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Transposable elements as plant transformation vectors for long stretches of foreign DNA

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Abstract The production of transgenic plants is now routine for most crops. However, using currently available transformation methods it is still difficult and time-consuming to obtain a collection of transformed individuals containing single or low-copy-number, intact transgenic inserts. Here we describe a set of broad-host-range transformation vectors based on the *Ac/Ds* transposition system that improve both transformation efficiency and the quality of transgenic loci. These vectors efficiently deliver long stretches of foreign DNA into the genome, leading to transgenic strains containing an intact single-copy insert of 10 kb. This type of vector could be an important additional tool for the production of transgenic plants with the well-defined, foreign DNA inserts required for biosafety approval and commercialisation.

Key words Transformation vectors · transposition · *Ac/Ds* · Plants

Introduction

Methods available for the production of transgenic crop plants can be divided into two categories: (1) the vectorless introduction of naked DNA and (2) tranformation mediated by *Agrobacterium tumefaciens*. The advantage of naked-DNA-mediated transformation is the absence of a restriction on host range, and this has resulted in its successful application to the most important crop species (for a review see Potrykus 1991). However, a high degree of rearrangement of the transformed DNA resulting in complex multicopy integration patterns of transgenes is still a serious obstacle to the broad application of this technology. Such composite transgenic loci tend to be physically instable and to vary in their expression due to silencing (for a review see Finnegan and McElroy 1994). In contrast, *Agrobacterium*-mediated transformation often results in a single or low-copy integration of foreign DNA, but its restricted host range excludes most cereal crops (for a review see Zambryski, 1988). A universal transformation system should meet the following criteria: (1) an easy and reproducible procedure leading to high transformation efficiencies, (2) precise transfer of large fragments of foreign DNA, (3) single or low-copy integration of transgenes, (4) predetermination of the chromosomal insertion site and (5) no host plant restrictions. Methods satisfying all of the above requirements probably need to be developed in stepwise manner.

In the present article we describe new DNA-delivery vectors based on transposon-carrying foreign genes and the extrachromosomal transposition of molecules into target genomes. For this purpose, we have adapted the *Ac/Ds* system of maize (for a review see Fedoroff 1989). The *Ac* (activator) element is 4.6 kb long and encodes a transposase required for its autonomous transposition as well as the activation of defective elements. Such defective derivatives of *Ac*, which are not able to produce transposase but are able to transpose due to transactivation, are called *Ds* (dissociation) elements. The *Ac/Ds* system is active in a wide tange of plants (Baker et al. 1986; Schmidt and Willmitzer 1989; for a review see Walbot, 1992) and the insertion of foreign DNA into *Ds* elements does not markedly hamper chromosomal transposition (Masterson et al. 1989; Fedoroff and Smith 1993). It was recently reported that extrachromosomal transposition from a viral replicon (Sugimoto et al. 1994) or plasmid molecules (Houba-Hérin et al. 1994) can mediate integration of foreign genes into chromosomes.

The broader application of extrachromosomal transposition for plant transformation demands: (1) size tolerance of foreign DNA inserts, (2) the establishment of

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experimental conditions favouring transposon-mediated transformation over unspecific insertions, (3) prevention of the integration of helper templates coding for transposase and (4) long, intact transgenic inserts, either single or at a low copy number. In the experiments presented here, extrachromosomal transposition as a delivery system for foreign DNA into plant chromosomes was assessed using the above criteria.

Materials and methods

Plasmid construction

Cloning methods used for plasmid construction were as described in Sambrook et al. 1989. The constructs are depicted in Fig. 1A and B.

