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Genetic Transformation of an *Ac/Ds*-based Transposon Tagging System in Carrot (*Daucus carota* L.)

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Summary

Maize transposable elements, *Activator* (*Ac*) and *Dissociation* (*Ds*) have been transformed into several heterologous plant species and they have been successfully used to tag and clone genes in *Arabidopsis*, flax, petunia, tobacco, and tomato. To investigate the possibility of transposon tagging for subsequent cloning of carrot genes, carrot calli were transformed with a modified maize transposable element *Activator*-transposase (*Ac*-TPase) and *Ds* using an *Agrobacterium*-mediated transformation method. *Ds* excision was observed in the tissue culture-regenerated plants carrying both *Ac*-TPase and *Ds*. Analysis of *Ds* footprints in six plants demonstrated the occurrence of small deletions at the donor site. Reinsertion of *Ds* into new chromosomal sites was detected in the transgenic plants analysed and the analysis of sequences flanking *Ds* at the insertion sites using TAIL-PCR demonstrated that 37.5 % of *Ds* insertions were into gene regions in the carrot genome. Although previous studies suggested that the autonomous *Ac* element may not be a suitable transposon for insertional mutagenesis in carrot, our results deduced that the *Ac/Ds* based two-element transposon tagging system can be another tool to mutagenise the carrot genome for the purpose of cloning carrot genes.

Key words. *Agrobacterium* – gene cloning – mutagenesis – tissue culture

Introduction

Transposon tagging has been demonstrated to be a powerful technique to identify and clone plant genes. Although transposons are ubiquitous throughout the plant kingdom, there are many species lacking active and well characterized transposable elements (Sundaresan 1996). To expand the possibility of transposon tagging into the heterologous plants, such as carrot, lacking active and well characterized transposons, heterologous transposon tagging was developed by Baker et al. (1986) with introduction and demonstration of *Ac* transposition in tobacco.

After the demonstration of trans-activation of the non-autonomous maize transposon, *Ds*, by *Ac* in transgenic tobacco plants (Hehl and Baker 1989), *Ac/Ds* and *Spm (En)/ dSpm (I)* based two-element transposon tagging systems were developed by a number of laboratories to increase the efficiency of heterologous transposon tagging (Bancroft et al. 1992; Fedoroff and Smith 1993; Tissier et al. 1999). In two-element based transposon tagging systems, a non-autonomous element such as *Ds* or *dSpm/I* was used as transposon tag while an autonomous element such as *Ac* or *Spm/En* was used to provide the transposase (TPase) required for activation of the non-autonomous element. In these systems, the transposition capability of autonomous elements was eliminated by modifying terminal inverted repeats (TIR) sequences and to ensure higher TPase expression, their TPases were expressed using a constitutive promoter such as the CaMV 35S or *nos* (Swinburne et al. 1992; Fedoroff and Smith 1993). The non-autonomous elements in these systems such as *Ds* or *dSpm/I* can carry antibiotic or herbicide resistance genes without effecting transposition capability of the element (Fedoroff and Smith 1993; Tissier et al. 1999). These resistance genes allow selection of plants carrying that element and verify the reinsertion of the excised element.

Carrot has several attributes that could make it a suitable species for developing a transposon tagging system. It is a diploid plant species and has a well-established tissue culture system and it can be transformed using *Agrobacterium* (Scott and Draper 1987). Although it has a biennial growth habit, one generation can be completed in a year and can produce up to 5000 seeds per plant from either self- or cross-pollination (Simon 1985), which increases the probability of detecting recessive mutations in transposon tagging studies.

Previous study suggested that the autonomous *Ac* element may not be an appropriate transposon for mutagenizing the carrot genome due to its low reinsertion rate (Van Sluys et al. 1987; Van Sluys and Tempe 1989). In this study, we introduced an *Ac/Ds* based two-element transposon tagging system developed by Fedoroff and Smith (1993) into carrot to investigated *Ds* transposition and

the possibility of using *Ds* for tagging and cloning carrot genes, since elevated transposition frequency of *Ds* by modified *Ac*-TPase is well documented in other plant species. Cloning and isolation of carrot genes with horticultural importance will help the plant breeders in future carrot improvement.

