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Characterization of the *Ac/Ds* behaviour in transgenic tomato plants using plasmid rescue

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Abstract

We describe the use of plasmid rescue to facilitate studies on the behaviour of *Ds* and *Ac* elements in transgenic tomato plants. The rescue of *Ds* elements relies on the presence of a plasmid origin of replication and a marker gene selective in *Escherichia coli* within the element. The position within the genome of modified *Ds* elements, rescued both before and after transposition, is assigned to the RFLP map of tomato. Alternatively to the rescue of *Ds* elements equipped with plasmid sequences, *Ac* elements are rescued by virtue of plasmid sequences flanking the element. In this way, the consequences of the presence of an (active) *Ac* element on the DNA structure at the original site can be studied in detail. Analysis of a library of *Ac* elements, rescued from the genome of a primary transformant, shows that *Ac* elements are, infrequently, involved in the formation of deletions. In one case the deletion refers to a 174 bp genomic DNA sequence immediately flanking *Ac*. In another case, a 1878 bp internal *Ac* sequence is deleted.

Introduction

DNA rearrangements, induced by the maize transposable elements *Ac* and *Ds*, have been studied, predominantly in the original host. The best characterized events are those associated with transposition of *Ac/Ds*. Analysis of DNA sequences immediately flanking the element showed that integration leads to an 8 bp target site duplication [21]. One or several basepairs may subsequently be deleted or substituted upon excision [21].

Integration of *Ac* and *Ds* elements is not an at random event. *Ac* preferentially transposes into hypomethylated DNA sequences [4]. These sequences are associated with transcriptional active regions [1]. Apart from the structure of target DNA, the transposition pattern of *Ac/Ds* is probably influenced by the structure of donor DNA. Experiments performed by Dooner *et al.* [6] demonstrate that *Ac* transposition patterns to linked sites in tobacco vary from locus to locus. Other than transposition, *Ac* and *Ds* elements can be involved in other kinds of DNA rearrangements such as deletions, inversions and duplications. Deletions can refer to both internal sequences of the transposable element and sequences immediately flanking the element [5].

Studies on the transposition process, including so-called 'aberrant' transposition events, may contribute to an understanding of the behaviour of *Ac/Ds* in the plant genome. To be able to perform these studies efficiently, it is important that transposon-plant DNA border fragments can be isolated easily and rapidly from the plant genome. The isolation of such fragments from the maize genome is complicated by the presence of 40 or more *Ac*-homologous copies. It may, therefore, be advantageous to perform these experiments using transgenic plants, in which *Ac/Ds* maintain their capacity to transpose [11]. Transgenic plants can then be selected which contain only one or a few copies of the transposable element.

Another advantage of the use of transgenic plants is that *Ac* and *Ds* elements can be introduced such that genetic and molecular analyses are facilitated. One way to do this is by incorporating the technique of plasmid rescue. This technique, which requires the presence of a plasmid origin of replication and a selectable marker gene functional in *E. coli*, has been used previously to isolate T-DNA border fragments from the genome of transgenic plants [14]. Originally, the applicability of plasmid rescue was restricted by the fact that high *E. coli* transformation efficiencies are needed that cannot be achieved consistently with conventional calcium chloride-based methods. Recently, however, alternative procedures, yielding high transformation efficiencies, were used successfully to rescue several T-DNAs from transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* plants [9, 16, 17, 19]. Based on these results, it can be expected that rescue systems for *Ac/Ds* allow a rapid isolation of transposon-plant DNA junctions, making it possible to accelerate studies on the transposition process.

In this work we demonstrate the applicability of rescue systems to study different aspects of the *Ac/Ds* transposition process in tomato. *Ds*-flanking DNA segments were placed on the RFLP map of tomato. Plasmid rescue is also used to analyse the consequences of the behaviour of *Ac* on the structure of DNA at the original site for *Ac*. The sensitivity of this method allows infrequently occurring 'aberrant' events associated with *Ac* to be identified. Based on the presented results, plasmid rescue is compared both to the construction and screening of phage λ libraries and to PCR techniques.

