

Transposition-Based Plant Transformation

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Agrobacterium T-DNAs were used to deliver transposable *Dissociation* (*Ds*) elements into the nuclei of potato (*Solanum tuberosum*) cells. A double-selection system was applied to enrich for plants that only contained a transposed *Ds* element. This system consisted of a positive selection for the neomycin phosphotransferase (*nptII*) gene positioned within *Ds* followed by a negative selection against stable integration of the cytosine deaminase (*codA*) gene-containing T-DNA. Sixteen of 29 transgenic plants were found to contain a transposed element while lacking any superfluous T-DNA sequences. The occurrence of this genotype indicates that *Ds* elements can transpose from relatively short extrachromosomal DNA molecules into the plant genome. The frequency of single-copy *Ds* transformation was determined at 0.3%, which is only about 2.5-fold lower than the potato transformation frequency for backbone-free and single-copy T-DNAs. Because of the generally high expression levels of genes positioned within transposed elements, the new transformation method may find broad applicability to crops that are accessible to Agrobacterium T-DNA transfer.

Agrobacterium-mediated plant transformation is based on the transfer of single-stranded plasmid DNA segments (T-DNAs) from the infecting pathogen to plant cell nuclei (Tinland et al., 1994; Yusibov et al., 1994). Although the virE2-coated linear DNA is temporarily protected from nuclease attack (Rossi et al., 1996), only about 1% of transferred T-DNAs escape eventual degradation by integrating into double-stranded chromosome breaks (Bravo-Angel et al., 1999; Chilton and Que, 2003; Rommens et al., 2004). The expression level of T-DNA-residing transgenes is often affected by position-dependent gene-silencing effects, which can be overcome by linking the transgenes to matrix-associated DNA regions (Peach and Velten, 1991; Mlynarova et al., 2003).

In contrast to the apparently passive integration of T-DNAs, mobile elements, such as maize (*Zea mays*) *Activator* (*Ac*), are generally believed to transpose via cut-and-paste DNA recombination. Excision is initiated by the assembly of an active synaptic complex in which the two ends of the element are paired and held together by bound *Ac*-transposase (*Tpa*) subunits (Gorbunova and Levy, 2000). In analogy with other transposition systems, reinsertion may occur when the 3' hydroxyl at each end of the excised element performs a nucleophilic attack on the host DNA (Reznikoff, 2003). In the final stage of the transposition process, noncomplementary ends of the broken donor DNA molecule are processed, rejoined, and filled. This repair process generates both a small excision site footprint, often comprising a few base pair of transposon-

end sequences and characteristic duplication of the target octonucleotide insertion site.

The *Ac* element encodes an 807-amino acid *Tpa* that mediates the initiation of transposition by specifically binding to multiple motifs positioned near the terminus of the element (Kunze and Starlinger, 1989). Separation of the two functions of *Ac* creates a two-component transposition system. An expression cassette for the *Tpa* gene represents the first component, whereas the second component consists of a nonautonomous *Dissociation* (*Ds*) element that contains the terminal sequences required for transposition (Rommens et al., 1992). Such two-component systems have been introduced into a variety of plant species by transforming plants with T-DNAs carrying both *Ds* and the *Ac-Tpa* gene (Hehl and Baker, 1989). As opposed to the unpredictability of T-DNA integration, *Ds* elements frequently transpose from stably integrated T-DNAs into gene-associated sequences that are adjacent to predicted matrix-associated DNA regions (Meissner et al., 2000; Koprek et al., 2001; Ito et al., 2002; Kim et al., 2004; Pan et al., 2005; Zhao et al., 2006). The site preference for *Ds* integration has been linked to generally high expression levels of genes positioned within the element (Koprek et al., 2001). Upon transposition, gene expression levels need to be stabilized by segregating the *Tpa* source away from *Ds* in progeny plants. This requirement may complicate breeding efforts, especially if the transposable element and its activator are tightly linked. Furthermore, the necessity to either self or cross fertilize transgenic plants sets hurdles to applications of this method in crops that are vegetatively propagated and suffer from in-breeding depression, such as potato (*Solanum tuberosum*).

The need to employ T-DNA transformation procedures for the introduction of transposons into plants can be circumvented by promoting the transposition of elements from extrachromosomal DNAs into the plant genome. Earlier methods were based on the use

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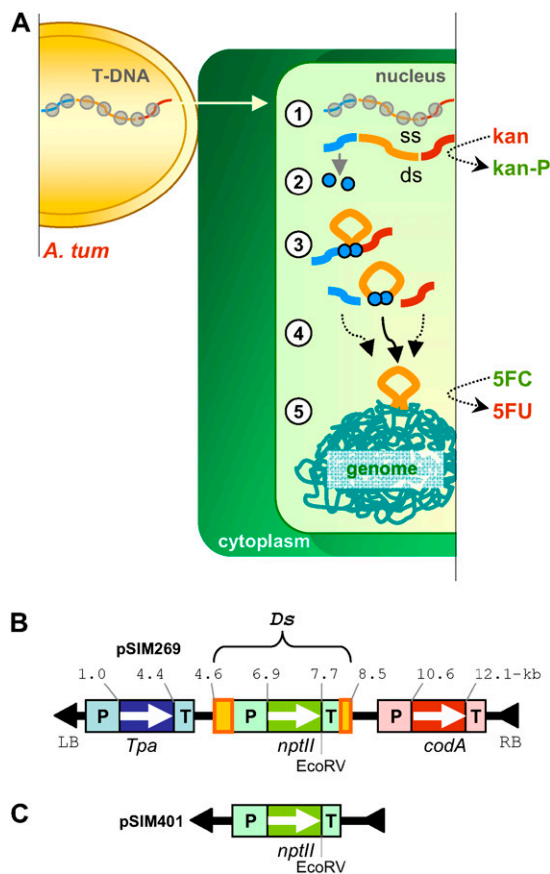


