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

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ABSTRACT

We have introduced a genetically marked Dissociation transposable element (Ds<sup>neo</sup>) into tomato. In the presence of Ac transposase, Ds<sup>neo</sup> excised from an integrated T-DNA and reinserted at numerous new sites in the tomato genome. The marker genes of Ds<sup>neo</sup> (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<sup>neo</sup>, we examined the genomic distribution of Ds<sup>neo</sup> receptor sites, relative to the location of the donor T-DNA locus. Restriction fragment length polymorphism mapping of transposed Ds<sup>neo</sup> elements was conducted in two tomato families, derived from independent primary transformants each bearing Ds<sup>neo</sup> within a T-DNA at a unique position in the genome. Transposition of Ds<sup>neo</sup> generated clusters of insertions that were positioned on several different tomato chromosomes. Ds<sup>neo</sup> 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<sup>neo</sup> transposition observed in tomato to be well suited for transposon mutagenesis. Our study made use of a novel, stable allele of Ac (Ac<sub>3</sub>) that we discovered in transgenic tomato. We determined that the Ac<sub>3</sub> element bears a deletion of the outermost 5 base pairs of the 5'-terminal inverted repeat. Though incapable of transposition itself, Ac<sub>3</sub> retained the ability to mobilize Ds<sup>neo</sup>. We conclude that a dual element system, composed of the stable Ac<sub>3</sub> trans-activator in combination with Ds<sup>neo</sup>, 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, FEDROFF 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 insertion mutants (FEDOROFF, FURTER and NELSON 1984; CHEN, GREENBLATT and DELLAPORTA 1987; THIBAUT, SCHEELE and STARLINGER 1987; HAKE, VOLBRECHT 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-

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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 h2-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 frequency, is consistent with linked transposition (Kermicle, Allemann 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 variants 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; 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, Allemann 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 variants 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, Dsneo, 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 Dsneo 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 Dsneo activity, we also investigated the genomic distribution of Dsneo 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 Dsneo. This approach exploits the existence of a detailed RFLP map of tomato (Bernatzky and Tanksley 1986; Tanksley
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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\textsuperscript{neo}.

Finally, we describe the isolation and characterization of a stable derivative of Ac (Ac\textsubscript{3}), that was identified in a tomato line harboring wild-type Ac. The Ac\textsubscript{3} allele, which bears a mutation in the 5' terminal inverted repeat sequence, serves as an effective trans-activator of Ds\textsuperscript{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\textsuperscript{neo} into the binary Ti-plasmid vector, pDW-Hph, at a unique Smal site in the untranslated leader region of a hybrid hygromycin phosphotransferase gene, P\textsubscript{apyr}HPT. In construction of Ds\textsuperscript{neo}, the HindIII interval of Ac was deleted and a chimeric neomycin phosphotransferase II gene, P\textsubscript{nos-NPTII} was inserted at the XhoI site. Figure 1 shows Ds\textsuperscript{neo} positioned within the P\textsubscript{nos} HPT gene fusion, as well as the uninserted P\textsubscript{nos-HPT} gene generated by Ds\textsuperscript{neo} excision. Transformants A90 and A106 were selected with kanamycin at 100 μg/ml. Excision of Ds\textsuperscript{neo} from the P\textsubscript{nos-HPT} gene construct was selected with hygromycin at 10 μg/ml. Generation of Ac transformant A61 by transformation of VF36 with plasmid pGV3850/HPT::pKUS has been described previously (OSBORNE et al. 1991).