For the production of the short *Ds* element, an *Eco*RI-*Bam*HI fragment of plasmid pKU56 (Coupland et al. 1989) was inserted between the *Eco*RI and *Bam*HI sites of pUC18 (Yanisch-Perron et al. 1985) to obtain plasmid p3'DS. Replacement of a *Eco*RI-*Bgl*II fragment of plasmid p3'*DS* with an *Eco*RI-*Bgl*II fragment of pCP20 (Coupland et al. 1989) resulted in plasmid pMDS. A linker containing *Bgl*II/*Bfr*I/*Sma*I/*Bfr*I/*Bgl*II sites was then inserted into the *Bgl*II site of pMDS giving plasmid pMDSL. As an excision marker, the coding region of the *bar* gene was included as a *Sma*I-*Hin*dIII fragment of pDHbar (Paszkowski 1993), resulting in plasmid pMDSbar. As an insertion marker, a 1.8-kb-long *Eco*RI fragment of plasmid pHP28 containing the hybrid *nptII* gene (Paszkowski et al. 1988) was inserted into the *Sma*I site of pMDSbar to produce plasmid pDSK. Plasmid pDJD containing a longer insert within *Ds* was constructed by inserting a 2.7-kb-long fragment of pESPB (Peterhans et al. 1990) containing a *hph* gene fused to the CaMV 19S promoter within the *Ds* element. To test the capacity of *Ds* to deliver even longer stretches of DNA, a 9.5-kb-long fragment (*Sma*I-*Mlu*I) of pTZA70 containing part of the tobacco nitrate reductase locus (Vaucheret et al. 1989) interrupted by the *nptII* gene of pHP28 was cloned into the *Sma*I site of pMDSbar, resulting in plasmid pDSNRd.

Plasmid pKU4bar was constructed by replacing the *Bam*HI-*Hin*dIII fragment of pKU4 (Baker et al. 1987), containing the *nptII* coding region, with the *Bam*HI-*Hin*dIII fragment of pDHbar, containing the coding region of the *bar* gene (Paszkowski 1993).

Plamid pSLJ1101 (Scoffield et al. 1992) containing the hybrid transposase gene was kindly provided by J. Jones, Norwich, UK. Plasmid pACMUT, containing the transposase gene with a frame shift mutation, was obtained by *Eco*RI restriction of pSLJ1101 within the transposase coding region, filling in protruding ends and religation. To produce transposase pre-mRNA *in vitro*, a *Sac*I-*Bam*HI fragment of pSLJ1101 was inserted into pBS3'UTR (a pBSKS$^+$ derivative from Stratagene) containing the 3' terminal untranslated sequence of TMV (Gallie and Walbot 1990). An analogous construct was obtained for the mutated transposase gene; these were named pBSAT and pBSMT, respectively.

Transcription in vitro

Transcripts were synthesised with T7 RNA polymerase (New England Biolabs) according to the supplier's instructions in the presence of the cap analogue m^{7}G(5')ppp(5')G(Pharmacia) using as a template *Eco*RV-linearised pBSAT or pBSMT.

Plant material and protoplast transformation

Plasmid DNA was introduced into leaf mesophyll protoplasts of *Nicotiana tabacum* cv 'Petit Havana' line SR1 (Maliga et al. 1973) and *Arabidopsis thaliana* line C24 by direct gene transfer (Negrutiu et al. 1987; Damm et al. 1990; Masson and Paszkowski 1992). Phosphinothricin (ppt)-resistant colonies could be scored after 3 weeks of culture under selective conditions, while kanamycin-resistant clones were clearly visible only after 6 weeks.