Materials and Methods

Plasmid constructs

The plasmids used to generate transgenic carrot were pCGN 1548 carrying *Ac*-TPase expressed using CaMV 35S promoter and pCGN 1549 carrying *Ds* (FEDOROFF and SMITH 1993). pCGN 1549 carrying the *Ds* element contains an *NPTII* gene as a selectable marker (Fig. 1). The synthetic *Ds* element on pCGN 1549 was created by replacing internal sequences of *Ac* with a β-glucuronidase (*GUS*) gene containing minimal promoter of CaMV 35S, for enhancer trapping, and an aminocyclitol phosphotransferase (*aph4*) gene with the CaMV 19S promoter, which confers hygromycin resistance to select *Ds* carrying plants. *Ds* was inserted upstream to the acetolactate synthase (*ALS*) gene coding region, which confers chlorsulfuron-resistance. This insertion separated *ALS* from its 2xCaMV 35S promoter and inactivated *ALS* expression until *Ds* excision. Therefore, chlorsulfuron resistant plants, resulting from *Ds* excision, allow phenotypic selection of plants carrying excised *Ds* element.

Plant materials and genetic transformation

Callus production was initiated from roots of USDA carrot inbred line B493 (SIMON et al. 1990) on MS medium (MURASHIGE and SKOOG 1962) containing 4.5 μM 2,4-D (Sigma, St Louis, MO, USA) and 0.1 μM kinetin (Sigma). Carrot calli were transformed with pCGN 1548 carrying *Ac*-TPase and pCGN 1549 carrying *Ds* using *A. tumefaciens* strain LBA 4404 (HOEKEMA et al. 1983) and the transformation method described by WURTELE and BULKA (1989). Transgenic carrot calli transformed with either *Ac*-TPase or *Ds* were selected on MS selection medium containing 4.5 μM 2,4-D, 0.1 μM kinetin, 500 μg ml^{-1} cephotaxime (Sigma) and 300 μg ml^{-1} kanamycin (Sigma). Kanamycin-resistant calli transformed with *Ac*-TPase were transformed for the second time with *Ds* and transgenic calli transformed with both *Ac*-TPase and *Ds* were selected on MS medium containing 4.5 μM 2,4-D, 0.1 μM kinetin, 500 μg ml^{-1} cephotaxime, and 50 μg ml^{-1} hygromycin (Sigma) since hygromycin resistance was conferred by the *aph*4 gene carried by the *Ds* element (Fig. 1). Somatic embryogenesis was initiated on B5 medium (GAMBORG et al. 1968) containing 1 μM kinetin, no 2,4-D and appropriate antibiotics under 95 μmol m^{-2} s^{-1} cool fluorescent lights for 16 hours day^{-1} at room temperature. Somatic embryos were transferred onto B5 medium with no hormones or antibiotics to develop into plants and regenerated plants were transplanted to the pots.

After transformation, each callus from independent transformation events was labelled. For example, AcDs#1 indicates callus number 1 transformed with both *Ac*-TPase and the *Ds* element, while Ds#1 is the callus number 1 transformed with *Ds* only. AcDs#1-1 indicates the tissue culture regenerated-plant number 1 from calli, AcDs#1. Similarly, Ds#1-1 was the tissue culture regenerated-plant number 1 from Ds#1.

Isolation of carrot genomic DNA

To sample the regenerated plants, at least three leaves were collected from each plant and used for DNA extraction. Lyophilized leaf samples were ground to powder and approximately 200 μl of leaf powders were used to extract DNA by using modified CTAB method described by FÜTTERER et al. (1995).

Polymerase Chain Reaction (PCR)

For all PCR, the following reaction and thermal cycler conditions were used. Each 25 μl PCR reaction contained 0.75 units of *Taq* polymerase (Promega, Madison, WI, USA), with supplied reaction buffer with 15 mM $MgCl_2$ at 1× concentration, 0.8 μM of each primer, dNTPs at 200 μM each, 90 ng template DNA. The reactions were heated to 94 °C for 2 minutes followed by 40 cycles of 94 °C for 30 sec, 56 °C for 1 min, and 68 °C for 4 min. Then, they were held at 4 °C. For these reactions a Perkin Elmer model 9600 Thermal Cycler was used. PCR products were separated on 1 % (w/v) agarose (Invitrogen, Carlsbad, CA, USA) gels in 1×TAE (40 mM Tris-acetate