Materials and methods

Plant material

Tomato plants (*Lycopersicon esculentum* HW61) were transformed with an *Agrobacterium* strain containing the binary plasmid vector pTT283 (with a 7.8 kb *Ds* element between the borders of the T-DNA) [28], as described previously [27]. To induce transposition of *Ds*, transformants were crossed with a transgenic tomato plant containing an active *Ac* element. Original sites of *Ac* were studied using a transgenic plant harbouring an intact T-DNA copy of pTT252 [27]. This T-DNA carries *Ac* with 18 and 35 bp of flanking maize *waxy* sequences [15] inserted between the NPT II gene (at the right-border side) and the bacterial plasmid pBR322 (at the left-border side). The presence of pBR322 allows the isolation of original *Ac* sites by plasmid rescue.

Plasmid rescue

Genomic DNA (10 μg) from a minipreparation of 2 g of greenhouse-grown plants [12] was digested with restriction enzymes according to the manufacturer's recommendations (BRL), circularized in 1.2 ml of ligase buffer (BRL) in the presence of 10 U T4 DNA ligase (BRL) and subsequently dialysed against distilled water. Speedvac centrifugation was used to obtain DNA concentrations of 1 μg/μl. Rescue experiments were performed by electrotransforming (20 kV/cm) 45 μl competent *E. coli* NM554 cells [24] with 2.5 μl DNA using the Gene Pulser apparatus from Bio-Rad. Transformation mixtures were selected on 35 μg/ml chloramphenicol (*Ds* rescue) or 100 μg/ml ampicillin (rescue of original *Ac* sites). The plasmid content in resistant clones was extensively characterized by restriction analysis.

Genetic mapping

Plant DNA segments of rescued *Ds* border fragments were used to localize *Ds* on the RFLP map of tomato. To identify RFLPs suitable for the mapping analysis, the plant DNAs were used as probes to filters containing *L. esculentum* and *L. pennellii* DNA isolates, digested with *Bam* HI, *Eco* RI, *Eco* RV, *Hin*d III and *Hin*f I. Restriction enzymes showing a polymorphism were subsequently used to digest the DNA of 38 F2 plants (laboratory of S. Tanksley) from a *L. esculentum* × *L. pennellii* F1 hybrid [2]. The F2 plants segregate for 64 RFLP markers (S.D. Tanksley *et al.*, in preparation). The segregation data for the plant DNA segments isolated from rescued clones were translated into map positions using the interactive computer package 'MAPMAKER' [18]. DNA isolation, restriction digests, Southern blotting, hybridizations and autoradiography were performed as described previously [26].

Sequence analysis

Ds plant DNA junctions were sequenced using the Applied Biosystems Model 373A DNA sequencing System. Sequences of *Ac* empty donor sites were determined from double-stranded plasmid DNA with Sequenase Version 2.0 (United States Biochemicals) according to the manufacturer's protocol.

Results

Recovery of Ds*-containing T-DNA border fragments*

Tomato plants were transformed with an *Agrobacterium* strain harbouring the binary plasmid vector pTT283 [28]. This vector contains a 7.8 kb *Ds* element within the HPT II gene between the borders of the T-region (Fig. 1A). The *Ds* element is equipped with bacterial plasmid sequences, allowing this element to be rescued from the genome. For our experiments, independent transformants, containing one (AAT6514-02/-30/-33/-64) or two (AAT6514-21) T-DNA insertions (as deduced from Southern blot analysis; data not shown) were used. To determine rescue efficiency, genomic DNA of four transformants containing a single T-DNA was digested with either *Xba* I, *Bam* HI or *Hin*d III, followed by self-circularization to generate plasmids with expected sizes of 5.1 kb, 5.8 kb and 8.2 kb (Fig. 1A). This DNA was subsequently electrotransformed to *E. coli.* The average number of clones obtained per microgram of plant DNA was 1617, 465 and 67 respectively.