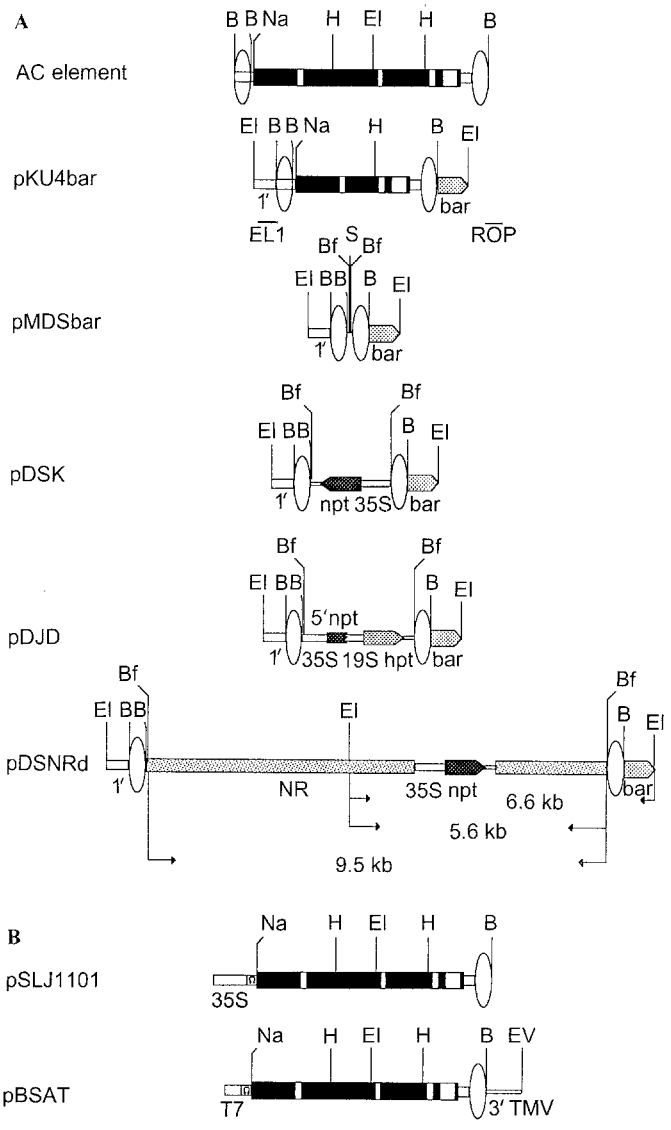


Fig. 1 A, B Structure of *Ac* and *Ds* derivatives (**A**) and transposase-encoding plasmids (**B**). The names of the constructs are given at the *left* of each drawing. *Ac* terminal structures required for transposition are presented at *ellipses*. *Open narrow boxes* represent expression signals and *broader boxes* protein-coding regions. In the transposase gene, *closed boxes* represent exons and *open boxes* introns. Other expression signals and coding regions used are indicated as follows: *1'* T-DNA promoter, *bar* coding region of the ppt gene, *35S* and *19S* promoters of the 35S and 19S transcripts of CaMV, respectively, *npt* coding region of the *npt* gene, *5'npt*, 5' region of the npt gene (till *Sph*I site), *NR* nitrate reductase gene of tobacco, *T7* promoter, a poly-A tail of the transposase transcript, *omega* and *3' TMV* regions of the Tobacco Mosaic Virus (TMV). Further details are given in the Materials and methods. Restriction sites are marked as follows: *B Bam*HI, *Bf Bfr*I, *EI Eco*RI, *EV Eco*RV, *H Hin*dII, *N Nae*I, *S Sma*I. Sizes of relevant restriction fragments of pDSNRd are marked

PCR analysis and DNA sequencing

Genomic DNA (1 µg) or plasmid DNA (10 ng) was mixed with 5 µl of 10 × PCR buffer (100 m*M* Tris-HCl, 15 m*M* $MgCl_2$, 0.5 *M* KCl, pH 8.3) and adjusted to a total volume of 50 µl. The reaction was performed in the presence of 0.2 m*M* dNTPs (Pharmacia), 0.5 m*M* of each oligonucleotide primer and 2.5 units of *Taq* polymerase (Boehringer). Incubation was carried out in a DNA Thermal Cycler

(Perkin-Elmer Cetus) under the following conditions: a denaturation of 5 min at 94 °C was followed by 34 cycles of 30-s denaturation at 94 °C, 30-s annealing at 60 °C and 30-s polymerisation at 70 °C. Primer EL1 'TCG'GAT'ACT'TAC'ACG'TCT'TGC'GC_3) annealing to the 1' poromoter region and primer ROP (^{5}'CCC'TTA'TCT'GGG'AAC'TAC'TCA'CAC_3') annealing to the 3' nontranslated area of the *bar* gene (Fig. 1) were used.

Amplified fragments were cloned into pCR™ vectors (Invitrogen). The sequences of cloned PCR products were determined by the dideoxynucleotide chain termination method (Sanger et al. 1977).

Southern blot analysis

DNA isolation and Southern blot hybridisation were performed as described previously (Peterhans et al. 1990).

Results

Assays of transposition

Transposition activity was determined by both an excision assay and an insertion assay. In both assays, *Ds*-containing plasmids (Fig. 1A) were usually cotransformed with a plasmid expressing a hybrid transposase gene (plasmid pSLJ1101, Scoffield et al. 1992, Fig. 1B) or, as a control, a frame-shift mutation of this gene (Plasmid pACMUT).

For the detection of excision events, transposons were placed between the 1' promoter of the octopine gene of *A. tumefaciens* T-DNA (Velten et al. 1984) and the coding region of the *bar* gene conferring resistance to ppt (phophinothricin). Upon *Ds* excision, a correct junction between the *bar* gene and the 1' promoter should lead to an active, restored gene and to the recovery of ppt-resistant clones. Since junctions between identical elements (1' promoter-bar gene) should be formed for all of the different *Ds* constructs, the efficiency of *Ds* excision can be directly compared by scoring the number of ppt-resistant clones.

