

Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells

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To analyze genomic changes resulting from double-strand break (DSB) repair, transgenic tobacco plants were obtained that carried in their genome a restriction site of the rare cutting endonuclease I-SceI within a negative selectable marker gene. After induction of DSB repair via *Agrobacterium*-mediated transient expression of I-SceI, plant cells were selected that carried a loss-of-function phenotype of the marker. Surprisingly, in addition to deletions, in a number of cases repair was associated with the insertion of unique and repetitive genomic sequences into the break. Thus, DSB repair offers a mechanism for spreading different kinds of sequences into new chromosomal positions. This may have evolutionary consequences particularly for plants, as genomic alterations occurring in meristem cells can be transferred to the next generation. Moreover, transfer DNA (T-DNA), carrying the open reading frame of I-SceI, was found in several cases to be integrated into the transgenic I-SceI site. This indicates that DSB repair also represents a pathway for the integration of T-DNA into the plant genome.

Keywords: end-joining/genome/illegitimate recombination/integration/transformation

Introduction

Double-strand breaks (DSBs) are critical lesions in genomes. A single genomic DSB is able to block cell division in mammalian cells (Huang *et al.*, 1996). Efficient repair of DSBs is therefore important for the survival of all organisms. In principle, DSBs can be repaired via illegitimate or homologous recombination. In higher eukaryotes illegitimate recombination seems to be the main mode of DSB repair (Sargent *et al.*, 1997). Various studies on illegitimate recombination have been performed in mammalian cells (for review see Roth and Wilson, 1988), *Xenopus* oocytes (e.g. Lehman *et al.*, 1994; Pfeiffer *et al.*, 1994) and yeast cells (e.g. Mezard and Nicolas, 1994). Studies on recombination in somatic plant cells are of particular biological significance since gametes may arise from meristem cells with mutated genomes (Walbot, 1996). Illegitimate recombination in plants has been investigated mainly via integration of transgenes, especially transfer DNAs (T-DNAs) (Matsumoto *et al.*, 1990; Gheysen *et al.*, 1991; Mayerhofer *et al.*, 1991; Hiei *et al.*, 1994; Ohba *et al.*, 1995; Papp *et al.*, 1996; Iglesias *et al.*, 1997; Takano *et al.*, 1997). Moreover, extrachromosomal illegitimate

recombination between plasmids (De Groot *et al.*, 1994; Gorbunova and Levy, 1997), circularization of extrachromosomal T-DNAs (Bakkeren *et al.*, 1989) and deletions at specific positions in the genome (Wessler *et al.*, 1990; Shirley *et al.*, 1992) were characterized at the molecular level. The main conclusion of these studies was that in many cases recombination occurred at short patches of sequences, homologous between both recombination partners—a phenomenon well known in other organisms (Roth and Wilson, 1988). Frequently short ‘filler’ sequences of unknown origin were found to be included in the new junctions. For plants, it was reported that DNA inserted between the ends of an extrachromosomal plasmid consisted mostly of scrambled sequences derived from the same plasmid and in one case from the tobacco genome. However, DSB repair was always accompanied by deletions whether or not filler DNA was incorporated in the newly formed junctions (Gorbunova and Levy, 1997).

Using highly specific restriction endonucleases for induction of breaks at specific loci in eukaryotic genomes (for review see Haber, 1995; Jasin, 1996) it has been possible to characterize DSB-induced homologous recombination in somatic plant cells (Puchta *et al.*, 1993, 1996; Chiurazzi *et al.*, 1996; Puchta, 1998a). However, the consequences of DSB-induced illegitimate recombination have not yet been analyzed. Therefore, we constructed transgenic tobacco lines which contain within a negative selectable marker (the *codA* gene, see Stougaard, 1993), an I-SceI site cleavable after *Agrobacterium*-mediated transient expression of the I-SceI open reading frame (ORF). When DSB repair resulted in genomic alterations associated with the loss of gene function, the cells became selectable by their resistance to 5-fluorocytosine (5-FC). From sequence analysis of these junctions we were able to identify the molecular structure of different genomic changes induced by DSB repair in plants.