Having rescued internal T-DNA fragments from the tomato genome, subsequent experiments were aimed at the rescue of T-DNA/plant DNA junction fragments. Genomic plant DNA was digested with *Bgl* II to generate such fragments (with expected sizes of at least 13.2 kb; Fig. 1A). Despite the inverse correlation between plasmid size and rescue efficiency, which is evident from the previous experiment, T-DNA border fragments ranging from 13.3 to 19.5 kb were rescued reproducibly (1–5 colonies per experiment). Restriction analysis of rescued clones with the enzymes *Bam* HI, *Bgl* II, *Eco* RI, *Hin*d III, *Pst* I, *Pvu* II, *Sac* II and *Sph* I showed that the T-DNA segments were intact, indicating that all five T-DNAs had maintained their structural integrity upon integration.

Characterization of the position of untransposed Ds *elements*

Genomic positions of untransposed *Ds* elements were determined by using T-DNA flanking plant DNA fragments as a probe on Southern blots prepared from DNA digests of *L. esculentum* and *L. pennellii.* The plant DNA fragments were obtained by digesting the rescued clones containing T-DNA borders with *Sac* II and *Bgl* II. A site for *Sac* II is present at only 181 bp from the junction between the right border of the T-DNA and the flanking plant DNA sequence (Fig. 1A). A site for *Bgl* II marks the end of plant DNA sequences in rescued clones (Fig. 1A). A *Sac* II/*Bgl* II dou-