Insertion activity was measured by the frequency of chromosomal integration of a marker gene placed within the *Ds* vector (i.e. the *nptII* gene coding for resistance to kanamycin or the *hph* gene coding for resistance to hygromycin; Fig. 1). Both excision and insertion activities can be expressed as relative transformation frequency (RTF).

A higher number of resistant clones in the presence of transposase would be indicative of the involvement of transposition in the restoration (excision) or integration of the selectable genes (insertion). Thus, the ratio between the number of transformed clones in excision and insertion assays using active or mutated transposase was used as a measure of transposition activity (TA).

Ds vectors of various sizes are equally active

It is known that terminal *Ac/Ds* sequences serve as *cis*-acting elements that are required for transposition (Coupland et al. 1988). For *Ds*-based vectors, it is of advantage to use the shortest sequences necessary for transposition to allow the transfer of long pieces of "passenger DNA" and the easy construction of an universal vector cassette. The minimal length of the active *Ds* termini has been determined by progressive deletion scanning of the *Ac* element (Coupland et al. 1989). Transposition activity of *Ds* derivatives deleted from the middle towards either end of the element showed that 238 bp at the 5' end and 209 bp at the 3' end were sufficient for activity (Coupland et al. 1989). It was, however, not determined whether a *Ds* consisting of two such short terminal sequences (minimal *Ds*) is as active as the longer elements. We have assembled such a minimal *Ds* (pMDSbar; Fig. 1) and compared its excision activity to a previously well-characterised highly active longer *Ds* (an internal *Hin*dIII deletion of *Ac*, Baker et al. 1987; Fig. 1, pKU4bar).

In the presence of pSLJ1101 (active transposase), a 3.5-to 4-fold increase in the number of ppt-resistant clones was observed for plasmids pMDSbar and pKU4bar (Fig. 2). The similar TA values for these two *Ds* elements suggest that the short *Ds* indeed contains all the signals required for efficient transposition.

In order to determine the ability of the constructs to transfer long stretches of foreign DNA, increasing sizes of passenger DNA were inserted into *Ds*. Plasmid pDSK contains the *nptII* gene as a 1.8-kb fragment, pDJD has an insert of 2.7 kb and plasmid pDSNRd has an insert of 9.5 kb (Fig. 1). Excision as well as integration assays for *Ds* elements of pMDSbar (470 bp), pDSK (2.3 kb), pDJD (3.2 kb) and pDSNRd (10 kb) (Fig. 1) gave similar transposition activities (Fig. 2), thus demonstrating that long foreign DNA stretches within *Ds* impair neither the excision nor insertion of *Ds* during the transformation process.

The background of unspecific integration can be lowered

Ds vectors need to be taken up by plant cells and activated by a cointroduced source of transposase. The uptake of any foreign DNA leads to unspecific chromosomal integration. Thus, a proportion of transformed clones within a population obtained with transposition vectors may contain plamid DNA of the *Ds* vector and/or molecules encoding the transposase. It is difficult to completely prevent such unspecific DNA integration, but it may be feasible to establish experimental conditions that favour transposition over unspecific random insertion. It is known that various structures of transformed DNA (circular, linear) as well as the presence of carrier DNA influence the efficiency of naked-DNA-mediated transformation (Paszkowski 1989). The most efficient combination is that of linear molecules in the presence of carrier DNA (Shillito et al. 1985). Thus, the application of carrier DNA and linear

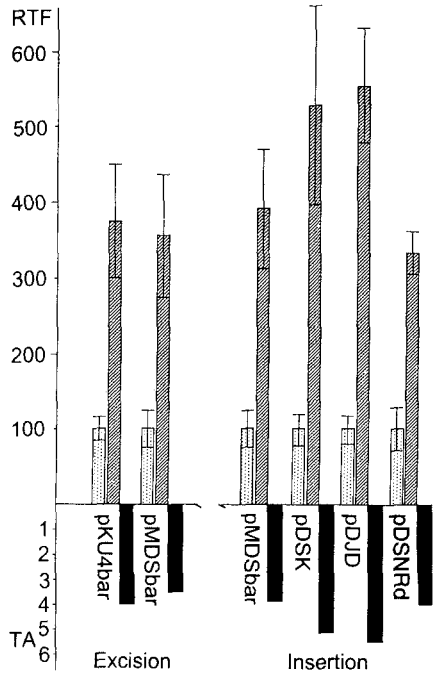
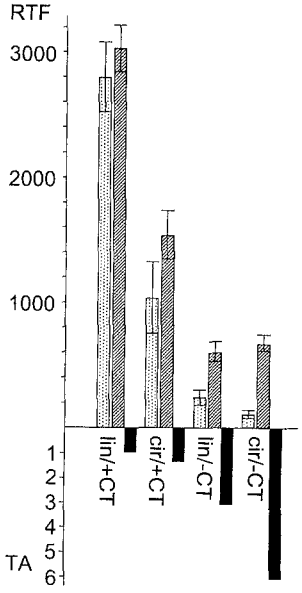


