we have developed an effective dual element system for transposon mutagenesis in tomato, consisting of a stable *Ac* trans-activator (*Ac3*) and a genetically marked *Ds* element (*Ds^{neo}*). Use of the immobile *Ac3* element to trans-activate *Ds^{neo}* facilitates genetic segregation of transposase and mutator components, allowing for stabilization of *Ds^{neo}* insertions. We have shown that *Ds^{neo}* transposes frequently in tomato, and possesses qualities that are desirable in an insertional mutagen: (i) *Ds^{neo}* receptor sites are often located on the same chromosome as the donor site of transposition, and (ii) *Ds^{neo}* receptor sites are not necessarily confined mainly to tight clusters around T-DNA donor loci. Viewed from the standpoint of transposon tagging, these findings suggest that effective use of *Ds^{neo}* as an insertional mutagen may best be achieved by first identifying transgenic plants bearing either an integrated T-DNA donor site, or a transposed copy of *Ds^{neo}*, on the same chromosome as a target gene of interest. However, it may not be necessary to utilize a transposition donor site that is tightly linked to the target gene.

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APPENDIX 1

Nucleotide sequences of *Ds^{neo}* 5' and 3' ends and genomic flanking regions (Figure 9). Sequences shown correspond to mapped IPCR isolates. Four 5'-flanking DNAs from *Ac3/Ds^{neo}* family 106 (#31, #32, #71, and #72) are displayed above four 3'-flanking DNAs from *Ac/Ds^{neo}* family 90 (#5, #301, #322, and #327). Underlines indicate *Ds^{neo}* termini.

Ds^{neo} 5'ends

#31 ACTACATGTTATAAGGAGAAGCAGGGATGAAAGTAGGATGGGAAAAT
 #32 GGTAAATACAAGCTCCAAAGCCAGGGATGAAAGTAGGATGGGAAAAT
 #71 AGTTCTATTCCGACCTCGGGCAGGGATGAAAGTAGGATGGGAAAAT
 #72 GGTACGAGAAGTACTTGACAACAGGGATGAAAGTAGGATGGGAAAAT

Ds^{neo} 3'ends

#5 GACCGTTACCGACCGTTTTCATCCCTACTTTCATGCATTGGAAATT
 #301 GACCGTTACCGACCGTTTTCATCCCTAATCTAGTAATGTGGAGAA
 #322 GACCGTTACCGACCGTTTTCATCCCTACGTGGTTCAGTCGCGCCT
 #327 GACCGTTACCGACCGTTTTCATCCCTAATCCATATATTTACTTAGA

FIGURE 9