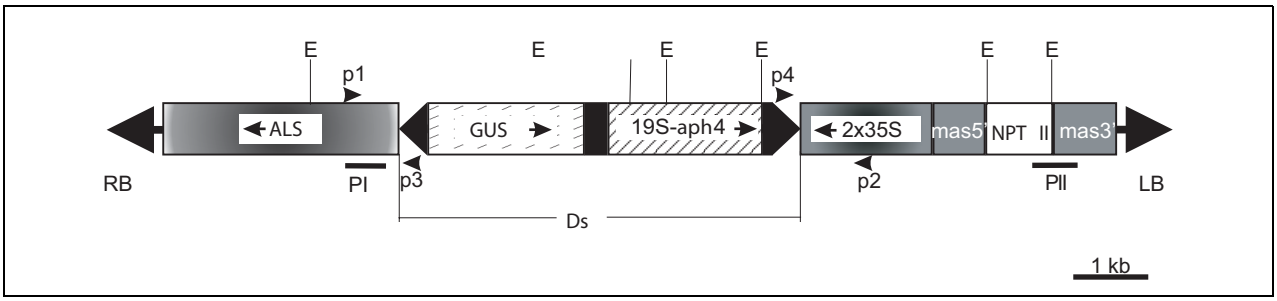


Fig. 1. pCGN1549:T-DNA constructs carrying *Ds*. RB: Right border; LB: Left border; *ALS*: Acetolactate synthase; E: *Eco*R I restriction sites; PI and PII: Southern hybridisation probes; p1 and p2: PCR primers for amplification of EDS; p3 and p4: sets of TAIL-PCR primers for 5' and 3' end of *Ds*, respectively; *GUS*: β-glucuronidase; 19S: CaMV 19S promoter; *aph*4: aminocyclitol phosphotransferase; 35S: CaMV 35S promoter; mas5': mannopine synthase promoter; mas3': mannopine synthase terminator; *NPT*II: neomycin phosphotransferase.

and 1 mM EDTA), stained with ethidium bromide (Sigma) and photographed.

Southern analysis

Five µg of genomic DNA was digested with *Eco*R I according to the protocol described by suppliers (Promega) and then size fractionated by electrophoresing through 1.5 % agarose gel along with a 1 kb DNA step ladder (Promega) as DNA molecular weight markers in 1×TAE buffer. For Southern blotting, modified protocols described by Bradeen and Simon (1998) were followed.

PCR-generated probes used in this study were amplified using plasmid pCGN 1549 carrying the *Ds* element as template and specific primers for each probe according to the PCR reaction and thermal cycler conditions described above. Probe DNA was purified from agarose gels using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) and 100 ng of eluted DNA was labelled with 5'-[α-^{32}P] deoxycitidine (Amersham Pharmacia Biotch, Inc., Piscataway, CA, USA) using the DECAprime II™ kit (Ambion, Austin, TX, USA) according to the methods described by the suppliers. Membranes were hybridised with labelled probe overnight in hybridisation buffer (30 ml hybridisation buffer: 10 ml 20 % SDS, 12 ml formamide, 60 µl of 0.5 M EDTA, and 8 ml 0.5 M Na_2HPO_4 pH 7.2) at 42 °C and washed with wash buffer (0.1×SSC and 0.25 % SDS) at 65 °C for 30 minutes. After rinsing with wash buffer, the membrane was exposed to a Packard Cyclone™ Storage Phosphor Screen overnight for visualization, and signal on the screen was detected by a Packard Cyclone™ Storage Phosphor System.

PII was used to confirm stable transformation and to estimate copy number of inserted T-DNA carrying *Ds* (Fig. 1). The following primers were used to generate PII: NPTII-UP 5'-ATC AGG ACA TAG CGT TGG CTA C-3' and MAS3'-DOWN 5'-GAC AGA TAA AGC CAC GCA CA-3'.

Excision of Ds

To test the excision of *Ds* from integrated T-DNA in the carrot genome using Southern blotting, PI probe was used (Fig. 1). Primers to generate probe PI were *ALS*-UP 5' CAT GGC GGC GGC AAC AA 3' and *ALS*-DOWN 5' ATC TAA CAA CGC ATC GGC TAA TCC 3'.

The following primers flanking the *Ds* element at the original integration site T-DNA were used to amplify Empty Donor Sites (EDS): 35S-up (p2) 5'-ACC TAA CAG AAC TCG CCG TAA AGA-3' and ALS-down (p1) 5'-ATC TAA CAA CGC ATC GGC TAA TCC-3' (Fig. 1). Twenty-five µl PCR reaction mixtures were prepared as described above using the same thermocycling conditions for amplification of EDS in plants transformed with both *Ac*-TPase and *Ds*. The products of PCR were separated by subjecting them to electrophoresis through 1.5 % agarose gels in 1×TAE buffer. Fragments were purified from agarose gels using the QIAquick gel extraction kit, cloned into the pGEM-T vector system (Promega), and sequenced. *Taq* DNA polymerase cycle-sequencing reactions were performed with fluorescent dye terminator reagents according to the conditions recommended by Applied Biosystems (Foster City, CA, USA), but using half volume reactions. DNA was sequenced with a PE-Biosystem 377 XL automated DNA sequencing instrument at the Biotechnology Center in the University of Wisconsin-Madison.