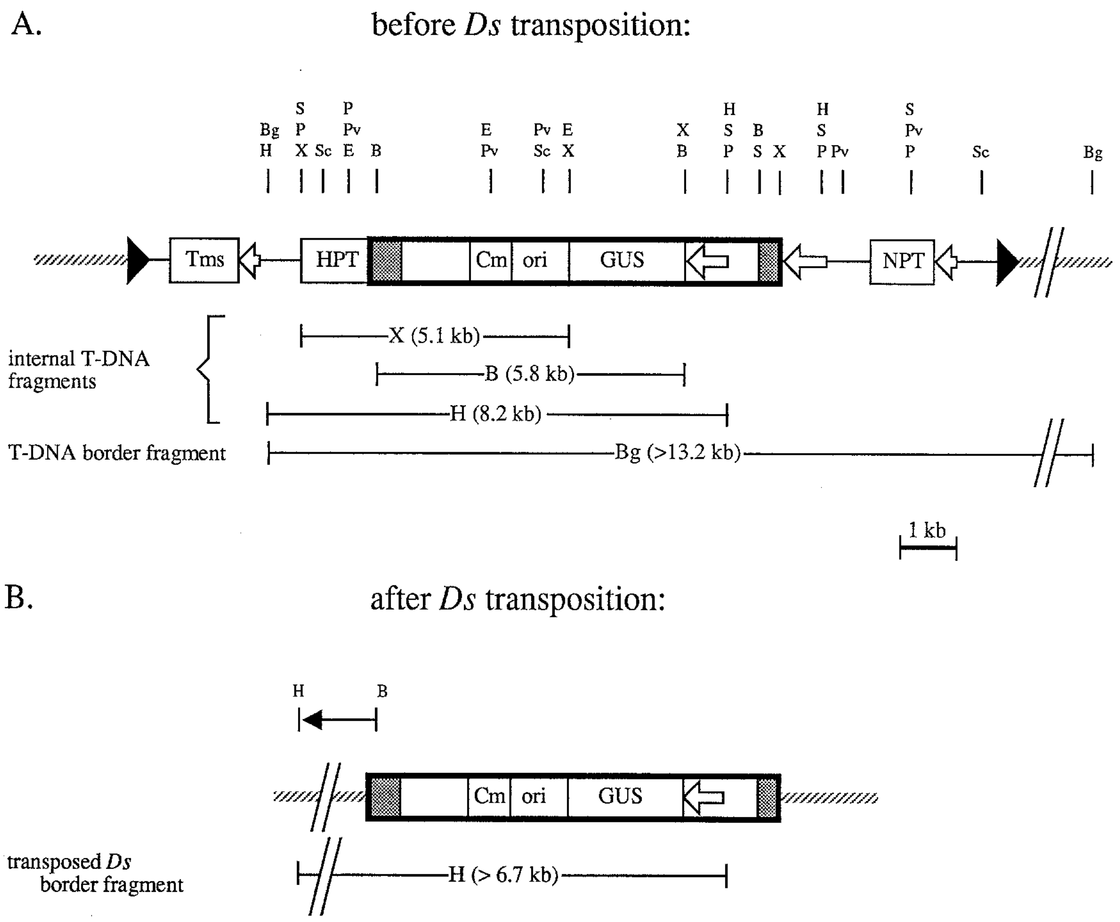

Fig. 1. Schematic structure on scale of *Ds* before (A) and after (B) transposition from the T-DNA insertions of plasmid pTT283. The *Ds* element (box indicated with a thick line) consists of the chloramphenicol resistance gene (Cm), the origin of replication of pACYC184 (ori) and the *β*-glucuronidase gene (GUS), inserted between 0.5 kb *Ac* terminal sequences (dashed boxes). Fragments generated for rescue experiments are indicated by thin lines. HPT, hygromycin phosphotransferase gene; NPT, neomycin phosphotransferase gene; Tms, T-DNA gene 2. Promoters driving these genes are indicated with white arrows. Black triangles refer to the left border (left) and right border (right) of the T-DNA. A dashed line represents plant DNA. The black arrow refers to sequenced *Ds*-plant DNA junctions. B, *Bam* HI; Bg, *Bgl* II; E, *Eco* RI; H, *Hin*d III; P, *Pst* I; Pv, *Pvu* II; *Sc*, *Sac* II; S, *Sph* I; X, *Xba* I.

ble digestion allows the isolation of plant DNA fragments with a minimal T-DNA sequence and it prevents the isolation of additional *Bgl* II fragments. Such additional and unrelated fragments could have been incorporated into the rescued plasmids during ligation of *Bgl* II-digested genomic DNA.

Out of six plant DNA fragments analysed, four (from plants AAT6514-02, -21 and -33) could be used to identify RFLPs between *L. esculentum* and *L. pennellii*. One fragment (from plant AAT6514-30) was too small to visualize any signal on a Southern blot and one fragment (from plant AAT6514-64) hybridized to a repetitive plant DNA sequence. The four plant DNAs revealing RFLPs were used as probes on filters prepared from DNA digests of 38 segregating F2 plants. Computer analysis of the data obtained from the resulting Southern hybridizations made it possible to place the *Ds*-containing T-DNAs of plants AAT6514-02 and -33 on chromosome 6, position 42 and chromosome 1, position 4 respectively (Table 1). The two *Ds* copies present in plant AAT6514-21 were mapped on chromosome 4, position 59, and on chromosome 2, position 4 (Table 1).

The fragment flanking the T-DNA insertion of plant AAT6514-64, which hybridized to a repetitive plant DNA sequence (Fig. 2, lanes 1, 3 and 5), has a size of 2.1 kb. To try and identify a region hybridizing to single-copy DNA, this 2.1 kb plant DNA fragment was digested with several restriction enzymes recognizing 4 or 5 bp sequences (*Dde* I, *Hae* III, *Hin*f I, *Mbo* I and *Taq* I). The subfragments generated in this way were hybridized with total genomic plant DNA as a probe. In most cases, hybridization lead to clearly visible bands on autoradiograms of Southern blots (results not shown). In the case of a 1.0 kb *Hae* III subfragment, however, no band could be observed, even after prolonged exposure times. This suggested that the 1.0 kb *Hae* III subfragment contains a less repetitive plant DNA sequence. A subsequent experiment showed that the subfragment can be used as a probe to visualize an RFLP between *L. pennellii* (Fig. 2, lane 2) and *L. esculentum* (Fig. 2, lane 4). An additional band present in DNA of AAT6514-64 (Fig. 2, lane 6) refers to the presence of the T-DNA insertion in this hemizygous plant. The segregation data for the RFLP visualized by the 1.0 kb subfragment could be used to place the untransposed *Ds* element of plant AAT6514-64 on chromosome 12, position 63 (Table 1).