**Activation of Ds\textsuperscript{neo} transposition:** Ds\textsuperscript{neo} transposition frequency and distance were assessed in two independent tomato families: family 90, Ac (Ac\textsubscript{61}) × Ds\textsuperscript{neo} (A90); and family 106, Ac\textsubscript{3} (D42-1) × Ds\textsuperscript{neo} (A106) (Figure 2, A and B). To generate family 90, Ac transformant A61 was used as the female parent in crosses to Ds\textsuperscript{neo} transformant, A90. To enrich for transposition events, F\textsubscript{1} seedlings resistant to hygromycin and kanamycin were selected. One such seedling, B60-2, was the F\textsubscript{1} progenitor of family 90. B60-2 carried, in addition to Ds\textsuperscript{neo} transposed Ac, but did not inherit the T-DNA of the Ac (A61) parent. F\textsubscript{2} progeny of B60-2, C205-2 to C205-30, were derived by selfing. Similarly, selfing of C205-6 to C205-30 yielded F\textsubscript{3} progeny. In the F\textsubscript{3}, selections for kanamycin resistance identified individuals containing transposed Ds\textsuperscript{neo}. F\textsubscript{2} plants C205-14 and C205-18 and their F\textsubscript{3} progeny contained Ac elements whereas C205-6, C205-21, C205-29, C205-30 and their F\textsubscript{3} progeny were void of Ac (data not shown). To generate family 106, R\textsubscript{6}, D42-1, an individual bearing stable Ac\textsubscript{3} (but no other Ac-hybridizing sequences detectable by Southern blotting) was used as the female parent in crosses to R\textsubscript{6}, C217-1 of Ds\textsuperscript{neo}-line 106. The stable Ac\textsubscript{3} allele arose in self progeny of Ac line 61. F\textsubscript{1} seedlings were subjected to selection with hygromycin and kanamycin. Similarly, R\textsubscript{6}, E11-2, another member of Ac line 61 bearing only stable Ac\textsubscript{2}, served as the female parent in separate crosses to R\textsubscript{6}, C218-1 of Ds\textsuperscript{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 nylon 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 PstI-BamHI fragment of the HPT gene, while Ds\textsuperscript{neo} excision and reintegration was assessed with a 1.3-kb EcoRI-BamHI 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 R\textsubscript{3}, C205-2 (line 90), while #106 was obtained from DNA of R\textsubscript{2}, B91-1 (line 106). Templates were prepared by digestion of genomic DNAs with TagI, followed by phenol extraction and ligation as described previously (OSBORNE et al. 1991). Ligated DNAs were heated briefly to 65°C, then cleaved with Asp718 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°C, 2 min/55°C, 5 min/72°C, 35 cycles. Reactions included 1.0 μg each of oligonucleotide primers that were complementary to sequences in the T-DNA right border: 5'-CAACAAGTGCGGATATTCG-3' (positions 459–478) and 5'-AACACCCACAGTTGACAGGAGTCC-3' (positions 330–311). IPCR products were cloned into either Smal-cut pUC19, or EcoRV-cut pKR (provided by J. JONES).

**Cloning of Ds\textsuperscript{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 PsI prior to the ligation step, and Acel was used for linearization. Primers were designed to incorporate EcoRI or BamHI sites. 5' flanking DNAs were amplified with primer, 5'-CAGGATCTCACGATAACGGTTGAGTCGGTACGGGA-3' (position 48 to position 26 of Ds\textsuperscript{neo}), and a P\textsubscript{nos-NPTII} gene primer, 5'-'CACGATCTGGTGATCTCGATCGACCAAAG-3' (position 1943 to position 1962 of Ds\textsuperscript{neo}). Similarly, 3' flanking DNAs were amplified with primer, 5'-CAGGATCTGGTGATCTCGATCGACCAAAG-3' (position 48 to position 26 of Ds\textsuperscript{neo}), and P\textsubscript{nos-NPTII} gene primer, 5'-CACGATCTCCACAGACATTCGCTGTC-3' (position 3722 to position 3703 of Ds\textsuperscript{neo}). IPCR products were digested with EcoRI and BamHI, and cloned into EcoRI- and BamHI-cleaved pUC19.