Reinsertion of Ds

Thermal asymmetric interlaced PCR (TAIL-PCR) was utilized to amplify sequences flanking *Ds* according to the protocol described by Tsugeki et al. (1996). One set of three nested primers was designed for the 5' end of *Ds*: Ds5-1 5'-ACG GTC GGG AAA CTA GCT CTA C-3', Ds5-2 5'-CCG TTT TGT ATA TCC CGT TTC CGT-3' Ds5-3 5'-TAC CTC GGG TTC GAA ATC GAT-3' (Fig. 1). Another set of three primers was also designed for the 3' end of *Ds*: Ds3-1 5'-ACC CGA CCG GAT CGT ATC GGT-3', Ds3-2 5'-CGA TTA CCG TAT TTA TCC CGT TC-3', and Ds3-3 5'-GTA TTT ATC CCG TTC GTT TTC GT-3' (Fig. 1). Two arbitrary degenerate primers were synthesized (Liu et al. 1995): AD2 5-(G/C)TTG(A/T/C/G)TA(G/C)T (A/T/C/G) CT(A/T/G/C)TGC-3' (256-fold degeneracy and T_m=47.9 °C) and AD5 5-(A/T)CAG(A/T/G/C)TG(A/T)T (A/T/G/C) GT(A/T/G/C)CTG-3' (256-fold degeneracy and T_m=47.9 °C). Primary, secondary, and tertiary PCR reactions and thermal cycling conditions were according to the protocols described by Tsukegi et al. (1996). Tertiary PCR products (50 µl) were separated by electrophores on 2 % agarose gels, and the DNA fragments were purified from gels, cloned into plasmid vectors and sequenced according to the method described above. The nucleotide sequence of each TAIL-PCR product was compared with the sequences in the GenBank databases using both the BLASTN and BLASTX program at NCBI and the alignments of these sequences were displayed with GENEDOC program (Nicholas and Nicholas 1997).

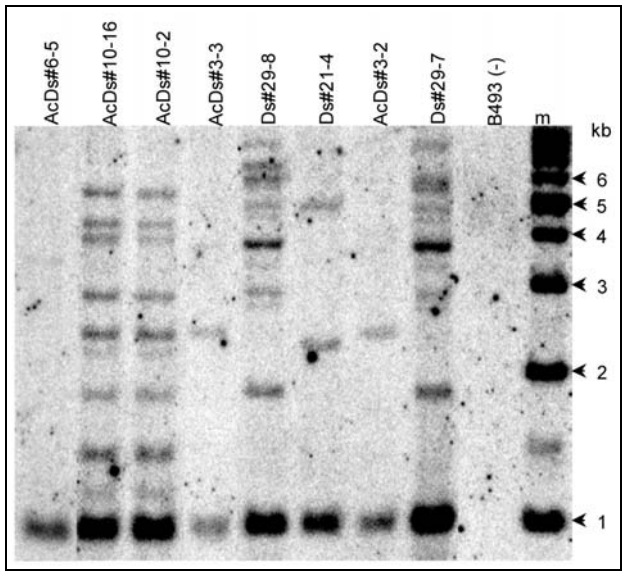


Fig. 2. Southern blotting with probe PII (NPT II-Mas 3' region) to confirm transformation and to estimate copy number of the T-DNA carrying *Ds* construct in transgenic carrots. The eight lanes from AcDs#6-5 to Ds#29-7 contained DNA samples from putative transgenic plants digested with *Eco*R I. Lanes AcDs#10-16 and AcDs#10-2, lanes AcDs#3-2 and AcDs#3-3, and lanes Ds#29-8 and Ds#29-7 were regenerated from the same transformation events and shared identical banding patterns. Lane B493 (-) was a DNA sample from a non-transgenic plant that was also digested with *Eco*R I. m: DNA molecular weight markers.

GUS assay

GUS expression in hygromycin resistant calli and somatic embryos were tested using the histochemical *GUS* assay described by (JEFFERSON et al. 1987).