Results

Experimental setup

To characterize illegitimate DSB repair, tobacco was transformed via *Agrobacterium* with the negative selectable marker gene cytosine deaminase (*codA*; Stougaard, 1993), carrying an I-SceI site between the promoter and coding region (Figure 1). The rationale for using this marker was that cutting the genomic I-SceI site would induce repair processes. Cells in which repair does not result in restoration of the functional gene can be selected by including 5-FC in the cultivation medium. Cells expressing a functional cytosine deaminase convert 5-FC into the highly toxic compound 5-fluorouracil, killing the cells, whereas cells lacking the enzyme grow and can be propagated (Stougaard, 1993). To exclude large deletions or conversions from the homolog resulting in

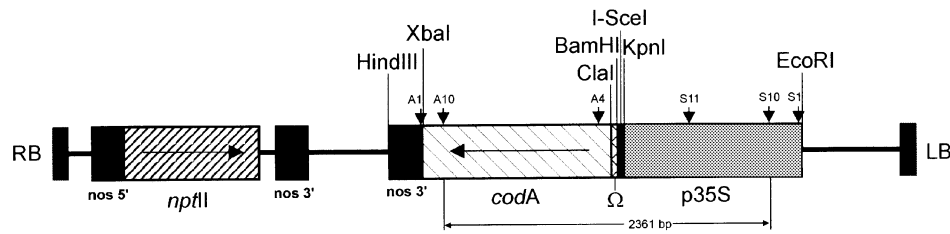


Fig. 1. Schematic map of the T-DNA from the binary plasmid pBNE3I inserted in the tobacco genome to study DSB repair. An I-SceI site was integrated between *codA* ORF and 35S promoter. The T-DNA used for the transformation also contained, in addition to the *codA* gene, a kanamycin gene as transformation marker. A4, A10, A1 and S1, S10 and S11 are primers used for the PCR amplification of the recombination junctions. The arrangement of genes on the T-DNA of the binary vector pCNE3I is identical to pBNE3I. RB, right border; LB, left border.

Table I. Compilation of 5-FC resistant tobacco cells obtained after induction of DSBs by transient expression of I-SceI

Transgenic line	Number of seedlings inoculated	Resistant calli	Tested	PCR positive ^a	I-SceI destroyed ^b	Sequenced
B9	644	90	64	36	13	11
C15	1254	99	63	23	15	14
C19	229	12	12	4	3	2
C25	349	15	6	6	2	1

^aFor a number of 5-FC resistant calli no PCR fragment could be amplified under the conditions used. The most likely explanation is that in these cases the DSB caused a deletion that included at least part of the primer binding sites. Alternatively, insertions too large to be amplified by PCR or other major rearrangements might be responsible for the lack of a PCR fragment.

^bDue to the relatively low concentration of 5-FC used for selection 'escapes' occurred (see Results).

loss of the entire transgene locus, kanamycin selection was applied simultaneously. The kanamycin-resistance gene resides on the same T-DNA but outside the *codA* gene (Figure 1). After DNA extraction from resistant calli, recombination junctions were amplified by PCR. The respective PCR products were then cloned and sequenced to elucidate the molecular nature of the event.

Induction of DSB repair, selection of calli and PCR analysis

We chose four different tobacco lines each containing a single copy of T-DNA of either the binary vector pBNE3I (line B9) or pCNE3I (lines C15, C19, C25) in their genome for this study. Both binaries carry on their T-DNA, in an identical arrangement, both a *codA* and a kanamycin-resistance gene (Figure 1). Seedlings of the selfed transgenic mother plants were inoculated via vacuum infiltration with an *Agrobacterium* strain carrying the binary pCISceI. This binary carries a plant expression cassette for I-SceI on its 2.4 kb long T-DNA (Puchta *et al.*, 1996) but no selectable marker gene. Thus, no selection for the integration of this T-DNA can be applied and the T-DNA may integrate or be lost in the transformed cells after DSB induction. After 3 days of cocultivation the seedlings were transferred to Murashige and Skoog (MS) plates containing 5-FC and kanamycin in the medium. Resistant calli could be detected after several weeks of cultivation. The results of all transformation experiments are summarized in Table I. DNA was extracted from the calli and recombination junctions were amplified by PCR for sequence analysis. PCR fragments could not be detected from all double resistant calli. Since the oligonucleotides used as PCR primers bind within a 2.3 kb region of the T-DNA (Figure 1), deletions involving at least one primer binding site, insertions too large to be amplified by PCR or other major genomic rearrangements might account for this fact. Surprisingly, the I-SceI site