**Construction of a λ EMBL4 library:** Pooled genomic DNAs from 18 F\textsubscript{2} progeny of family 90 (Table 1) were partially digested with MboI 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 BamHI and SalI (FRITSCH and SAMBROOK 1982). Ligations were packaged according to the manufacturer's protocol (Promega) and bacteriophage were plated on Escherichia coli host Q538 (MANIATIS, FRITSCH and SAMBROOK 1982). Duplicate filters from a library of approximately 5 × 10\textsuperscript{8} 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\textsuperscript{neo}.
in place" in the T-DNA. Three positive clones were identified which hybridized to the P\textsubscript{nos} probe, but not to the HPT probe. Purified λ DNAs were digested with EcoRI and HindIII, and analyzed by Southern blotting using the end of genomic DNAs digested with each of six different restriction enzymes: BstNI, DraI, EcoRI, EcoRV, HaeIII, HindIII. Enzymes that produced polymorphic restriction fragments between the two species were used to digest genomic DNAs of 56 F\textsubscript{2} progeny of an L. esculentum cv. VF36 and Lycoperisicon pennelli hybrid (Bernatzky and Tanksley 1986). Southern blots of digested F\textsubscript{2} 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\textsuperscript{exo} 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, \( P = 0.00009 \).

For statistical analysis of data from family 106 (Figure 8), we again assumed that Ds\textsuperscript{exo} 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 \( Ds\textsuperscript{exo} \) insertions. Thus, for the data of Figure 8, \( P = 0.07 \).

**Cloning and characterization of stable Ac termini:** Sequences flanking Ac and \( Ac \) were isolated by PCR from EcoRI-digested genomic DNAs of \( R_c, C201-17 \) and \( R_c, C203-29 \), respectively, each a member of Ac line 61 containing a single stable \( Ac \) element. Following linearization of the template DNA with HindIII, amplification of \( Ac \) 5' and 3' ends was performed using primers designed to include Psfl and BglII sites. Primer pairs for the 5' end of stable \( Ac \) were as follows: 5'-CACCTGCAAGATTCGATCTGTTAAGGCTGCAAGG-3' (position 47 to position 28 of \( Ac \)), and 5'-GGTGAAAGATGCTGAGTCTAAAGGAG-3' (position 2456 to position 2475 of \( Ac \)). Primer pairs for the 3' end were as follows: 5'-CACCTGCAAGATTCGATCTGTTAAGGCTGCAAGG-3' (position 4531 to position 4550 of \( Ac \)), and 5'-CACCTGCAAGATTCGATCTGTTAAGGCTGCAAGG-3' (position 2475 to position 2456 of \( Ac \)).

**RESULTS**

Characterization of tomato transformants bearing a marked Ds element within a T-DNA: A Ds element carrying a chimeric, P\textsubscript{nos}-NPTII, kanamycin resistance (Kn\textsuperscript{b}) gene was introduced into tomato cultivar VF36 by Agrobacterium-mediated transformation using plasmid pH2 (McCormick et al. 1986). The marked Ds (\( Ds\textsuperscript{exo} \)) of pH2 is positioned within the untranslated leader of a gene fusion (P\textsubscript{nos}-HPT), that confers hygromycin resistance (Hyg\textsuperscript{b}) only upon excision of the element (Figure 1).

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**Figure 1:** Restriction map of \( Ds\textsuperscript{exo} \) at its donor location in the T-DNA, and map of the predicted empty donor fragment following excision. Panel A depicts \( Ds\textsuperscript{exo} \) that is \"in place\" in the T-DNA, while panel B depicts the uninterrupted P\textsubscript{nos}-HPT gene created by excision of \( Ds\textsuperscript{exo} \). Open boxes showing positions of the NPTII and HPT marker genes are indicated. Speckled regions represent \( Ds \) sequences. 5' and 3' termini of \( Ds\textsuperscript{exo} \) 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 P\textsubscript{nos} probe, while the open box below the map of panel A represents the HPT probe. Numbers refer to sizes of EcoRI-PstI restriction fragments, in kilobase pairs (kb), detected by P\textsubscript{nos} or HPT probes. E, EcoRI; P, PstI; H, HindIII.