Results and Discussion

Southern analysis indicated that T-DNA carrying *Ds* was introduced into the carrot genome successfully. *Eco*R I digestion of T-DNA carrying *Ds* generates a 1kb fragment of *NPTII* gene, detectable by probe PII (Fig. 1). This internal *Eco*R I fragment was detected with PII in each of eight putative transgenic plants examined (Fig. 2). Additional DNA fragments greater than 1 kb in length were also detected by PII in all transgenic carrots. These fragments represent *mas* terminator (mas 3' in Fig. 1), left border (LB), and DNA fragment flanking LB of the T-DNA. The exact sizes of these fragments will depend on the distance between LB and the next *Eco*R I site in the carrot genome at the insertion site. The number of *Eco*R I fragments detected by Southern analysis suggested that transgenic carrot plants could contain from one to at least nine copies of T-DNA carrying *Ds* integrated into the genome (Fig. 2). HARDEGGER and STURM (1998) also reported high copy numbers of T-DNA transferred by *Agrobacterium tumefaciens* strain LBA 4404 in carrot. The authors indicated that strain LBA 4404 transferred higher copies of T-DNA to the carrot genome than strain GV3101. WURTELE and BULKA (1989) detected from 1 to at least 15 copies of T-DNA integrated into the carrot genome by *Agrobacterium* strain GV3850. The authors concluded that the high number of T-DNA copies integrated into the carrot genome may be due, in part, to the higher level of kanamycin concentration (300 μg ml^{-1}) used in the selection medium.

The carrots evaluated in Fig. 2 are a subset of plants regenerated from seven independent calli transformed with *Ds* only, and 13 with both *Ac*-TPase and *Ds*. Primary transformed plants regenerated from calli AcDs#3, AcDs#6, and AcDs#10 carrying both *Ds* and *Ac*-TPase and calli Ds#15, Ds#18, Ds#21, and Ds#29 carrying only *Ds* were analysed for *Ds* transposition in this study.

Excision of Ds

Southern blot analysis of *Eco*R I digested genomic DNA from tissue culture-regenerated plants, co-transformed with both *Ac*-TPase and *Ds,* indicated that the *Ds* element was excised from its original cloning site on T-DNA within the carrot genome. If *Ds* did not excise, probe PI should detect only one 4.5 kb *Eco*R I fragment of nuclear DNA between the first and second restriction sites in Fig. 1, whereas a 3.6 kb fragment is expected if *Ds* excised. DNA samples transformed with both *Ac*-TPase and *Ds* had either two bands with the sizes of 4.5 kb and 3.6 kb or only the 3.6 kb band (Fig. 3) indicating that *Ds* had excised from its original cloning site in either some or all of the cells, respectively. Only a 4.5 kb PI-hybridising DNA fragment was present in transgenic plants transformed with *Ds* only, indicating no *Ds* excision. Sequencing of empty donor sites (EDSs) in plants carrying excised *Ds* revealed that small deletions of flanking genomic sequences occurred during *Ds* excision (Table 1). In two plants, there was a 3-bp-deletion (C-GC) while four plants had an 8-bp-deletion in the EDS (C-GCGTGAC).

The sizes of deletions were as of expected according to the previous reports in tobacco (BAKER et al. 1986), maize (SCOTT et al. 1996), *Arabidopsis* (RINEHART et al. 1997) and barley (SCHOLZ et al. 2001) indicating that similar excision and repair mechanisms were responsible for generation of footprints in carrot. *Ds* footprints in AcDs#10-2 and AcDs#10-16 are probably generated by the same ear-