Fig. 2 Relative transformation frequency (*RTF*) and extrachromosomal transposition activity (*TA*) obtained with *Ds* vectors of different length. *Dotted bars* represent RTF in the presence of pACMUT; *cross-hatched bars* represent RTF in the presence of pSLJ1101. Standard deviations are shown as error bars. Each bar represents the mean relative numbers, expressed in percentage, of ppt-(pKU4bar and pMDSbar) or kanamycin-(pDSK, pDJD, pDSNRd) resistant clones from at least three independent transformations, without carrier DNA, involving 1.2×10^6 protoplasts each with the *Ds* vector on a circular plasmid. Samples cotransformed with pACMUT are equivalent to 100%; the mean absolute value for these samples was 89 transgenic clones. *Black bars* represent the TA calculated as described in the Results section. The TA value for excision and insertion assays were always very similar (compare data in Fig. 5)

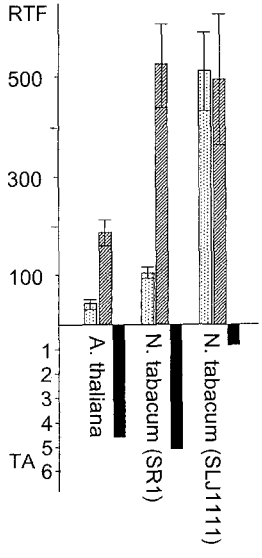
Fig. 3 Relative transformation frequency (*RTF*) and extrachromosomal transposition activity (*TA*) of the pDSK vector in linear (*lin*) or circular form (*cir*) in the presence (+*CT*) or absence (−*CT*) of carrier calf thymus DNA. Symbols are as in Fig. 1. Each RTF bar represents the mean relative number of kanamycin-resistant clones from at least three independent transformations of 1.2×10^6 protoplasts each. A sample of circular *Ds* vector cotransformed with pACMUT in the absence of carrier DNA is equivalent to 100%



Ds vectors should result in a high background of unspecific inserts. This was indeed observed, and the presence of transposase in these transformations did not result in an increased number of transformed clones (Fig. 3). The most pronounced effect of transposase was observed with circular molecules without carrier DNA, where the presence of transposase increased the relative transformation frequency more than sixfold (Fig. 3).

It has been observed that the activity of transposon chromosomal material is related to the amount of available transposase. It has also been reported that in tobacco transposition frequency increases with an increasing amount of transposase production (Jones et al. 1989; Hehl and Baker 1990), although the converse was observed in maize (McClintock 1951). Using the integration assay, we examined the effect of variation in the amount of *Ds* vector and transposase-encoding activator plasmid. The lowest, effective amount of activator plasmid should be used in order to limit its chromosomal cointegration. Thus, we used a high-expression transposase hybrid gene construct that had been shown to be most suitable for chromosomal transposition in tobacco and *Arabidopsis* (Scoffield et al. 1992; Swinburne et al. 1992). Equimolar amounts of the activator plasmid and the vector yielded a 5.2- and a 4.6-fold transposase-dependent increase of transformation frequency in tobacco and *A. thaliana*, respectively (Fig. 4). However, transposase had no measurable effect with a tenfold lower amount of activator plasmid (data not

Fig. 4 Relative transformation frequencies (*RTF*) and extrachromosomal transposition activities (*TA*) of pDSK in *A. thaliana*, pDJD in transgenic line SLJ1111 expressing a chromosomal copy of transposase, and both pDSL and pDJD in the *N. tabacum* control line. RTF values for the two vectors in the control line were very similar (compare also Fig. 2) and were thus combined. Symbols are as in Fig. 1. Each RTF bar represents the mean relative number of antibiotic-resitant clones from at least three independent transformations of 1.2×10^6 protoplasts each. A sample of circular *Ds* vector cotransformed with pACMUT into control *N. tabacum* protoplasts in the absence of carrier DNA is equivalent to 100%