\[
\text{AGGCTTATAAATAAGGGC-3'} \quad \text{(position 2531 to position 2511 of Ac)}
\]

IPCR products were cloned into PstI-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 \( Ac \) 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 EcoRI and BamHI sites to facilitate cloning. The 5' end of \( Ac \) was amplified with primer, 5'-GATGGATCCGGTGTGAATGCTGC-3' (position 376 to position 358 of \( Ac \)), and flanking primer, 5'-GACGAATTCCGTTTATAATGTTGAC-3' (the 3' end of \( Ac \)) as well as amplified with primer, 5'-GATGGATCCGGTGTGAATGCTGC-3' (position 4186 to position 4204 of \( Ac \)), and flanking primer 5'-GACGAATTCCGTTTATAATGTTGAC-3'. PCR products were digested with EcoRI and BamHI, and cloned into EcoRI- and BamHI-cleaved pUC19 prior to nucleotide sequencing.
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Family 90

\[ \text{Ac(A61)} \times \text{Ds}^{\text{neo}}(\text{A90}) \]

\[ \text{F1} \]

\[ \text{B60-2} \]

\[ \text{F2} \]

\[ \text{C208-6, C208-18, C205-21, C205-29, C205-30, C205-14, C205-2, C205-5} \]

\[ \text{D31-1, D31-4, D31-5, D31-6, D31-7, D32-1, D32-2, D32-3, D32-4, D32-5, D32-7, D32-9, D32-11} \]

Family 106

\[ \text{D}^{\text{neo}}(\text{A106}) \]

\[ \text{B91-1, B91-2, B91-5} \]

\[ \text{P Ac3(D42-1)} \times \text{Ds}^{\text{neo}}(\text{C217-1}) \]

\[ \text{Ac2 (E11-2)} \times \text{Ds}^{\text{neo}}(\text{C218-1}) \]

\[ \text{F1} \]

\[ \text{D48-1, D48-2, D48-3, D48-7, D48-9, D47-2, D47-10} \]

Kanamycin selection yielded several independent primary (R₀) transformants, two of which, A90 and A106, were judged to carry single T-DNA loci. KnK segregated roughly 3:1 (102 KnK:35 KnS) in self-progeny of A106. Furthermore, Southern blot analysis of A106 DNA, using a fragment of the HPT gene as a probe, detected a unique 5.8-kb HindIII 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 EcoRI-PstI junction fragment (not shown). Southern blot analysis of restriction enzyme-digested DNA isolated from R₀ lines using Ds, NPTII, HPT, and PNeo fragments as 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\textsuperscript{neo} transposition in two independent tomato families, 90 and 106, derived from A90 and A106. These families were produced by crossing each Ds\textsuperscript{neo}-bearing line, to a plant containing either an autonomous or a stable Ac trans-activator (Figure 2, A and B).

Ds\textsuperscript{neo} transposition from T-DNA 90 is trans-activated by autonomous Ac: In the presence of Ac, Ds\textsuperscript{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\textsuperscript{neo} continued to transpose, in plants containing Ac, for at least three generations.

Figure 3A depicts a Southern blot analysis of Ds\textsuperscript{neo} transposition in F₁, F₂ and F₃ progeny generated from a cross between line 90 and a plant harboring an autonomous Ac element (see Figure 2A). Selection for HygK and KnK allowed us to identify F₁ seedlings in which transposition of Ds\textsuperscript{neo} to new genomic sites had occurred. F₂ and F₃ offspring were generated by selfing of F₁ and F₂ individuals, respectively, followed by selection for KnK in the F₃. The PNeo fragment used as a probe (Figure 1) detects two fragments (2.0 and 2.7 kb) in EcoRI- and PstI-digested genomic DNA that are diagnostic of Ds\textsuperscript{neo} that is "in place" within the T-DNA of line 90 (Figure 3A, lane a). In an F₁ individual bearing an Ac trans-activator (lane b), PNeo also detects a 1.1-kb empty donor fragment created by Ds\textsuperscript{neo} excision from the T-DNA. The empty donor fragment is absent in the self F₂ and F₃ offspring shown in lanes c-g, as these plants did not inherit the T-DNA from their F₁ parent. Additional PNeo-hybridizing fragments...
detected in F₁, F₂, and F₃ progeny denote transposed Dsnro elements (Figure 3A, lanes b–g).