Figure 1. Transposon-based transformation. A, Model of *Ds* transposition from an extrachromosomal T-DNA into the genome of a plant cell. The T-DNA contains a *Tpa* gene expression cassette (blue), a *Ds* element carrying an expression cassette for the *nptII* gene inserted between the ends of the maize element *Ac* (orange), and a *codA* gene inserted between regulatory sequences (red). Plant transformation is initiated by the transfer of single-stranded (ss) and virE2-coated (gray circles) T-DNA from *Agrobacterium* (*A. tum*) to the plant cell nucleus (step 1). After obtaining its double-stranded (ds) form, expression of the *Tpa* and *codA* genes will result in the production of *Tpa* (blue circles) and the conversion of 5FC to toxic 5-fluorouracil, respectively (step 2). *Tpa* will bind to the *Ac*-derived sequences and catalyze excision of *Ds* (steps 3 and 4). The excised element, possibly together with broken T-DNA fragments, may integrate into the plant cell genome (step 5). Application of a sequential selection on kanamycin and 5FC was predicted to yield transformants that contained a *Ds* element and lacked T-DNA. B, Diagram of the T-DNA of pSIM269 and pSIM401. Black triangles represent the left and right border of the T-DNA.

of either plasmids or geminiviruses that contain a *Ds* element. These methods yielded very low transformation frequencies for wild tobacco (*Nicotiana plumbaginifolia*) protoplasts (Houba-Herlin et al., 1994) and rice (*Oryza sativa*; Sugimoto et al., 1994). They may be difficult to apply to plant species that are not as accessible to protoplast transformation and regeneration. Here, we describe a new method that employs *Ds*-containing T-DNA. Transformed cells were selected for the presence of *Ds* and against preservation of T-DNA. The majority of regenerated plants contained a single *Ds*

insertion without additional foreign DNA. The frequency of single-copy and backbone-free transformation events was only about 2.5-fold lower than that determined for conventional T-DNA transformation of potato. Importantly, our studies indicate that transposable elements can excise from nonreplicating and relatively short linear DNA segments for efficient integration into the plant genome.

RESULTS

Production of Transformed Plants

Previous studies indicated that T-DNAs often degrade within days upon transfer to plant cells (Bravo-Angel et al., 1999; Rommens et al., 2004). As modeled in Figure 1A, we hypothesized that such linear DNA molecules could function as transient vehicles for nuclear delivery of transposable *Ds* elements. Early transposition events might coincide with loss of T-DNA, thus resulting in the regeneration of plants only containing *Ds*. This theory was tested by employing the T-DNA shown in Figure 1B. The *Ds* element of this T-DNA consisted of an expression cassette for the neomycin phosphotransferase (*nptII*) gene inserted between the minimal *Ac*-derived sequences that are required for nonautonomous transposition (Weil and Kunze, 2000). The element was positioned next to the *Ac*-*Tpa* gene fused to the asparagus (*Asparagus officinalis*) *Pr1* (AoPr1) promoter. By rapidly responding to both wounding and infection (Warner et al., 1993), the AoPr1 promoter was expected to drive early *Tpa* gene expression and allow *Ds* excision prior to T-DNA degradation. To select against stable T-DNA integration, an expression cassette for the negative selectable marker gene cytosine deaminase (*codA*) was placed on the other side of *Ds*. The protein encoded by this gene converts 5-fluorocytosine (5FC) into toxic 5-fluorouracil (Perera et al., 1993). A binary vector carrying *Ds* flanked by *Tpa* and *codA* gene expression cassettes, designated pSIM269, was introduced into *Agrobacterium* LBA4404, and the resulting strain was used to infect three groups of 1,500 potato stem explants. Control transformations were performed with a binary vector carrying the *nptII* gene inserted between T-DNA borders (Fig. 1C).

After a 2-d cocultivation period, infected explants were subjected to kanamycin to select for proliferating cells that contained *Ds*. A subsequent transfer to medium supplemented with 5FC allowed selection against stable integration events of *codA*-carrying T-DNA. Figure 2 shows that this double-selection procedure yielded $0.65\% \pm 0.02\%$ shoots explant⁻¹. Much higher frequencies were obtained by either omitting the 5FC step ($22.2\% \pm 1.1\%$ shoots explant⁻¹) or employing the conventional T-DNA vector pSIM401 for standard transformation and selection ($19.5\% \pm 1.6\%$ shoots explant⁻¹; Fig. 2). These results indicate that approximately 97% of kanamycin-resistant cells were

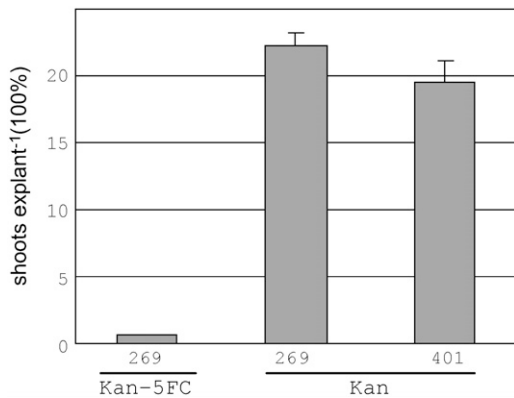


Figure 2. Potato transformation frequencies (transformed shoots explant⁻¹) × 100% for *Agrobacterium* strains containing pSIM269 and pSIM401, respectively. After cocultivation, explants were placed on medium containing kanamycin (kan). After 20 d, one group of explants infected with *Agrobacterium*:pSIM269 was transferred to 5FC (Kan-5FC). Data represent the average ± SE of three experiments.

eliminated during the negative selection procedure, apparently because these cells contained stably integrated T-DNAs that expressed the *codA* gene.