Table II. Compilation of DSB repair events associated with simple deletions at the I-SceI site

Line	Deletion (bp)	Length of deletion at		Homology (bp)
		35S promotor	<i>codA</i> gene	
C15-13 ^a	2	2	–	0
C15-531	3	3	–	0
B9-39	5	1	4	1
C15-23	8	8	–	7 8
C15-671	15	14	1	6 8
B9-48	236	236	–	0
C15-67	305	260	45	6 8
C15-44	1055	1047	8	0
B9-3	1060	1057	3	2
C19-5	1218	390	828	4

The top strand (see Figure 2) was taken as reference for the nick. ^aIn this case the bottom strand was taken as reference for the nick as nucleotides of the 3' end of this strand are found next to the 35S promoter in the junction.

was destroyed in only about half of the PCR products. This indicates, that due to the relatively low concentration of 5-FC used for selection, some calli survived in spite of an intact *codA* gene. Similar results were reported previously when the *codA* gene was used as negative selectable marker in plants (Schlaman and Hooykaas, 1997). PCR products without I-SceI site were cloned and sequenced for further analysis.

Classification of recombination junctions

In total 28 recombination junctions were sequenced. Surprisingly, only 18 of these were reduced in size. In the other 10 cases the size of the locus was increased after DSB repair. The class of junctions with reduced size could be subdivided into those with simple deletions (Table II) and those having deletions associated with insertion of small filler sequences (Table III). The class of junctions

Table III. Compilation of DSB repair events associated with deletions and insertion of small filler sequences

Line	Deletion (bp)	Length of deletion at		Homology		Length of filler (bp)	Origin of filler sequence
		35S promoter (bp)	<i>codA</i> gene	35S (bp)	<i>codA</i>		
B9-62	88	2	86	nt	nt	72	tobacco sequence ^b
C15-131	117	76	41	nt	nt	4	-CATT-
C15-51	262	227	35	nt	nt	19	-ATTTTTTCTACAATATCC-
B9-50	395	290	105	3	3	32	35S promoter sequence
B9-46	458	439	19	nt	nt	121	tobacco sequence ^b
C15-29 ^a	1152	–	1152	nt	nt	13	-GAAATCTGCAGGA-
B9-88	1287	1281	6	nt	nt	5	-TTCAT-
C15-22	1322	1294	30	nt	nt	13	-GATGCGCATCAAA-

The top strand (see Figure 2) was taken as reference for the nick.

^aIn this case the bottom strand was taken as reference for the nick as nucleotides of the 3' end of this strand are found next to the 35S promoter in the junction.

^bAs determined by PCR analysis.

nt: not testable.

Table IV. Compilation of DSB repair events associated with the insertion of genomic sequences into the I-SceI site

Line	Deletion (bp)	Length of deletion at		Homology		Length of filler (bp)	Origin of filler sequence
		35S promoter (bp)	<i>codA</i> gene	35S (bp)	<i>codA</i>		
B9-86	1	–	1	0	3	90	T92 gene (auxin-binding prot.)
C15-36 ^a	0	–	–	nt	7 9	200	sub-telomeric repeats
B9-44	2	–	2	nt	nt	330	low repetitive tobacco sequence
C19-1	11	6	5	nt	nt	359	high repetitive tobacco sequence
B9-82	1	1	–	nt	nt	582	polygalacturonase-like sequence
B9-1	4	4	–	nt	nt	795	retrotransposon Tnt1-like sequence

The top strand (see Figure 2) was taken as reference for the nick.

^aIn this case the bottom strand was taken as reference for the nick as nucleotides of the 3' end of this strand are found next to the 35S promoter in the junction.

nt, not testable.