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

Dsnro transposition from T-DNA 106 is trans-activated by stable Ac derivatives: Two stable Ac elements, Ac2 and Ac3, each effectively activated transposition of Dsnro 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 fragment, show several HygR and KnR F₁ progeny of crosses between line 106 (lane a) and plants bearing either Ac2 or Ac3 (see Figure 2B). As in Figure 3A, Pnos detects fragments specifying Dsnro that is “in place” in the T-DNA of line 106, transposed Dsnro, as well as the empty donor site. In most individuals, some Dsnro remained “in place” (2.0- and 2.7-kb Pnos-hybridizing fragments). However, in every F₁ individual examined, somatic excision of Dsnro was signaled by the presence of the 1.1-kb Pnos-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 Dsnro (Figure 3B, lanes d–f). Approximately one to two new Dsnro insertions were detected per plant. In one F₁ plant, substantial excision of Dsnro from the T-DNA occurred (lane g), though no reintegrated copies of Dsnro were detected with the Pnos probe. Insertion of Dsnro 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 Dsnro 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 Ac3.

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 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 Dsnro. 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) which are deleted from the 5’-inverted repeat of Ac3.

![Figure 4](image_url)—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](image_url)—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.
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 Dsneo transposition distance: We investigated patterns of Dsneo transposition in each of two independent tomato families described above: family 90, Ac × Dsneo (A90), and family 106, Ac3 × Dsneo (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 Dsneo. Toward this end, we isolated genomic sequences flanking the T-DNA and transposed copies of Dsneo. To amplify only regions flanking Dsneo, we designed primers that would be amplified from genomic DNA 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 Dsneo insertions were recovered by IPCR, as described in MATERIALS AND METHODS. Oligonucleotide primer pairs were chosen to amplify only regions flanking Dsneo termini. Ac flanking regions were not amplified, since one primer of each pair anneals to sites present in the P neoNPTII marker gene of Dsneo.

In family 90, regions flanking the 3' end of Dsneo 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 regions enabled us to determine whether IPCR products that were obtained in separate reactions, especially products of similar size, actually comprised different Dsneo insertions. For example, similar size products (#301 and #15) were recovered from an F3 individual (C205-1), 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 obtained from two F3 individuals (D32-4 and D36-1). Thus, these products comprise a single, inherited transposition event rather than two different events.

### Table 1

<table>
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The bacteriophage λ library was constructed from pooled genomic DNAs of 18 F2 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.
In total, six unique IPCR products, each representing a distinct \textit{Ds}^{neo} insertion site, were obtained from \textit{Ac}/\textit{Ds}^{neo} family 90 (Table 1). Nucleotide sequences of genomic regions flanking the 3' ends of several of these \textit{Ds}^{neo} insertions are displayed in Appendix 1.

Genomic regions flanking three additional transposed \textit{Ds}^{neo} elements in family 90 were isolated from a bacteriophage \textit{\lambda} library constructed from pooled F\textsubscript{2} progeny DNAs (see Table 1 and MATERIALS AND METHODS). These \textit{\lambda} clones (\#1, \#10, and \#11) hybridized to a \textit{P}_{\text{Nos}} probe, but not to an HPT gene probe (which detects \textit{Ds}^{neo} that is “in place” within the T-DNA). Southern analysis of purified \textit{\lambda} DNAs using a 5' \textit{Ac} probe identified fragments containing the junction between genomic DNA and transposed \textit{Ds}^{neo} (Table 1).