Our results show that large T-DNA border fragments recovered from the genome of primary transformants can be used to deduce the position

Table 1. Assignment of *Ds* elements to the tomato RFLP map.

Plant number	Size of plant DNA segment (kb)	Chromosome number, position (cM)
Original sites		
AAT6514-02	0.5	6, 42
AAT6514-21	6.3	4, 59
	0.5	2, 4
AAT6514-30	0.1	[a]
AAT6514-33	5.5	1, 4
AAT6514-64	2.1 (1.0[b])	12, 52
New integration sites		
AAT6514-02 × *Ac* line		
Clone ST233	1.1	3, 75
Clone ST223	0.1	12, 63
Clone ST202	0.6	10, 83

[a] The size of the plant DNA fragment flanking the T-DNA insertion present in the genome of plant AAT6514-30 did not allow detection of bands on Southern blots prepared from DNA digests of *L. esculentum* and *L. pennellii.*

[b] Hybridization of the entire T-DNA flanking plant DNA fragment to a repetitive plant DNA sequence made it necessary to use a subfragment for RFLP mapping.

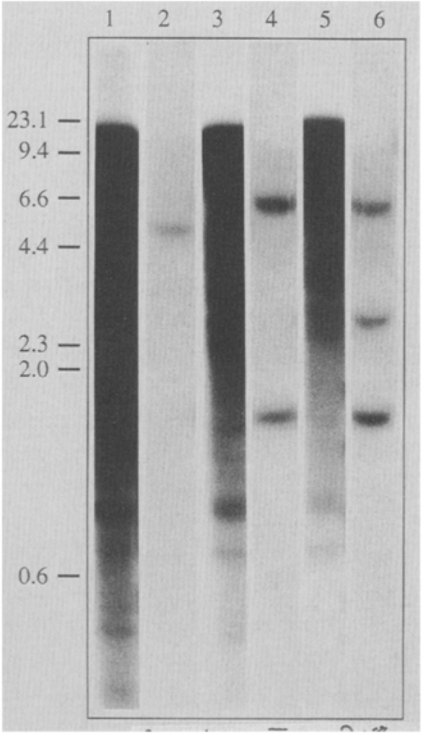

Fig. 2. Identification of an RFLP using a T-DNA border fragment rescued from the genome of plant AAT6514-64. A Southern blot prepared from *Hin*d III-digested DNA of *L. pennellii* (lanes 1 and 2), *L. esculentum* (lanes 3 and 4) and plant AAT6514-64 (lanes 5 and 6) was hybridized with a 2.1 kb plant DNA fragment flanking the T-DNA insertion present in the genome of AAT6514-64 (lanes 1, 3 and 5) and a 1.0 kb *Hae* III subfragment (lanes 2, 4 and 6). The 1.0 kb subfragment could be used to visualize an RFLP between *L. esculentum* and *L. pennellii.* The band in DNA of AAT6514-64, which is absent in DNA of *L. esculentum*, refers to the hemizygous state of the transformant. Fragment sizes were determined from λ DNA size markers.

of *Ds*. In five cases established, the *Ds*-containing T-DNAs are located on different chromosomes.

Rescue of transposed Ds *elements*

Transposition of *Ds* from the T-DNA reduces the minimal expected size for *Ds*/plant DNA junctions and makes it easier to rescue such junctions. Chimaeric F1 plants containing both *Ds* and an active *Ac* can, therefore, be used to obtain 'libraries' of transposed *Ds* border fragments. Genomic DNA isolated from one F1 plant, obtained from a cross between AAT6514-02 and an 'activator' line, was digested with *Hin*d III, self-circularized and transformed to *E. coli.* The minimum expected size for generated plasmids is in this case 6.7 kb (Fig. 1B). Restriction analysis of ten clones with the enzymes *Bam* HI, *Bgl* II, *Eco* RI, *Hin*d III, *Xba* I and *Sac* II showed that the *Ds*-flanking DNAs are different from DNA flanking the untransposed element and that they are different from each other, indicating that the clones refer to independent transposition events. This experiment also showed that the rescued part of *Ds* is intact in all cases, implying that the modified *Ds* element maintains its integrity after transposition. The results obtained from restriction analysis were confirmed by sequencing *Bam* HI-*Hin*d III fragments of seven clones containing *Ds*-plant DNA junctions (Fig. 1B): the sequenced plant DNAs were different in each case and no mutations were detected in the 5′ ends of *Ds* (Table 2).