Table V. Compilation of DSB repair events associated with the insertion of T-DNA into the genomic I-SceI site

Line	Genomic deletion (bp)	Length of genomic deletion at		Homology (bp)		T-DNA length (bp)	Length of T-DNA deletion (bp)	
		35S promoter (bp)	<i>codA</i> gene	35S	<i>codA</i>		LB	RB
C25-1	36	5	31	1	9 15	1745	23	620
C15-47	6	6	–	0	4 7	2292	83	13
C15-52	8	8	–	2 3	0	2355	30	3
C15-53	7	7	–	5 10	5 8	2282	95	11

The top strand (see Figure 2) was taken as reference for the nick. LB, left border; RB, right border.

with increased size could be subdivided into genomic insertions (Table IV) and T-DNA insertions (Table V).

Endonuclease I-SceI cuts within an 18 bp sequence producing 3'-overhangs of four nucleotides (Figure 2). For general compilation of all recombination events the top strand with the nick next to the 35S promoter was taken as reference for the position of the cut in Tables II–V. The 3'-end of the top strand was conserved within most newly formed junctions. There were only three clear exceptions (noted in Tables II–IV) in which the 3' end of the bottom strand was conserved upon repair.

Junctions reduced in size

Simple deletions. In 10 cases, part of the transgene sequence was lost without further change in the locus (Table II). We detected deletions between 2 and 1218 bp. The small deletions of 2–15 bp do not seem to be

responsible for the FC-resistant phenotype of the respective cells by themselves. These calli probably escaped the selection together with a number of other calli that maintain the functional I-SceI site (see Table I). In four cases only the end adjacent to the 35S promoter was deleted, whereas in the other six, both ends of the break were involved in the deletion. While in four cases no homology was found between the rejoined sequences (e.g. Figure 2, junctions C15-531 and B9-48), in one case one identical base pair and in five other cases small, partly interrupted homologies from 2 to 7 bp could be detected (e.g. Figure 2, junctions C15-23 and C19-5).

Deletions associated with the insertion of small filler sequences. In eight cases the junctions reduced in size contained extra sequences between the rejoined ends (Table III). With one exception (C15-29) all deletions

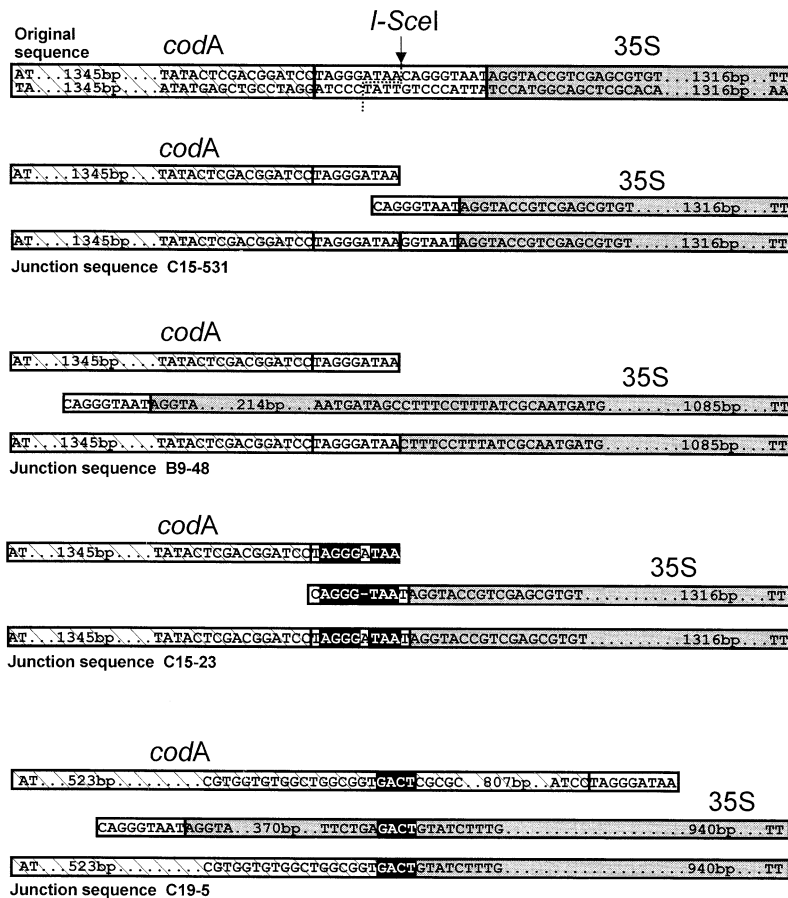


Fig. 2. Representative junctions of simple deletion events (see also Table II). In the upper part the original I-SceI-site (white box) between the *codA* gene (shaded box) and 35S promoter (gray box) is shown. The staggered I-SceI cut is indicated in the double strand. For the sake of simplicity the junction sequences are only shown as top strands. In case of C15-531 and B9-48 no terminal homology was involved in the religation, whereas short patches of homology between the rejoined strands (marked by black boxes) are found in the junctions of C15-23 and C19-5.