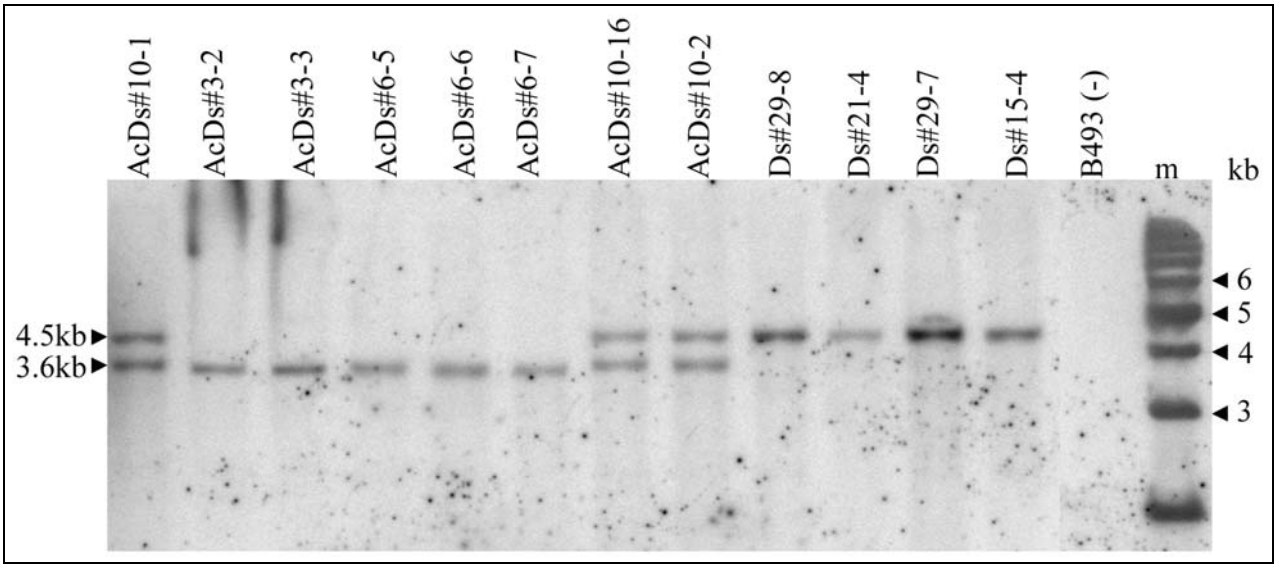


Fig. 3. Southern blotting with probe PI (for *ALS* gene) to test excision of *Ds*. Lanes from AcDs #10-1 to AcDs #10-2 contained *Eco*R I digested DNAs from plants transformed with both *Ac*-TPase and *Ds*. Lanes from Ds#29-8 to Ds#15-4 contained *Eco*R I digested DNA from plants transformed with *Ds* only. Lane B493 (-) contained *Eco*R I digested DNA from a non-transgenic plant as a negative control. m was DNA molecular weight markers.

Table 1. Analysis of *Ds* footprints after excision from T-DNA in six transgenic carrot plants. The last line is the original flanking sequence of the *waxy* locus of maize in the transformation construct carrying *Ds*.

Plants	Empty donor site
AcDs#6-5	G C G T G A C . - . . G T G A C C C G
AcDs#6-7	G C G T G A C . - . . . . . C C G
AcDs#3-3	G C G T G A C . - . . G T G A C C C G
AcDs#3-2	G C G T G A C . - . . . . . C C G
AcDs#10-16	G C G T G A C . - . . . . . C C G
AcDs#10-2	G C G T G A C . - . . . . . C C G
waxy locus	G C G T G A C C - G C G T G A C C C G

"." shows deleted sequences during *Ds* excision. "-" indicates sequences of the *Ds* element.

ly excision event in the callus line before the regeneration of these plants. Although AcDs#3-2, AcDs#6-7 and AcDs#10-16 were regenerated from independent transformation events, an 8-bp-deletion pattern was observed in all three transgenic plants. Similarly, a 3-bp-deletion event was observed in two independent transgenic plants, AcDs#3-3 and AcDs#6-5. SCHOLZ et al. (2001) reported occurrence of two specific footprint sequence patterns in more than one independent barley transformant. The same footprint sequences were also shared by several independently transformed callus lines of rice (IZAWA et al. 1991). SCOTT et al. (1996) proposed that the sequences flanking *Ds* in maize can influence the host (maize) repair mechanism, which is an important factor in the process of generating excision patterns. In this study, the *Ds* element was transformed with *Agrobacterium tumefaciens* and shared identical T-DNA sequences flanking *Ds*. It is likely that the T-DNA sequences flanking *Ds* may have influenced the excision or repair mechanism to generate the same footprints in carrot plants regenerated from independent transformation events.

Although *Ds* footprint sequences were analysed in six plants, using primers p1 and p2 (Fig. 1), we were able to amplify EDS in all 58 plants regenerated from AcDs#3, AcDs#6 and AcDs#10 indicating *Ds* excision. However, EDS was not amplified in any of 26 plants regenerated from Ds#15, Ds#18, Ds#21, and Ds#29 carrying only *Ds* (data not presented). VAN SLUYS et al. (1987) reported that autonomous *Ac* excised in five of 18 independently transformed carrot root cultures, but in that study, the *Ac*-TPase gene was driven by its natural promoter. In our study, the *Ac*-TPase gene was under the control of CaMV 35S promoter to increase the transcription of *Ac*-TPase. The higher activity of *Ac*-TPase could explain the higher rate of *Ds* excision (100 %) in our study than of *Ac* by VAN SLUYS et al. (1987) because increased transcription of *Ac*-TPase was also correlated with higher rates of *Ds* transposition in *Arabidopsis* (SWINBURNE et al. 1992).