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

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

\textbf{Isolation of genomic sequences flanking T-DNA loci:} The integrated T-DNAs of transformants A90 and A106 each served as starting points for \textit{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 \textit{HindIII}-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 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 \textit{EcoRI} -\textit{PstI} digest of A90 DNA, as a T-DNA right border probe (not shown). Similarly, when used as a hybridization probe on \textit{HindIII}-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).

\textbf{RFLP map positions of flanking regions:} We assigned IPCR or \textit{\lambda} clones from each family, corresponding to regions flanking the T-DNA and transposed copies of \textit{Ds}^{neo}, to positions on the RFLP map. Our method involved the use of a tomato \textit{F\textsubscript{2}} mapping population, and a database of RFLP markers (both gifts of S. TANKSLEY) in combination with the MapMaker computer program (LANDER \textit{et al.} 1987) (see MATERIALS AND METHODS). As reported previously for \textit{Ac} (OSBORNE \textit{et al.} 1991; BELZILE and YODER 1992), nearly all \textit{Ds}^{neo} target sites in tomato were single copy genomic DNA sequences (not shown).

Figure 7 illustrates the distribution of \textit{Ds}^{neo} receptor sites and the location of the integrated T-DNA of \textit{Ac}/\textit{Ds}^{neo} family 90. Nine sequences flanking \textit{Ds}^{neo} insertions were assigned to the RFLP map. These insertions were recovered from self \textit{F\textsubscript{2}} and \textit{F\textsubscript{3}} progeny derived from a single \textit{F\textsubscript{1}} individual. Since \textit{Ds}^{neo} remained active in tomato for several generations, we
Transposition of a Marked Ds

FIGURE 7.—RFLP map positions of the T-DNA and transposed Dsneo elements in Ac/Dsneo 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 Dsneo 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 Dsneo would serve as a new donor site for further transposition events. Here, we observed both linked and unlinked transposition of Dsneo. 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 Dsneo 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 Dsneo did not insert at all genomic sites with equal probability. If each Dsneo 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 $10^6$ trials.

Figure 8 depicts the distribution of Dsneo receptor sites and the location of the integrated T-DNA of Ac3/Dsneo family 106. Five flanking DNAs, obtained from three different F1 siblings, were placed on the RFLP map. Transposed copies of Dsneo were located on 3 of the 12 tomato chromosomes. No Dsneo 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 Dsneo elements located on chromosome 8 (#31, #71 and #91), originated in a different F1 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 Dsneo insertion sites on chromosome 8 (Figure 8) using a binomial distribution (see MATERIALS AND METHODS). If Dsneo 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.
hybridization probes, we demonstrated that three of these \( Ds^{neo} \) insertions (D32-1, D32-2, D32-4) were germinally transmitted to \( F_1 \) 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 1983).

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 segre gated 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

**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
Ds\textsuperscript{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\textsuperscript{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\textsuperscript{neo}.

**Preferential insertion of Ds\textsuperscript{neo} on the same chromosome as the T-DNA donor locus:** In family 106, we isolated transposed Ds\textsuperscript{neo} elements from three F\textsubscript{1} offspring. Insertions that arose in different F\textsubscript{1} 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 were not determined precisely. We, on the other hand, determined precise RFLP map positions for all Ds\textsuperscript{neo} insertion loci regardless of their linkage status with respect to the T-DNA.

In family 106, transposed copies of Ds\textsuperscript{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 F\textsubscript{1} individual. Statistical tests showed that this arrangement was unlikely to occur by chance (P = 0.007), if Ds\textsuperscript{neo} transposed randomly in the tomato genome. Therefore, we consider that the probability of recovering Ds\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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 F\textsubscript{1} plant (#31 and #32, or #71 and #72), no linkage between receptor sites was observed.