Three plant DNA segments flanking *Ds* (transposed from chromosome 6) were used to visualize an RFLP. New integration sites were subsequently established on the chromosomes 3, 10 and 12 (Table 1).

Table 2. Structure of *Ds*-plant DNA junctions. *Bam* HI-*Hin*d III fragments of seven rescued clones were subcloned into phage m13mp18 to determine 185 bp 5′ *Ds* terminal sequences from the *Bam* HI site and flanking plant DNA sequences. The 11 bp *Ds* terminal inverted repeat (TIR) and 30 bp of adjacent plant DNA are shown.

TIR	Flanking plant DNA sequence
Clone ST202 TTTCATCCCTG	tacattactcatgttaatttgtcagcagca
Clone ST203 TTTCATCCCTG	gcatggacaacttccagcagatgtaggcct
Clone ST204 TTTCATCCCTG	cgtttggattaacataaccatagagattat
Clone ST225 TTTCATCCCTG	cttatggaaacaatactaatgttgatggct
Clone ST233 TTTCATCCCTG	gctacatgcacttaattgcatcgtattaaa
Clone ST239 TTTCATCCCTG	gcttatgctataaaaaatcagtaagaaaag
Clone ST244 TTTCATCCCTG	gttaaggccatcttaatattctctccccag

The Ac *element can be involved in the formation of deletions*

Apart from studying reintegration of a transposable element, plasmid rescue can be used to study excision of such an element. Here, an *Ac* element, flanked at the original site by a bacterial origin of replication and an ampicillin resistance gene, was used to study the behaviour of this element in a primary transformant. A DNA isolate was di-

Table 3. Structure of *Ac* excision sites in transgenic tomato plants.

		DNA sequence																
Original *wx-m7*		G	G	T	C	A	C	G	C	(*Ac*)	G	G	T	C	A	C	G	C
Empty donor site fragment	1	G	G	T	C	A	C	G	C		C	G	T	C	A	C	G	C
	2	G	G	T	C	A	C	–	–		–	–	T	C	A	C	G	C
	3	G	G	T	C	A	C	G	–		–	G	T	C	A	C	G	C
	4	G	G	T	C	A	C	G	–		–	G	T	C	A	C	G	C