Plants Transformed with *Ds* Only

The 5FC-insensitive plants obtained from infections with the pSIM269 strain were genotyped by carrying out PCR on each of at least three leaves per plant. As shown in Figure 3, this analysis identified four different genotypes. The first and largest genotypic group consisted of 16 plants that were positive for *Ds*, but lacked other parts of the T-DNA and backbone of pSIM269 (plants 269-3, 4, 5, 6, 7, 12, 13, 16, 18, 19, 20, 21, 22, 23, 24, and 27). The consistency of PCR results indicated that the group I (*Ds*) plants represented stably transformed rather than chimeric events. Further evidence for the genetic uniformity of these plants was obtained from DNA gel-blot analysis. This experiment demonstrated that the majority of transformants, 15 of 16, contained a single *Ds*-hybridizing band that displayed a similarly strong signal intensity to that of a control pSIM368 T-DNA plant, which contains expression cassettes for the *nptII* and *codA* genes (Fig. 4; data not shown). Hybridization of stripped DNA filters with probes derived from *Tpa* and *codA* genes confirmed the absence of T-DNA sequences that had originally flanked *Ds* (Fig. 4; data not shown). Collectively, our results support the model that *Ds* elements can transpose from nonintegrating T-DNAs into the progenitor cells of transformed plants (Fig. 1A).

The recovery of 15 *Ds*-only plants from 4,500 explants indicates a *Ds* transformation frequency of 0.3% ± 0.02%. This frequency is much lower than the above-described 19.5% for conventional pSIM401 transformation. In this latter case, however, PCR analysis found 84% ± 1% of plants contained backbone DNA (Fig. 5A), whereas approximately 73% of plants rep-

resented multiple T-DNA integration events (Fig. 5B). Transgenic plants carrying such superfluous DNA are more difficult to characterize molecularly and are often discarded early on during the commercial line selection process. Thus, 0.8% of control pSIM401 plants contained backbone-free and single-copy T-DNA insertions. This frequency is only 2.5-fold higher than that for single-*Ds* transformation.

Employment of the pSIM269 Strain Can Give Rise to Aberrant *Ds* Transposition Events

Three transformed plants contained the *Ds* element together with (1) a junction fragment comprising *Ds* and downstream DNA; and (2) the *codA* gene (269-9, 11, and 17; Fig. 6). The absence of T-DNA sequences upstream from *Ds* in these group II plants can be explained most easily by assuming that the element dissociated from its T-DNA at the 5' side only. The same hypothesis was used before to explain the frequently

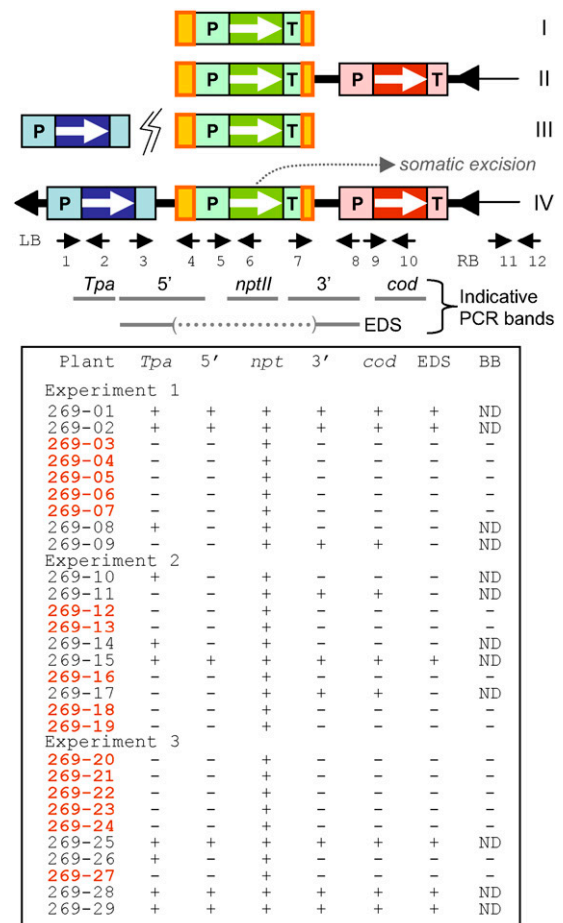


Figure 3. PCR-based genotyping of transgenic plants. Primers are indicated as arrows with sequences shown in “Materials and Methods.” Roman numerals indicate the four different genotypes that were identified. 5', Junction of upstream T-DNA and flanking 5' end of *Ds*; 3', junction of the 3' end of *Ds* and flanking downstream T-DNA; *cod*, *codA* gene; BB, pSIM269 plasmid backbone.