involved both ends of the break. The size of the deletions varied from 88 to 1322 bp, and that of the filler sequences from 4 to 121 bp. In five cases the sequences were too short (4–19 bp) to allow valid conclusions about their origin. With the 32 bp filler B9-50 we could identify the sequence as part of the deleted region of the 35S promoter, inserted in inverse orientation. Short patches of homology were found between recombination partners at both filler-break junctions (Figure 3). In the other two cases we performed PCR on wild-type tobacco DNA, using primers specific for the ends of the filler sequences. In both cases we obtained specific bands of the expected size, demonstrating that both sequences are present in the wild-type tobacco genome (data not shown).

Junctions enlarged in size

In 10 cases the PCR fragment obtained after DSB repair was bigger than from the original construct.

Genomic insertions. In six cases this was due to the fact that an insertion of a larger genomic sequence (90–795 bp) was accompanied by no or very small (1, 1, 2, 4 and 11 bp) deletions (Table IV). The insertions were characterized (i) by sequence comparison with known sequences in databases, (ii) by Southern blotting to wild-type tobacco DNA (and as negative control to mouse DNA) to test their abundance in the tobacco genome, and (iii) by PCR

with wild-type tobacco DNA to test whether inserts were copied uninterruptedly into the DSB. The 90 bp insert B9-86 was practically identical (85/90) to the promoter region of the unique T92 gene coding for an auxin-binding protein (DDBJ/EMBL/GenBank accession No. X70903). Whereas the break-insert junction close to the *codA* gene contained three homologous base pairs, no homology could be detected at the other end (Figure 3). In case of the 795 bp insert B9-1 significant homology (311/411) to the tobacco retrotransposon Tnt1 (Grandbastien *et al.*, 1989) was detected. Analysis of the inserted sequence revealed that the ORF coding for the reverse transcriptase domain of Tnt1 is interrupted by stop codons. Therefore, the inserted sequence is not itself derived from an active retrotransposon, although we cannot exclude that the inserted sequence originates from a cDNA produced by trans-complementation of an active Tnt1 element. The sequence is, as expected, repetitive in tobacco (see Figure 4 for Southern blot). Computer analysis indicated that the 200 bp insert C15-36 contains two repeats of a sequence fully matching the consensus sequence of a tobacco chromosomal subterminal repeat (Suzuki *et al.*, 1994). Interestingly, an interrupted 7 bp homology could be detected between the consensus sequence and the break site close to the *codA* gene (Figure 3). The Southern blot of tobacco DNA restricted with *HindIII* (Figure 4) revealed a pattern, in which, in contrast to other repetitive DNAs,