Reinsertion of Ds into new chromosomal sites in the carrot genome

New chromosomal insertion sites of *Ds* in the carrot genome were amplified by using TAIL-PCR (LIU et al. 1995; TSUGEKI et al. 1996). The sizes of the TAIL-PCR amplified DNA fragments ranged from 200 bp to 1000 bp (data not presented). Sequence alignment of these TAIL-PCR amplified DNA fragments demonstrated that at least four different genomic sequences of carrot flanking the 5' end of the *Ds* and four different sequences flanking the 3' end of *Ds* were amplified from transgenic plants indicating that at least two independent *Ds* insertion events occurred in both AcDs#6-5 and AcDs#3-3 (Fig. 4). Although these plants originally carried only a single copy of the T-DNA carrying *Ds* (Fig. 2), detection of more than one *Ds* insertion site could be due to the secondary *Ds* transposition in the subgroup of cells carrying transposed *Ds* within the sampled leaves, suggesting that *Ds* remained mobile in these plants. Alternatively, it is also possible that *Ds* increased its copy number in carrot during transposition since tendency of *Ac* to increase its copy number in tomato is well-documented (YODER 1990).

Sequence analysis of *Ds* insertion sites had not been reported previously in carrot and we found that they included intact terminal inverted repeats (TIRs), except for DsIS-1 (Fig. 4). The nucleotide sequence of DsIS-1 with missing TIRs indicated the occurrence of imperfect *Ds* transposition in carrot. BLAST searches in GenBank

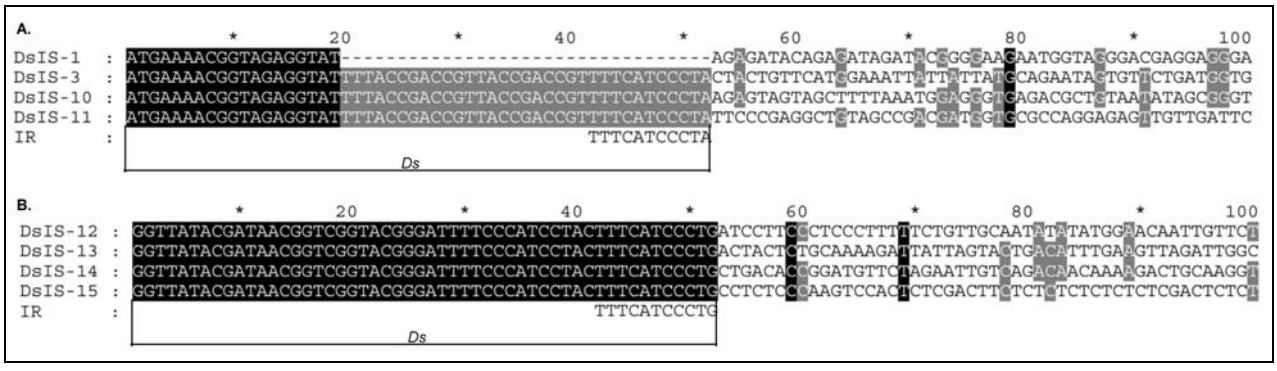


Fig. 4. Sequences analysis of DNA flanking (A) 3' and (B) 5' ends of *Ds* at the insertion sites in the carrot genome. IR: terminal inverted repeat sequences of *Ds*. DsIS-1, DsIS-10, DsIS-13 and DsIS-15 were cloned from AcDs#6-5 while DsIS-3, DsIS-11, DsIS-12 and DsIS-14 were flanking *Ds* in AcDs#3-3. Black, 100 % identity; grey, 75 % identity; white, less than 75 % identity.