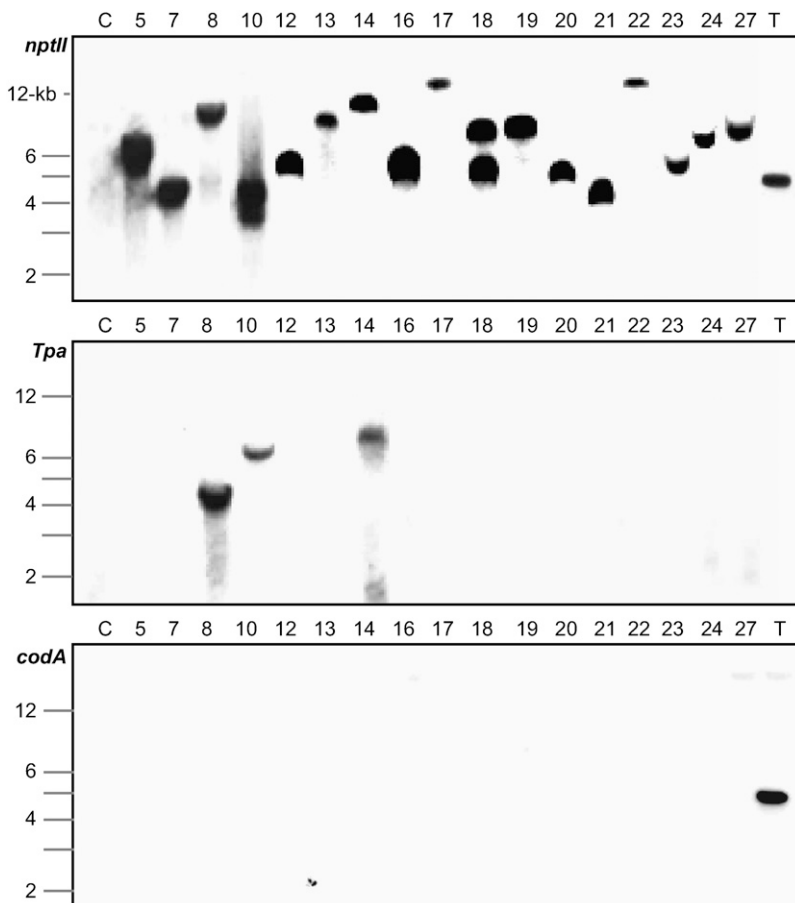


Figure 4. DNA gel-blot analysis of group I and group III plants. Lanes contain 10 μ g each of *EcoRV*-digested plant DNA. Filters were hybridized with internal fragments of *nptII* (top) and *Tpa* (bottom). Plant DNA fragments hybridizing with *nptII* represent independent insertions of *Ds*. These fragments have a size of at least 3.1 kb, which is the distance from one end of *Ds* to the internal *EcoRV* site. C, Untransformed controls; T, pSIM368 T-DNA plant.

occurring deletion of transposon-flanking DNA sequences in both plants and bacteria (Dooner et al., 1988; Roberts et al., 1991; Jilk et al., 1993). Indeed, sequence analysis of plant 269-9 demonstrated that this plant contained new sequences upstream from the transposable element (data not shown).

The genotype of four additional plants, 269-8, 10, 14, and 26, resembled that of the second group, but, interestingly, lacked the junction of *Ds* and the downstream T-DNA (Fig. 3). These group III plants may have originated from extrachromosomal excision followed by independent integration of both *Ds* and one of the broken T-DNA fragments. Evidence for this hypothesis came from DNA gel-blot analyses, which demonstrated that the four plants contained the *nptII* and *Tpa* genes on separate *EcoRV* fragments (Fig. 4; data not shown). For instance, plant 269-8 contained the *nptII* gene on an 11-kb fragment, whereas the *Tpa* gene was located on a fragment with a size of 5 kb. An alternative explanation for the molecular organization of this third group of plants is that a transposed *Ds* element cointegrated with independently transferred and severely truncated T-DNA. Such a course of events is unlikely, however, because truncated T-DNAs usually contain the 5' part of the T-DNA rather than the 3' part (Young and Nester, 1988). All *Ds* bands displayed high signal hybridization intensities, which are indic-

ative of initial integration events. The absence of weak *Ds* bands suggests that the *Ac-Tpa* gene of group III plants was not functionally active (Fig. 4). This inactivity may be due to either position-integration effects or truncation of the *Tpa* gene expression cassette.

Some Plants Derived from Transformation with the pSIM269 Strain Represent Conventional T-DNA Integration Events

The last group of plants (269-1, 2, 15, 25, 28, and 29) were positive for internal fragments of not only the *nptII* and *Tpa* genes, but also the *codA* gene. Position integration effects may, in these exceptional cases, have limited expression of the negative selectable marker gene, allowing the plants to survive subjection to 5FC. The six group IV plants also contained both upstream and downstream junction fragments for *Ds* and flanking T-DNA sequences. Such junctions are designated here the 5' and 3' full donor site (FDS) and indicate the presence of *Ds* at its original position in the T-DNA. However, the elements were not entirely dormant because primers located about 0.2 kb upstream and downstream, respectively, from the original site of *Ds* (Pr3 and 8) could in all cases be used successfully to amplify an 0.4-kb empty donor site (EDS) fragment (Fig. 3). Further evidence for this