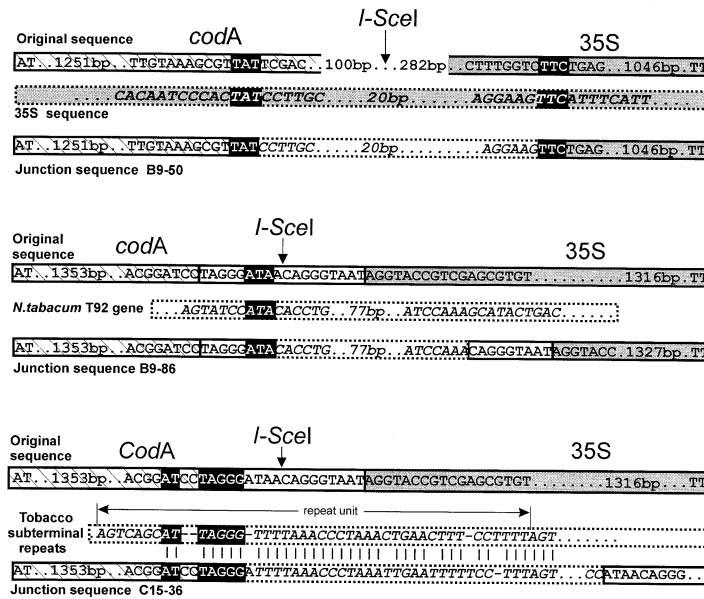


Fig. 3. Junctions of a filler sequence (B9-50; see also Table III) and two genomic insertions (B9-86 and C15-36; see also Table IV). Only top strands are shown. The inserted sequences are marked by dotted boxes. In most cases short patches of homology (marked by black boxes) are found between both recombination partners at the newly formed junctions. With C15-36 only the junction homologous to the core sequences of the tobacco subterminal repeat is shown. Note that in the junction C15-36 the bottom strand was involved in the recombination reaction, as the four nucleotides of the staggered cut site of *I-SceI* (see Figure 2) are found close to the 35S promoter in the junction.

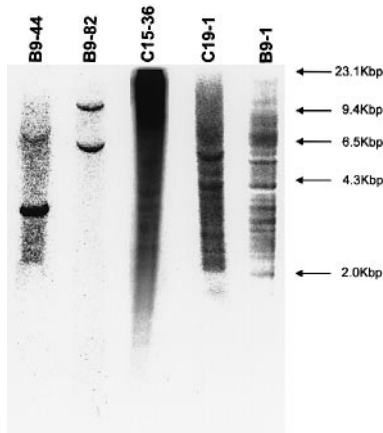


Fig. 4. Southern blots revealing that repetitive and unique genomic sequences are inserted in DSBs. *HindIII*-digested tobacco DNA was used for hybridization with the respective inserts as probes. As a control, *HindIII*-digested mouse DNA was used, which never produced a signal (data not shown). B9-44 (unknown sequence of 330 bp), B9-82 (582 bp partly homologous to 5' sequences of a polygalacturonase gene), C15-36 (200 bp homologous to chromosomal subterminal repeat) C19-1 (unknown sequence of 359 bp) and B9-1 (795 bp homologous to Tnt1 retrotransposon).

the strongest signals are shifted to the top of the gel, indicating an under-representation of *HindIII* sites within the chromosomal regions which contain subterminal repeats. A limited region of homology (49/65) was also found between the 582 bp insert B9-82 and 5' sequences of a polygalacturonase gene (Tebbutt *et al.*, 1994). The insert B9-82 lacks recognition sites for *HindIII* or *EcoRI*, yet Southern blots (Figure 4) of tobacco DNA restricted with *HindIII* or *EcoRI* (data not shown) and probed with the inserted sequence, revealed two bands without any background. This indicates that two different genomic loci within the allo-tetraploid tobacco genome are homologous to the insert B9-82. No significant homologies to sequences

in databases could be detected for the remaining two inserts. From the results of Southern blotting of tobacco DNA we were, however, able to demonstrate that the 359 bp insert C19-1 is a repetitive sequence and has a similar pattern of distribution as the Tnt1 specific insert B9-1 (Figure 4). With the 330 bp insert B9-44, a single strong band was detected in the Southern blot. However, the significant background indicates that there might be sequences related to B9-44 which occur in high copy number in the tobacco genome. Using insert-specific primers and wild-type tobacco DNA we obtained a PCR fragment of the expected size for all inserts, indicating that the inserts were copied in a correct and uninterrupted fashion into the break (data not shown). Our results thus unambiguously demonstrate that different classes of unique and repetitive genomic sequences can be inserted into a DSB in plant cells. Notably, all inserts contained stop codons in all possible reading frames.

T-DNA insertions. In four cases T-DNA molecules used for the transient expression of the *I-SceI* gene were found to be inserted into the break (Table V), again accompanied by small deletions (6–36 bp) at one or both ends of the break. As often found in transgenic plants the T-DNAs were not integrated completely. Although at the left border from the nicking site 23, 30, 83 or 95 nucleotides and at the right border 3, 11, 13 or 620 nucleotides were missing in the individual cases, most of the genetic information of the T-DNA (1.7 and 3 × 2.3 kb) was integrated into the break. We found in five of eight cases small partly interrupted homologies of 2–9 bp between the T-DNA and the flanking ends of the DSB of transgene locus (Figure 5).