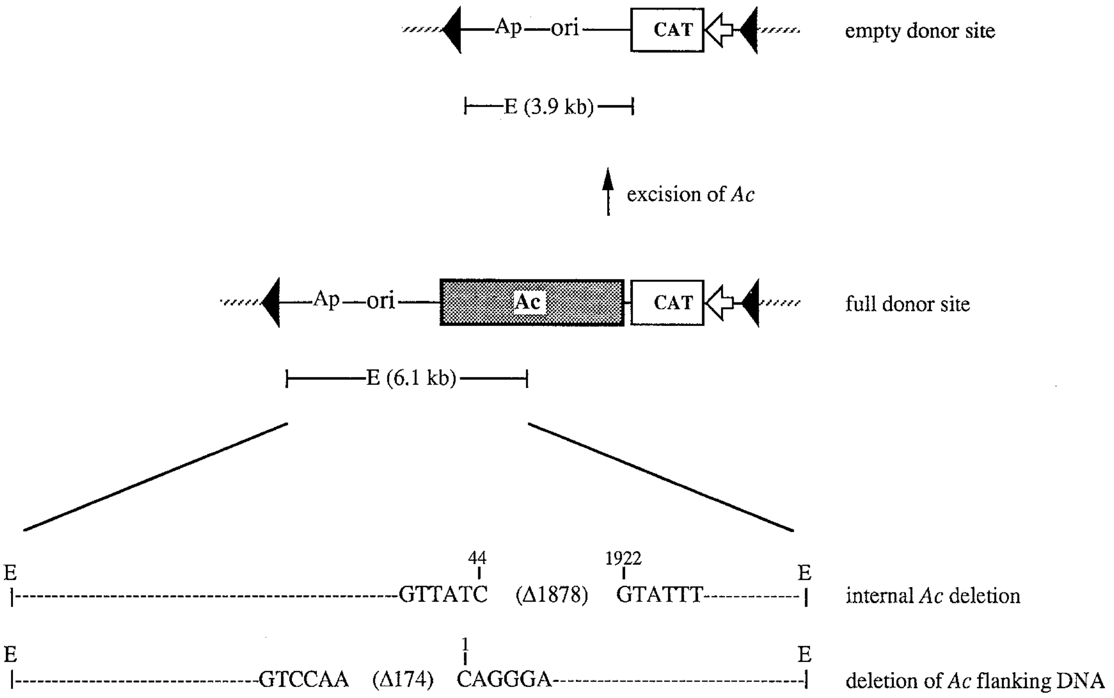

Fig. 3. Analysis of original *Ac* integration sites rescued from the genome of a chimaeric transformant. Apart from full (55) and empty (22) donor sites (A), a rearranged full donor site, lacking (Δ) an *Ac* flanking sequence (B) and a full donor site lacking an internal *Ac* sequence (C), were isolated from the genome of a primary transformant.

gested with *Eco* RI to generate full donor site fragments with an expected size of 6.1 kb and empty donor site fragments with a size of 3.9 kb (Fig. 3). After self-circularization, the DNA was used to generate a library consisting of original sites for *Ac*.

As expected, most rescued plasmids, which were restriction-analysed, represented the full (55 out of 79) or empty (22 out of 79) donor site fragments for *Ac*. Sequence analysis of four empty donor site fragments showed that excision had affected 2–4 bp immediately flanking the original site for *Ac* (Table 3). In two cases, the two central nucleotides of the target site duplication (CG) were deleted. Deletion of these nucleotides was described previously as a consequence of excision of *Ac* flanked by the same target site duplication in rice [20].

Interestingly, two rescued clones, containing an untransposed *Ac* element, refer to possible aberrant transposition events. In one case a 174 bp sequence, originally flanking *Ac*, is deleted. This deletion includes the 8 bp duplicated target site sequence. In the other case an 1878 bp internal *Ac* sequence was removed (Fig. 3).

The results show that, due to the high efficiency of plasmid rescue, which enables large numbers of clones to be recovered and analysed, infrequent rearrangements at the original site of *Ac* can be rapidly isolated from a chimaeric transformant.

Discussion

The efficiency of plasmid rescue

We show that *Ds* elements, equipped with the pACYC184 origin of replication and the chloramphenicol resistance gene, can be rescued efficiently from the genome of transgenic tomato plants. Restriction and sequence analysis shows that the integrity of this large and complicated *Ds* element is maintained upon transposition.

Generated plasmids with a relatively small size (5.1 kb) were rescued most efficiently. In four independent experiments, on average, 1617 clones were rescued per 2.5 μg of genomic DNA. Since the genome size of tomato is calculated to be 7.1×10^5 kb [13], 2.5 μg of genomic DNA will contain approximately 18 pg of the particular 5.1 kb fragment. The rescue efficiency is, thus, $9 \times 10^7/\mu$g DNA fragment. Although the efficiency of plasmid rescue decreases with increasing plasmid size, T-DNA border fragments were isolated reproducibly from *Bgl* II digests of five transgenic plants. The largest fragment isolated has a size of 19.5 kb. In some cases, *Bgl* II fragments may, however, be too large to be rescued efficiently from the genome. Other enzymes, which do not cut between the right or left T-DNA border and the bacterial plasmid sequences (like *Sal* I, *Nde* I, *Apa* LI or *Aat* II), can then be used to generate smaller T-DNA border fragments.