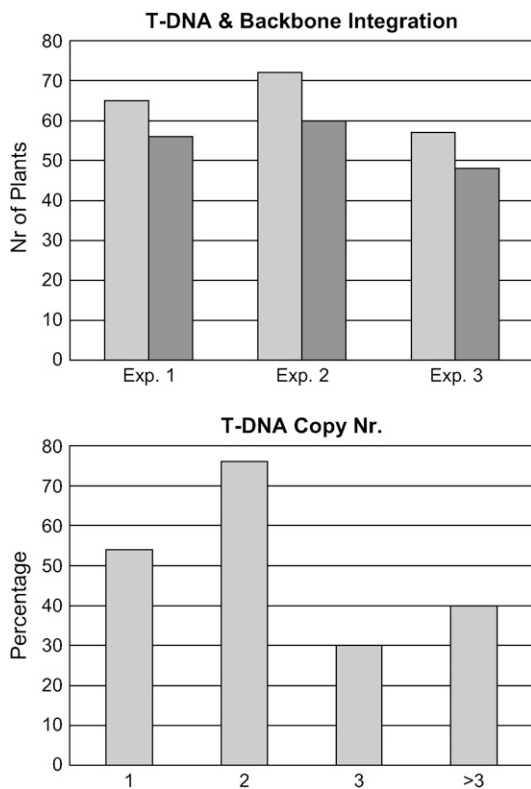


Figure 5. Frequencies for backbone DNA integration and multiple-copy T-DNA integration for conventional potato transformation. A, Three groups of kanamycin-resistant transgenic plants (light gray bars) were PCR analyzed for the presence of backbone DNA (dark gray bars). B, Copy numbers were defined by hybridizing *EcoRV*-digested DNA with an *nptII* gene-derived probe.

somatic excision of *Ds* in some, but not all, plant cells came from DNA gel-blot analysis. In addition to bands that hybridized strongly to both *Tpa* and *nptII* (*Ds*) genes, all plants of this group contained additional minor bands that hybridized only to *nptII* (Fig. 6; data not shown). This activity of *Ds* has been described before for a variety of plant species transformed with *Ds*-containing T-DNAs, including potato (Haring et al., 1989; Pavingerova et al., 2001).

Expression Levels of the *Ds*-Residing *nptII* Gene

The integration sites of *Ds* elements that had transposed from extrachromosomal T-DNAs were characterized by carrying out inverse PCRs for the randomly chosen group I and group III plants 269-4, 5, 7, and 8. Sequence analyses of recovered junction fragments demonstrated that the elements had maintained their integrity during transposition from extrachromosomal T-DNAs into the plant genome. Furthermore, the integration events were in all cases unique and associated with typical 8-bp target site duplications (Fig. 7). In plant 269-4, the insertion is 1.5 kb downstream of the 1,3- β -glucan glucanhydrolase gene AJ586738. Plant 269-5 contained an insertion in the 5'-untranslated

leader sequence of a putative CCCH-type zinc finger protein (GenBank accession no. BG889316), and the insertion of plant 269-8 was positioned immediately downstream of the start codon of an unknown potato gene (DV627343). These integration events provide new examples for the previously observed tendency of transposable elements to often insert near the 5' end of genes (Pan et al., 2005). In contrast, plant 269-7 contained the element positioned in the terminator region of an unknown gene (CV498058) at a distance of 438 bp downstream from the 3'-transcript cleavage site. We also found that sequences within approximately 200 bp from the integration site consisted of AT-rich (64%) DNA comprising at least one putative motif with the purine-rich consensus sequence 5'-AGATNTAGAG-AGAAA (Fig. 7; data not shown). Collectively, our results demonstrate that *Ds* elements can excise from extrachromosomal T-DNAs to subsequently integrate into plant genomes.

To test our initial hypothesis that transposon-based transformation would support higher frequencies of transgene expression than conventional T-DNA transformation, we determined the *nptII* RNA levels in both *Ds* plants and pSIM401 controls by real-time quantitative reverse transcription (RT)-PCR. Figure 8 shows that expression levels of the 15 *Ds* plants were, on

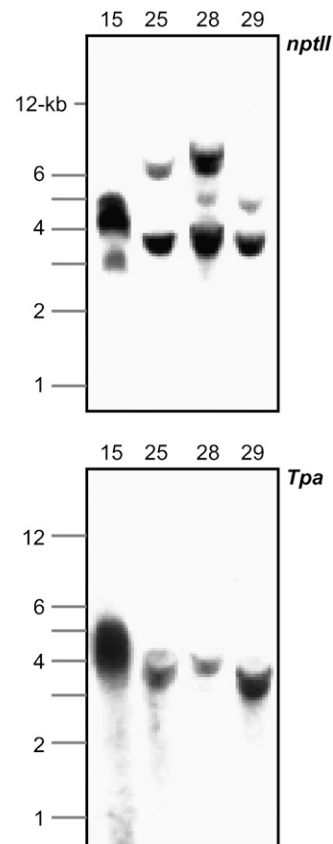


Figure 6. DNA gel-blot analysis of pSIM269 T-DNA (group IV) plants. See the legend of Figure 3 for details.