shown). In order to provide a stable high supply of transposase, *Ds*-containing vectors were introduced into protoplasts of the transgenic *N. tabacum* line (SLJ1111), which had been previously selected for the efficient production of transposase and a high rate of *Ds* excision (Scoffield et al. 1992). Transposition in protoplasts of this line was compared to that mediated by cotransformation of transposase-encoding plasmid into the corresponding nontransgenic line. The effects of the two sources of transposase were similar (Fig. 4). Further increase in the transposase levels was attempted by cotransformation of *Ds* vectors into protoplasts of the line SLJ1111 together with an additional 10 µg of activator plasmid. This approach did not increase integration efficiencies further (data not shown), suggesting that a surplus of transposase does not lead to an increase in the efficiency of transposition. There are two alternative explanations of this result: (1) the system is saturated and addition of transposase has no effect or (2) the interaction between introduced activator and the chromosomal copy of the transposase gene results in silencing, thereby preventing an overproduction of transposase. Since, there was no significant reduction of integration frequency in the line SLJ1111 when the construct coding for a mutated transposase was used, it is possible to exclude silencing.

A further attempt was made to increase integration through the transposition pathway by reducing the number of *Ds*-vector molecules while leaving the amount of activator unchanged. This yielded only slightly higher TA values, but the absolute number of transformants was significantly lower (data not shown).

Transposase mRNA activates transposition

In order to overcome the drawback of cointegration of activator plasmid, transposase mRNA produced in vitro was used for transient activation of transposition. Transposase transcript was produced by in vitro synthesis of RNA using plasmid pBSAT as the template (Fig. 1), thus obtaining unspliced pre-mRNA. To enhance mRNA stability and the efficiency of its translation, the coding region of the transposase gene was flanked by the TMV ω-leader and the 3′ terminal untranslated region of TMV (plasmic pBSAT, Fig. 1). The transcription was terminated 250 bp after the end of the coding region, behind the untranslated fragment of TMV that can functionally replace a poly-(A) stretch (Gallie and Walbot 1990). An analogous mRNA was obtained for the mutated transposase gene (plasmid pBSMT). The efficiency of transposition measured by the excision assay was reflected in a 2.5-fold increase in *bar* gene restoration in the presence of the transposase transcript as compared with the addition of pre-mRNA copied from the mutated gene (Fig. 5). This observation was confirmed in the insertion assay, where a 2.7-fold increase in RTF was observed in the presence of the correct pre-mRNA (Fig. 5).

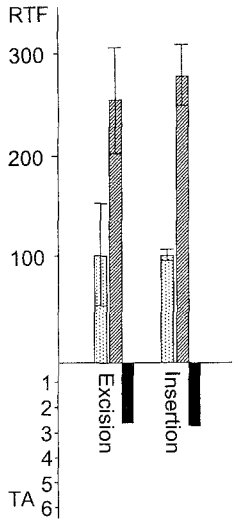


Fig. 5 Relative transformation frequency (*RTF*) and extrachromosomal transposition activity (*TA*) of pDSK in excision and insertion assays after activation of transposition with cotransformed transposase pre-mRNA. Symbols are as in Fig. 1

Specificity of *Ds* excision and insertion

Excision as well as insertion of a *Ds* vector should lead to the formation of predicted DNA junctions at both excision and insertion sites. The excision site was rescued from ppt-resistant clones by the polymerase chain reaction (PCR) using primers annealing in the 1′ promoter and the *bar*-coding regions (Fig. 1). After perfect excision of *Ds*, a 883-bp-long fragment was expected. Of four clones obtained in the presence of active transposase, three amplified a fragment that approximated the expected size (data not shown). Two ppt-resistant clones obtained from a control treatment (pACMUT) did not result in PCR amplification. Since the junction between the 1′ promotor and the *bar* gene was not restored in PCR-negative clones, resistance to ppt was probably due to the accidental integration of the *bar* gene behind plant sequences with promoter-like activity. This could also be the case for one PCR-negative but ppt-resistant clone that resulted from transformation in the presence of active transposase. Sequence analysis of 1′ promoter *bar* gene junctions revealed short deletions (up to 28 bp) around the excision sites. Such deletions are commonly observed (Fedoroff 1989; Shen and Hohn 1992).