The efficiency of plasmid rescue makes it possible to generate sub-genomic 'libraries' of transposed *Ds* border fragments, using chimaeric F1 plants. Previously, several transposed *Ac*s were isolated by screening phage λ libraries, containing DNA of transgenic tomato plants [22]. Compared to this procedure, the generation of transposon libraries with plasmid rescue is easier and faster. The difference in the number of recovered clones, furthermore, implies that plasmid rescue is more efficient.

As another alternative to plasmid rescue, *Ds* border fragments can, like *Ac* borders [22], be isolated with modified PCR techniques [7, 23]. These techniques do, however, not imply a direct cloning of the isolated fragments. Another disadvantage of modified PCR can be that mainly small fragments are amplified efficiently, limiting the possibilities to characterize both the integrity of the transposable element and the structure of target DNA.

The efficiency of *Ds* rescue is demonstrated by the high frequencies in which *Ds* elements can be rescued from the plant genome. For the rescue of the original *Ac* site, the efficiency is demonstrated by the ability to identify the infrequent occurrence of deletions within or adjacent to *Ac*. Fragments referring to these events were not visible on Southern blots.

Applications of transposon rescue

The ability to obtain relatively large border fragments with plasmid rescue is shown to facilitate the identification of RFLPs required for mapping. In one case, a T-DNA is flanked by a 2.1 kb plant DNA fragment which hybridizes to a repetitive plant DNA sequence. Further analysis of subfragments showed, however, that one subfragment, with a size of 1.0 kb, contains a unique plant DNA sequence.

Current experiments are aimed at determining transposition patterns of *Ds* elements. For this purpose, libraries prepared from several digests are used. In this way, it is prevented that rescued clones represent a non-random selection of the entire pool of transposed *Ds* elements. Based on studies on *Ac* transposition in tobacco [6], a locus-to-locus variation in the *Ds* transposition pattern is expected. *Ds* elements which preferentially transpose to linked sites can be used for targeted tagging experiments; elements displaying a more dispersed transposition pattern can, on the other hand, be used for a non targeted tagging approach [3].

The ability to isolate large numbers of transposed *Ds* border fragments from chimaeric F1 plants may allow a rapid and extensive analyses on the structure of target DNA. For instance, by hybridizing hundreds of independent clones with either total genomic plant DNA or RNA, frequencies in which *Ds* transposes into repetitive DNA sequences or into active genes respectively can be determined easily and accurately. Studies on the nature of target DNA may be important to assess the potential of (*Ds*) transposon tagging.

The technique of plasmid rescue has also been used to isolate modified *P* elements, containing a plasmid origin of replication and a carbenicillin resistance gene, from the genome of *Drosophila* [10]. It was shown that rescued sequences could be used to screen for transposon insertions into cloned genes. This application could, in principle, also be extended to tomato provided the availability of a large number of independent cDNA clones.

Apart from the use of plasmid rescue to study (*Ds*) transposition, it can also be used to examine other DNA rearrangements in which transposable elements are involved. Here, we describe an internal *Ac* deletion and a deletion of an *Ac* flanking sequence. Interestingly, the end-point of the internal deletion in *Ac* is only 31 bp distant from the endpoint of a deletion previously isolated from the maize genome [5]. Internal deletions of the structural similar *P* element of *Drosophila* are believed to be a consequence of incomplete double-strand gap repair [8]. It is, however, unknown whether in this case the plant has employed a similar mechanism as in *Drosophila*.

The observed deletion of *Ac*-flanking DNA is similar to the 789 bp deletion in the maize *bz-s:2114* (*Ac*) allele [5]. In both cases, the adjacent deletions occurred at the proximal site of *Ac* and include the 8 bp direct repeat of the target sequence. The formation of adjacent deletions may be generated as a consequence of intra-replicon transposition [5]. Alternatively, it can be explained by considering a model for *Tam3* transposition put forward by Robbins [25]. This model proposes that double-strand breaks occur at one end of the transposable element and at the recipient site. Only after association of the free ends, a double-strand break occurs at the other side of the element, followed by ligation to the recipient site and resealing of the donor site. Possibly, a short distance between initial breaks might lead to loss of the intervening sequence.

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