**Linked and unlinked transposition of Ds\textsuperscript{neo}:** In family 90, we isolated transposed Ds\textsuperscript{neo} elements from self F\textsubscript{2} and F\textsubscript{3} progeny, all descended from the same F\textsubscript{1} 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\textsuperscript{neo} at a frequency comparable to that reported previously for Ac (roughly 50%) in tomato, would yield clustered Ds\textsuperscript{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\textsuperscript{neo}. The most striking feature of this array was the arrangement of insertions in small clusters on at least one chromosome. 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\textsuperscript{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\textsuperscript{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\textsuperscript{neo} receptor sites in close proximity to the T-DNA. The data of Figure 7, in which Ds\textsuperscript{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\textsuperscript{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\textsuperscript{neo} in close proximity to the T-DNA donor site. In family 90, two Ds\textsuperscript{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 Dsneo 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 Dsneo insertions linked to the T-DNA may reflect a substantive difference between modes of transposition employed by Ac and our marked Dsneo element. It is formally possible that Ac sequences removed in construction of Dsneo 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 Dsneo 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 Dsneo (Belzile and Yoder 1992). Instead, we consider the more likely possibility that the observed distributions of transposed Dsneo 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 Dsneo 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 Dsneo receptor sites in family 90 result from rounds of unlinked and linked transposition. However, another plausible explanation for the observed clustering of Dsneo is the existence of insertion hotspots. No obvious target site specificity was revealed by inspection of nucleotide sequences of genomic regions flanking the 5' or 3' ends of several transposed Dsneo elements (Appendix 1). However, our data do not eliminate the possibility that Dsneo 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 Dsneo 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 Dsneo 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 Dsneo 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 Dsneo 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 Dsneo 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 Bretell 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. 1998; 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**

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**mut**). Use of the immobile Ac3 element to trans-activate Ds**mut** facilitates genetic segregation of transposase and mutator components, allowing for stabilization of Ds**mut** insertions. We have shown that Ds**mut** transposes frequently in tomato, and possesses qualities that are desirable in an insertional mutagen: (i) Ds**mut** receptor sites are often located on the same chromosome as the donor site of transposition, and (ii) Ds**mut** 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**mut** 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**mut**, 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 \( Ds^{exo} \) 5’ and 3’ ends and genomic flanking regions (Figure 9). Sequences shown correspond to mapped IPCR isolates. Four 5’-flanking DNAs from \( Ac/\overline{Ds}^{exo} \) family 106 (\#31, \#32, \#71, and \#72) are displayed above four 3’-flanking DNAs from \( Ac/\overline{Ds}^{exo} \) family 90 (\#5, \#301, \#322, and \#327). Underlines indicate \( Ds^{exo} \) termini.

\[ Ds^{exo} \text{ 5' ends} \]

\[
\begin{align*}
\#31 & : & \text{ACTACATTTATAGGGAGACGGCGTTACGGATACGAAGTATGCTGTTGGAAAT} \\
\#32 & : & \text{GTTAACACCGGCTCAAGACCGGTACACTACACATGGTGAGAAAT} \\
\#71 & : & \text{AGTCTACCGGACTGTGCGGAGACGCGTTACGGATACGAAGTATGCTGTTGGAAAT} \\
\#72 & : & \text{GTTAACACCGGCTCAAGACCGGTACACTACACATGGTGAGAAAT} \\
\end{align*}
\]

\[ Ds^{exo} \text{ 3' ends} \]

\[
\begin{align*}
\#5 & : & \text{GACCGTTACCCAGTCACACTACACACGGGATGAAAGTAGGATGGGAAAAT} \\
\#301 & : & \text{GACCGTTACCCAGTCACACTACACACGGGATGAAAGTAGGATGGGAAAAT} \\
\#322 & : & \text{GACCGTTACCCAGTCACACTACACACGGGATGAAAGTAGGATGGGAAAAT} \\
\#327 & : & \text{GACCGTTACCCAGTCACACTACACACGGGATGAAAGTAGGATGGGAAAAT} \\
\end{align*}
\]

Figure 9