Upon excision, the *Ds* vector should free itself of plasmid DNA flanking the excision site; thus, upon integration, transposon borders should be directly linked to host chromosomal DNA. This is in contrast to the random integration of plasmids containing *Ds* vectors (e.g. in the presence of mutated transposase) in which case plasmid DNA neighbouring the *Ds*, the 1′ promotor and the *bar* gene should be present in the transgenic locus. All 5 randomly chosen clones obtained after cotransformation with pACMUT indeed revealed the presence of *bar* gene sequences flanking the *Ds* vector. In contrast, 13 out of 16 kanamycin-resistant clones obtained in the presence of active transposase lacked *bar* sequences flanking *Ds*. While further mapping of the junctions to plant DNA using Southern blotting and PCR suggested that the insertion of *Ds*

vectors often occurred as predicted (Houba-Hérin et al. 1994; Sugimoto et al. 1994) at the terminal *Ds* sequences, the structures of some transgenic inserts suggested short terminal deletions of DS vectors (data not shown).

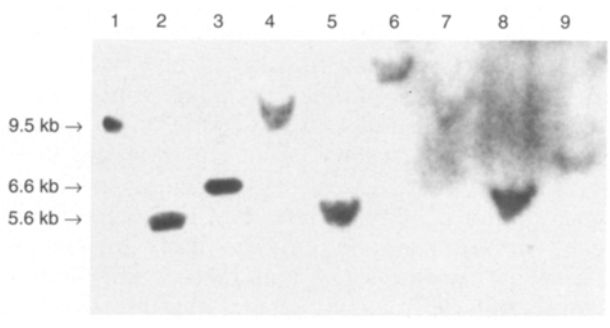
Structure of foreign DNA inserts

The fidelity of foreign DNA integration using *Ds*-based vectors was examined by Southern blot analysis using the internal *nptII* gene as a hybridisation probe (Fig. 1). Digestion with *Bfr*I should reveal the presence of an intact 9.5-kb insert of vector pDSNRd (Fig. 1), while restriction with *Eco*RI should reveal junction fragments(s) with plant DNA and thus allow the determination of the number of inserts. Double digestion with *Eco*RI and *Bfr*I may reveal possible internal rearrangements within the transgenic locus. Of 16 clones resulting from cotransformation with the active transposase, 9 showed single-copy chromosomal insertions (Fig. 6 and data not shown). None of these clones contained plasmid sequences flanking the *Ds*, which suggests a pathway of extrachromosomal transposition from the plasmid molecule into chromosomal DNA. 2 resulted in integration of an intact 9.5-kb insert of passenger DNA (Fig. 6), while inserts of the other clones were terminally shortened.

Interestingly, out of 5 clones obtained after cotransformation with the mutated transposase, only 1 revealed a single-copy integration of the entire stretch of transforming DNA (data not shown). Junctions to the *bar* gene were present as the result of random integration into host chromosomes. This confirms previous observations that circular plasmids used for transformation without carrier DNA can often produce simple integration patterns (Paszkowski 1989), but at a low efficiency of transformation (Fig. 3), and that they yield unpredictable vector/chromosomal DNA junctions.

Kanamycin-resistant clones obtained after transposition-mediated transformation of *A. thaliana* protoplasts revealed that 4 of the 15 clones had an intact single-copy insert of the *Ds* element without flanking plasmid sequences, 3 clones had integrateed two copies and that the remaining 8 clones had more complex transgenic loci (data not shown). In control treatments with the mutated transposase gene, 9 out of 10 transformed clones had complex integration patterns.

Fig. 6 Southern blot analysis of two independent kanamycin-resistant clones transformed with an activated element of pDSNRD. DNA was digested with *Bfr*I (*lanes 1,4,7*), *Bfr*I and *Eco*RI (*lanes 2,5,8*) or *Eco*RI alone (*lanes 3,6,9*). *Lanes 1–3* control pDSNRd DNA, *lanes 4–6* genomic DNA of clone 1, *lanes 7–9* genomic DNA of clone 2



Discussion

We have examined the properties of transformation vectors based on extrachromosomal transposition of engineered *Ds* elements. Similar successful transposition vectors have been developed for *Drosophila* using *P* elements (Spradling and Rubin 1982). This system is widely used for efficient transformation since engineered *P* elements can serve as a carrier for foreign DNA fragments of 8.1 kb and 11.1 kb. Molecular analysis has shown that such modified transposons undergo a highly precise integration (Rubin and Spradling 1982). *P*- element vectors are able to transpose from extrachromosomal nonreplicating plasmid molecules, resulting in one to five integrations per genome (Spradling and Rubin 1982).