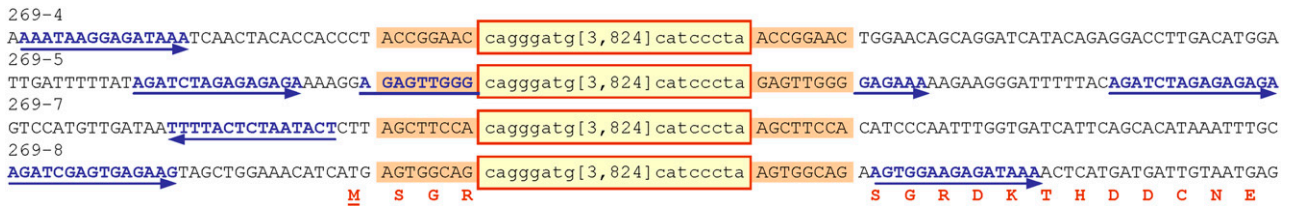


Figure 7. *Ds* integration sites. Intact transposable elements are boxed with terminal octanucleotides (shown in lowercase) and omitted nucleotides (shown between brackets). Target site duplications are highlighted. Sequences resembling the 5'-AGATCTAGAGAGAAA consensus are shown in blue with arrows indicating their orientation. Amino acids encoded by the gene that is disrupted in plant 269-8 are shown below the DNA sequence in red and bold with the first Met (M) underlined.

average, twice as high as those of nine pSIM401 controls ($P = 0.03$). This difference was due, in part, to very low expression levels in plants pSIM401-4 and 5. Apparently, these *nptII*⁺ plants had undergone transgene silencing after surviving kanamycin selection. Three *Ds* plants that contained unusually high amounts of RNA also contributed to the generally higher gene expression levels of transposed *nptII* genes. Although further studies are needed to elucidate this link, our data indicate that the *nptII* gene is generally expressed at higher levels if positioned within a transposed *Ds* element than if part of an integrated T-DNA. Our results also demonstrate that *Ds* elements can excise from extrachromosomal T-DNAs for subsequent integration into regions that often promote high levels of gene expression.

DISCUSSION

We have shown that T-DNAs containing both a *Ds* element and *Ac-Tpa* gene can be used as vehicles for transposition-based plant transformation. Excision from nonintegrating T-DNAs is followed by *Ds* integration into the plant genome, often without cointegration of T-DNA sequences. The resulting *Ds* plants are not chimeric, indicating that transposition occurred from nonintegrating T-DNAs into the genomes of progenitor cells. Previous studies demonstrated that *Ds* elements can excise from chromosomes, plasmids, and geminiviruses. Our results now also indicate that linear and short (approximately 4.5-kb) flanking DNA segments are sufficient to support cut-and-paste recombination processes of *Ds*. Such excision events are likely to have occurred after the originally single-stranded and *virE2*-coated T-DNAs converted into their double-stranded form (Tzfira et al., 2003) prior to T-DNA degradation.

Given the relatively low frequency of early transposition, it is not surprising that almost all group I plants contained a single copy of the *Ds* element. However, our results contradict earlier findings on *Ds* transposition from plasmid DNA into the genome of wild tobacco (Houba-Herlin et al., 1994). Although these earlier studies indicated very low frequency of *Ds* transformation (approximately 0.001%), more than one-third of plants obtained through this method con-

tained three copies of *Ds*. This discrepancy between plasmid- and T-DNA-based delivery systems may be explained by the assumption that the copy number of plasmids in the nuclei of polyethylene glycol-treated protoplasts is higher than that of T-DNAs in the individual plant cells of *Agrobacterium*-infected explants. The abundance of plasmids may have given rise to multiple simultaneous transposition events.

Ds elements that excised from integrated T-DNAs were often shown to integrate into DNA regions that support high gene expression levels (Koprek et al., 2001). We found this tendency also to be linked to transposition from nonintegrating T-DNAs. Thus, transposable elements may be used as efficient vehicles for the high-level expression of genes of interest. Further studies need to be performed to determine whether such high gene expression levels can also be obtained with plant genes, which may be more easily silenced (Garcia-Perez et al., 2004). Infrequently, *Ds* integration may result in the inactivation of agronomically important genes. Such mutations are not likely to cause mutant phenotypes because potato suffers from inbreeding depression and is generally not self fertilized to produce homozygotes.

It may be possible to further increase *Ds* transformation frequencies by modifying either the construct

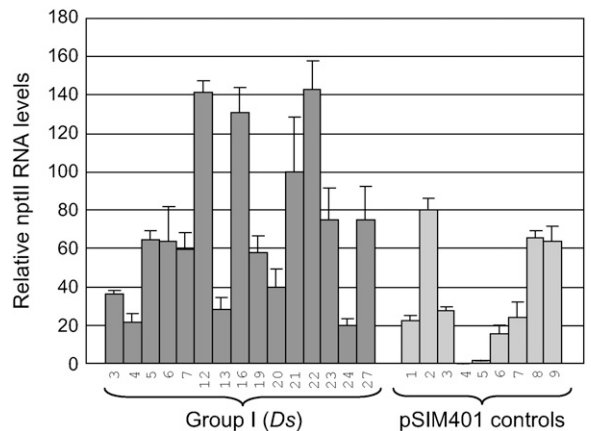


Figure 8. Expression levels of the *nptII* gene. Data represent the mean \pm SE of three independent experiments as determined by quantitative real-time RT-PCR.

or the selection procedures. For instance, the timing of *Tpa* gene expression may be optimized by employing promoters, such as the 35S promoter of the *Cauliflower mosaic virus*, that are known to trigger early *Ds* excision if fused to the *Tpa* gene (Scofield et al., 1992). Alternatively, identification of plants only containing *Ds* may be facilitated by employing different kinds of selection strategies. Insertion of *Ds* between a promoter and the *nptII* gene would make it possible to apply transient selection procedures that were developed to generate marker-free transformed plants (Rommens et al., 2004). Proliferating cells that survive this treatment would contain excised elements. A negative selection system can then be used to eliminate most T-DNA integration events, whereas a visual marker within *Ds* would facilitate the identification of regenerated shoots that contain a transposed element.