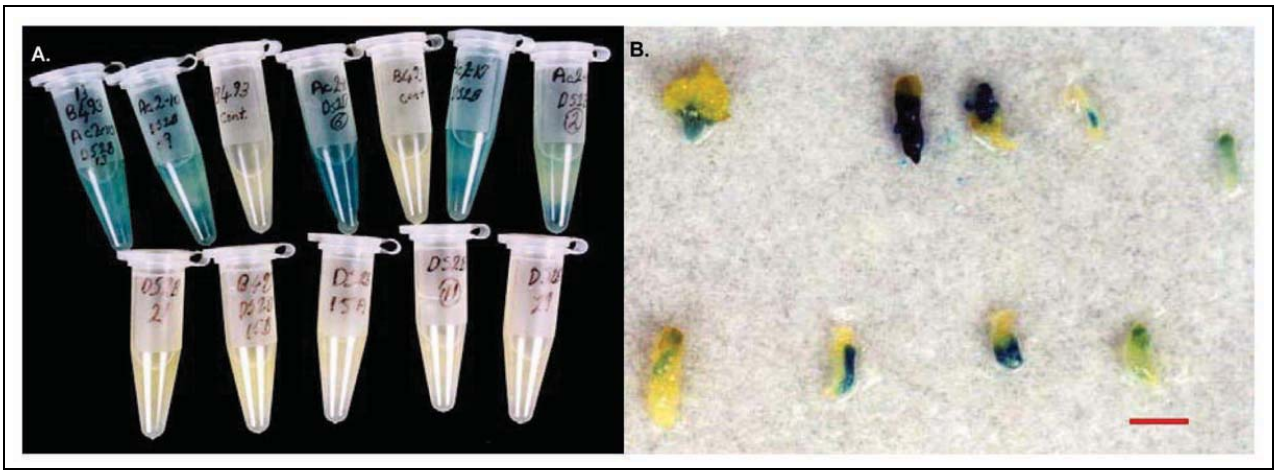


Fig. 5. (A) *GUS* expression in transgenic carrot callus. Yellow tubes in the upper row contained samples from un-transformed calli. Yellow tubes in the lower row contained calli from Ds#11, Ds#15, Ds#21, and Ds#29, transformed with *Ds* only. Blue tubes in the upper row contained calli from AcDs#1, AcDs#2, AcDs#6, AcDs#9, and AcDs#13 and transformed with both *Ac*-TPase and *Ds*. (B) Somatic embryos, produced from calli, AcDs#10, transformed *Ac*-TPase and *Ds*, were expressing *GUS* in various tissues. The red bar is 3 mm long.

yielded no significant databases matches with the carrot flanking sequences at the *Ds* insertion sites at the nucleotide level. However, putative amino acid sequences derived from translation of the three nucleotide sequences of flanking carrot DNA had significant homology with the amino acid sequences of proteins in other plants including polyproteins of *ty1/copia* like LTR retroelements, tomato verticillum wilt disease resistance gene, and vacuolar sorting receptor protein in the GenBank protein database, using the BLASTX program (data not shown). No significant match was found for the other five sequences (62.5 %). These five sequences could represent yet-unsequenced intergenic or intron regions of the carrot genome or carrot gene segments not found in other species. The low percentage of GenBank matches for the carrot sequences flanking *Ds* is probably due to the limited amount of ESTs or gene sequences of carrot. SCHOLZ et al. (2001) found that two of nine barley sequences matched with GenBank sequences, a level of matching similar to our study. Although the authors concluded that the low percentage of GenBank matches was due to short flanking sequences amplified with AIMS-PCR, five sequences flanking *Ds* which were larger than 22 bp were identified. These sequences were long enough to generate significant GenBank matches if homologous sequences of these AIMS-PCR products were present in the GenBank databases.

The *Ds* element construct contains a *GUS* gene whose expression is dependent upon *Ds* insertion within or near an active carrot gene since the promoter of the GUS gene lacks enhancer elements. In Fig. 5A, *GUS* expression was evident by the blue colour of five calli transformed with both *Ac*-TPase and *Ds* while untransformed control calli and five calli transformed with *Ds* only had no demonstrable *GUS* activity. Expression of the *GUS* gene was also observed in somatic embryos produced from calli co-transformed with both *Ac*-TPase and *Ds* and its expression pattern differed among the embryos (Fig. 5B) suggesting that the *Ds* element reinserted into or near diverse active genes in carrot since the *GUS* expression without transposition due to the T-DNA integration in the scope of a carrot genomic enhancer element was expected to generate uniform *GUS* expression pattern in all somatic embryos.

Previous studies indicated that about half of the transposed *Ac* element did not reinsert (DOONER and BELACHEW 1989) and only one out of five excised autonomous *Ac* element reinserted in carrot root cultures (VAN SLUYS et al. 1987; VAN SLUYS and TEMPE 1989). Although low reinsertion rate of autonomous *Ac* element in carrot in previous studies suggested that it may not be a suitable for transposon to mutagenize the carrot genome, the *Ac/Ds* system in our study allows selection of plants carrying *Ds* since the *aph4* gene conferring hygromycin resistance was located on the *Ds* element and any cells or somatic embryos which were not carrying a *Ds* element would be hygromycin sensitive. Since selection of transgenic carrot cells carrying *Ds* and initiation of somatic embryogenesis were done on hygromycin containing medium, all hygromycin resistant plant regenerated from these somatic embryos should have at least one *Ds* element. Indeed, analysis of transgenic plants transformed with single copy of T-DNA carrying *Ds* showed that this construct was successful for selection of carrot plants containing *Ds*. Therefore, the *Ac/Ds* system used in this study could be a useful tool for cloning and isolation of the carrot genes and we are pursuing that goal.

We have successfully introduced an *Ac/Ds* based two-element transposon tagging system in carrot and demonstrated that *Ds* will transpose in carrot if the *Ac*-TPase is provided in the same cell. About 37.5 % of *Ds* insertion into gene regions in the carrot genome as indicated by sequence analysis of DNA flanking *Ds* using TAIL-PCR suggested that this system has the potential for targeting carrot genes of interest.

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