For the plant *Ac/Ds* system, a tight association of transposition with DNA replication has been indicated. This was concluded from a genetic analysis of transposition at the maize *P* locus (Greenblatt and Brink 1962) and recently supported by detailed molecular studies (Chen et al. 1992) that suggested the occurrence of transposition between DNA synthesis (S phase) and mitotic anaphase. This situation has restricted the use of extrachromosomal transposition to viral replicons (Laufs et al. 1990; Shen and Hohn 1992). In addition, excision events have been mostly studied, and the chromosomal integration of a transposon-containing hygromycin resistance gene supplied on a viral replicon into the rice cells has been described only recently (Sugimoto et al. 1994). *Ds*/viral vectors were cotransformed together with two additional plasmids: transposase-encoding plasmid and a plasmid coding for functions necessary for viral replicaton. Against all expectations, the presence of transposase and/or viral replication functions did not influence the rate of recovery of the transgenic clones. Nevertheless, the authors suggested that replication was beneficial to *Ds* transposition.

The use of nonreplicating plasmids as *Ds* carriers also resulted in the recovery of an empty donor site on the chromosome. It was, however, not possible to determine if transposition occurred before or after integration of the plasmid DNA into a chromosome (Shimamoto et al. 1993). Only recently it was shown that *Ds* elements are not only able to excise from extrachromosomal nonreplicating plasmid molecules but also to insert into host chromosomes (Houba-Hérin et al. 1994). Thus, the presence of the replication origin on extrachromosomal DNA containing a transposon seems not to be absolutely necessary for transposition, although it cannot be excluded that a higher transposition efficiency might be achieved in association with DNA replication.

We have examined properties of transformation vectors based on such an extrachromosomal *Ac/Ds* transposition system and established conditions favouring transposon-mediated transformation over unspecific insertions. In plant species where naked-DNA-mediated transformation is at present the only method available for the production of transgenic material, it is difficult to obtain transgenic loci with single or low-copy inserts of long stretches of foreign DNA. Thus, it is necessary to screen by the molecular analysis of transgene structres for lines with intact transgenes. The recovery rate of simple transgenic loci is strongly reduced with the increasing length of transgenes. Houba-Hérin et al. (1994) showed that short foreign DNA inserts (approximately 2 kb) within a long *Ds* element (approximately 3 kb) could be successfully inserted into the genome of *N. plumbaginifolia* using extrachromosomal transposition. It was, however, not determined if longer inserts could be faithfully delivered and at what frequency relative to standard transformation methods. In addition, the assessment of this new gene transfer system required the development of a method for transient supply of transposase, which would eliminate incorporation of its template into the chromosomes of transformed cells. Here we have shown the presence of frequent, intact inserts of foreign DNA upto 9.5 kb long into host chromosomes using *Ds*-elements as a transfer system. It seems that we have not reached the upper limit of insert length since the longest stretches used did not impair the efficiency of *Ds*-mediated transformation. This efficiency was significantly higher than that of standard carrier-free DNA delivery.

It will probably be necessary to tune this gene transfer system to suit a particular target plant species by changing the proportions of vector and activator plasmid (or the amount of transposase mRNA). We observed, however, that elevated levels of transposase did not further increase gene transfer efficiency. This could be due to limiting amounts of the host factors thought to be involved in *Ac/Ds* transposition (Schwartz and Dennis 1982; Feldmar and Kunze 1991) or to a negative effect of transposase molecules when present at high concentration. Therefore, the competence and the sensitivity of different plant species may differ significantly, although the species used in this present study, tobacco and *Arabidopsis*, both supported similar levels of extrachromosomal transposition under comparable experimental conditions.

A transient supply of transposase can be achieved by providing DNA or RNA templates or the protein directly. It has been shown that mRNA produced in vitro can be efficiently translated after the transformation of protoplasts (Callis et al. 1987; Gallie et al. 1989). Therefore, in addition to the DNA templates we examined the ability of cotransformed transposase pre-mRNA to activate extrachromosomal transposition. The observed stimulation of transposition activity in the presence of transposase RNA suggests that the supply of transposase as a transcript seems to be an attractive possibility.

In conclusion, transposon-mediated transformation leads to a high transformation efficiency and intact, single-copy, long inserts of foreign DNA. This method should be useful, in combination with a variety of DNA uptake techniques (for review see Potrykus 1991), in the improvement of both transformation efficiency and the quality of transgenic loci in commercially important plant species.

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