Plant 269-18 is a unique group I plant in that it contains two *Ds* elements. These elements may have independently transposed from two cotransferred and nonintegrating T-DNAs. However, it is also possible that copy number was doubled by the occurrence of a second transposition event from replicated into unreplicated DNA in a similar manner as shown before for *Ds* transposition in maize (Chen et al., 1992; Duval-Valentin et al., 2004). In that case, the similar intensity of the two visualized *Ds* bands would indicate that the second transposition occurred very early during cell proliferation.

Because *Ac-Tpa* sources have only been identified in maize, *Ds* elements are likely to remain stably integrated at their original positions in the potato genome. In fact, there have been no reports on *Ac-Tpa*-independent transposition of *Ds* in any heterologous plant system. Expression levels of genes that reside within the transposed *Ds* elements are also expected to remain stable. One argument for this assumption comes from the finding that transposon methylation has only been associated with nontransposed elements that reside in T-DNAs located within highly repetitive genomic regions (Koprek et al., 2001). Furthermore, studies on the progeny of transgenic rice plants containing transposed *Ds* elements indicate that expression levels are not likely to be affected by a gradual methylation of *Ds* (Szeverenyi et al., 2006).

Our studies also identified transformation events that were associated with aberrant transposition. Group II plants contained *Ds*, *codA*, and the intermediary 3' FDS, but lacked the 5' FDS and EDS. Most likely, this genotype was generated by insertion of truncated T-DNAs. Alternatively, the absence of upstream sequences was a consequence of *Ds* excision attempts. Such activities would result in adjacent deletions that have been reported for both plant and bacterial transposons (Dooner et al., 1988; Roberts et al., 1991; Jilk et al., 1993). The third group consists of plants that contained both an intact *Ds* element and at least part of the *Ac-Tpa* gene. However, these plants lacked the junction between the two elements. They may have arisen from the independent integrations of

an excised *Ds* and a resulting broken T-DNA fragment. Further studies will be required to elucidate the precise molecular basis of both the *Ds* 5' FDS *codA* and *Tpa-Ds* genotypes.

In contrast to the early transpositions that yielded group I to group III plants, transpositions from integrated T-DNAs resulted in the development of chimeric group IV plants. Even if such late transpositions would be followed by rare recombination events that triggered T-DNA deletion, the multiple resulting cell lines would be extremely unlikely to eventually give rise to plants resembling any of the other groups of plants.

The two-component *Ds-Ac-Tpa* system described here may not represent the only tool kit for transposon-based transformation. Various plant species have been shown to contain elements that belong to the *Ac-Ds* family. Such elements include *Tip100* of common morning glory (*Ipomoea purpurea*; Ishikawa et al., 2002), *Pac1* of pearl millet (*Pennisetum glaucum*; MacRae et al., 1994), and various elements in sugar cane (*Saccharum officinarum*; de Araujo et al., 2005). Furthermore, it may be possible to employ other transposable element systems, such as Arabidopsis (*Arabidopsis thaliana*) *Tag1* (Frank et al., 1997) and maize *En-Spm* (Kumar et al., 2005).

The two different protoplast-based *Ds* transformation systems that were described more than a decade ago have found little or no utility in commercial crop transformation programs. Drawbacks of these early methods include low transformation frequencies and

Table I. Primers

Name	Sequence
Pr1	5'-CGTTCCTGAATCAAAGTGACCTGTAC
Pr2	5'-CTCTTGTGGTAGACATCAACTTGGC
Pr3	5'-GCATGCTAAGTGATCCAGATG
Pr4	5'-CTGCAGTCATCCCGAATTAG
Pr5	5'-AGGAAGGAATTCCTCCGGATCAGC
Pr6	5'-AGGAGCAAGGTGAGATGACAGG
Pr7	5'-GGAATTCGCGTAGACTTATATGGC
Pr8	5'-TGATGACCAAATCTTGTCATCCTC
Pr9	5'-GAATCAGCTAATCAGGGAGTGTG
Pr10	5'-GCCATGCGCGTTGTTTACATCG
Pr11	5'-CACGCTAAGTGCCCGCCGTCGGAG
Pr12	5'-TCCTAATCGACGGCGCACCGGCTG
Pr13	5'-TGGGTGGAGAGGCTATTCGGCTATGA
Pr14	5'-ATGTCCTGATAGCGGTCCGCCACA
Pr15	5'-CGTTCCTGAATCAAAGTGACCTGTAC
Pr16	5'-CTCTTGTGGTAGACATCAACTTGGC
Pr17	5'-ACGAAAACGGAACGGAAACGGGATATAC
Pr18	5'-ATCGGTTATACGATAACGGTCCGGTACG
Pr19	5'-GTAGAGCTAGTTTCCCGACCGTTTTCAC
Pr20	5'-GATTTTCCCATCCTACTTTTCATCCCTG
Pr21	5'-CGACCGGATCGTATCGGTTTTTCGATTAC
Pr22	5'-GCACATAAGTGATAAGTCTTGGGCTC
Pr23	5'-CGGTAGAGGTATTTTACCGACCGTTAC
Pr24	5'-CTAACATAAGAAGCCATATAAAGTCTAC
Pr25	5'-CGTCGATTCAGATTATCCA
Pr26	5'-ACGAAGAAAACGACAATCCCAAC
Pr27	5'-TGCAAGACGAGGCAGCGCGGCTAT
Pr28	5'-AGGAGCAAGGTGAGATGACAGG

cumbersome procedures for the proliferation and regeneration of protoplasts from crop plants. The use of T-DNAs as *Ds* delivery systems makes it possible now to use explants rather than protoplasts for transposition-based transformation. The ease of use and broad applicability of an explant transformation/regeneration system may encourage broader exploitation of transposable elements as tools for plant transformation. Transposition-based plant transformation provides new means of genetically modifying plants without inserting bacterial DNA into their genomes. A previously developed method employs plant-derived transfer DNAs as alternatives to *Agrobacterium* T-DNAs (Rommens et al., 2004, 2005). Application of any of these two methods may alleviate some of the public concerns about the permanent introduction of bacterial, viral, and synthetic DNA into the genomes of food crops (Rommens, 2004).

MATERIALS AND METHODS

Plant Transformation

Ten-fold dilutions of overnight-grown *Agrobacterium* LBA4404 cultures were grown for 5 to 6 h, precipitated for 15 min at 2,800 rpm, washed with Murashige and Skoog liquid medium (PhytoTechnology Laboratories) supplemented with Suc (3%, pH 5.7), and resuspended in the same medium to 0.2 OD₆₀₀ nm. The resuspended cells were mixed and used to infect 0.4- to 0.6-mm internodal segments of the potato (*Solanum tuberosum*) variety Ranger Russet. Infected stems were incubated for 2 d on coculture medium (one-tenth Murashige and Skoog salts, 3% Suc, pH 5.7) containing 6 g/L agar A111 (Phytotechnology) at 22°C in a Percival growth chamber (16 h light) and subsequently transferred to callus induction medium (Murashige and Skoog medium supplemented with 3% Suc, 2.5 mg/L of zeatin riboside, 0.1 mg/L of naphthalene acetic acid, and 6 g/L of agar) containing timentin (150 mg/L) and kanamycin (100 mg/L). After about 3 weeks, proliferating explants would start to develop shoots. This shoot formation was then enhanced by transfer, 1 week later, to shoot induction medium (Murashige and Skoog medium supplemented with 3% Suc, 2.5 mg/L of zeatin riboside, 0.3 mg/L of GA₃, and 6 g/L of agar) containing timentin, kanamycin, and, for most explants infected with *Agrobacterium*:pSIM269, 5FC (500 mg/L). The resulting shoots were harvested 1 month after transfer and placed on Murashige and Skoog medium with 3% Suc, 6 g/L of agar, and timentin (150 mg/L) for root development.

Plant Genotyping

Plant DNA was isolated by using a robust and reliable method described previously (Rommens et al., 2004). The primer pairs Pr1-Pr2 were used to visualize a junction fragment for the AoPR1 promoter and the linked *Tpa* gene, Pr3-Pr4 for 5' FDS, Pr5-Pr6 for the promoter-*nptII* gene fusion within *Ds*, Pr7-Pr8 for 3' FDS, Pr9-Pr10 for promoter-*codA* gene junction, and primers Pr3-Pr8 for EDS. The primer pair used to determine the presence of vector backbone sequences was Pr11-Pr12 (Table I).

DNA Gel-Blot Analysis

Plant DNA isolation was conducted using the DNeasy kit (Qiagen). *EcoRV* digests were run on gel, blotted, and hybridized with radioactively labeled probes as described previously (Rommens et al., 1992). Primers for amplification of fragments that were used to generate probes to visualize the presence of the *nptII* (*Ds*) and *Ac-Tpa* genes (remaining T-DNA) were Pr13-Pr14 and Pr15-Pr16, respectively.

Isolation of Target Sites

To amplify the junctions of *Ds* and upstream flanking plant DNA, inverse PCR was carried out using *PstI*, *XbaI*, and *AsnI* digests (2 μg). After 1 h of

DNA circularization, DNA ligase treatment, and removal of salts using the QIAquick PCR purification kit (Qiagen), DNA digests were used as templates for PCR (30 cycles) with primers Pr17 and Pr18. The amplified products (1 μL) were used as a template for subsequent PCR (35 cycles) with a set of nested primers (Pr19 and Pr20). The *Ds* junctions with downstream flanking DNA were obtained by using *EcoRI*, *AflIII*, and *EcoRV* digests with Pr21-Pr22 for the first PCR, and Pr23-Pr24 for the second PCR. DNA fragments were separated by agarose gel electrophoresis, isolated using Qiagen's gel isolation kit, cloned into plasmid pGEMT-Easy (Invitrogen), and sequence analyzed using Big Dye Terminator, version 3.0 sequencing chemistry (Applied Biosystems) and an ABI 3730 DNA sequencer.

RNA Analysis

For quantitative real-time RT-PCR, RNA was isolated from pSIM269 and pSIM401 tobacco (*Nicotiana tabacum*) leaves and treated with RNase-free DNaseI (Invitrogen). Reactions were performed using 25 ng RNA and the QuantiTech SYBR Green RT-PCR kit (Qiagen) according to the manufacturer's instructions. Internal control primers (Pr25 and Pr26) were designed to amplify the cytochrome oxidase (*Cox*) gene. Primers for the *nptII* gene were Pr27 and Pr28.

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