Plants were regenerated from several calli representing all different classes of recombinants. Segregation and PCR analysis of the seedlings obtained demonstrated that the

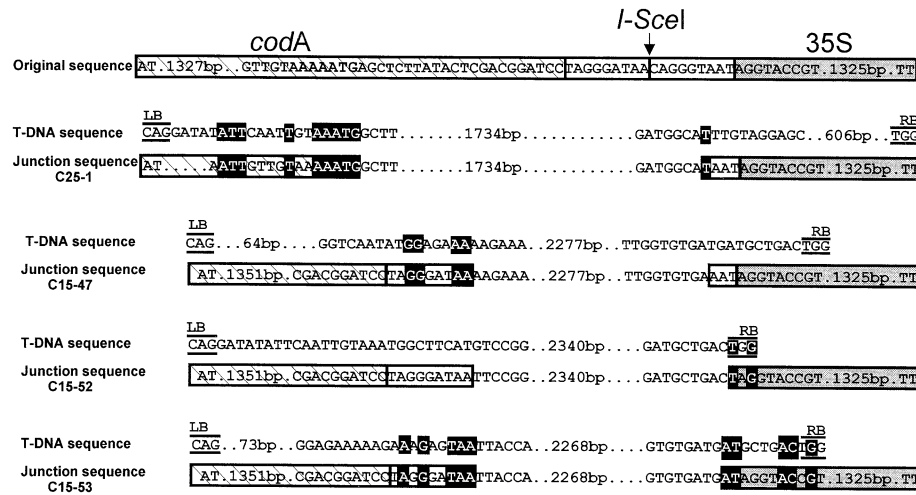


Fig. 5. Junctions between T-DNAs and the break site in the genome (see Table V). Only the top strand is shown. Homologous base pairs are highlighted by black boxes. RB, right border; LB, left border.

induced alterations were stably transferred to the next generation (data not shown).

Discussion

DSB repair in somatic plant cells

Different transgenic tobacco lines which harbored a single artificial *I-SceI* site between promoter and ORF of a cytosine deaminase gene (Stougaard, 1993) were used to study consequences of illegitimate DSB repair in plants. Major alterations were detected via loss of function of the cytosine deaminase, selected with 5-FC in the medium. However, neither DSB repair by correct ligation nor cells in which a non-repaired DSB results in cell death can be analyzed with the assay system. Simultaneous selection for kanamycin resistance was applied to exclude gene conversion events from the homolog in hemizygous plantlets. In contrast to a recent report (Risseuw *et al.*, 1997) we found no indication of instability of the *codA* gene in our transgene lines. In control experiments in which the *I-SceI* gene was not expressed a small number resistant calli was obtained. These calli had escaped the selection, as we did not find changes in the transgene (data not shown).

Sequence analysis of the modified junctions allowed us to elucidate the processes that occurred during somatic DSB repair. We could define two main classes, one in which the size of the locus was reduced due to DSB repair and the other in which the size was enlarged. Reduction in size was due to simple deletions or deletions associated with the insertion of short filler sequences; an increase in size was due to insertions of genomic sequences or T-DNAs harboring the *I-SceI* gene used for transient expression of the enzyme.

Two different pathways are involved in the formation of simple deletions

In 10 events the transgene locus was reduced in size without concomitant insertion of new sequences (Table II). Thus a simple deletion of sequences occurred before or during the joining of the two broken ends. We found two subclasses of such junctions. In one (five events) none or only 1 bp of homology was involved in the rejoining,

whereas in the other (five events) 2–7 homologous base pairs present in both recombination partners could be detected at the junction borders. This is strongly reminiscent of data found in other eukaryotic systems (Lehman *et al.*, 1994; Mason *et al.*, 1996) and for T-DNAs in plants (Bakkeren *et al.*, 1989; Gheysen *et al.*, 1991; Mayerhofer *et al.*, 1991), and confirms data recently reported for DSB repair of plasmid molecules in plants (Gorbunova and Levy, 1997).

For cells of higher eukaryotes, junctions without homologies were explained by simple ligation, whereas small patches of homologous nucleotides were considered to be a prerequisite for the operation of a single-strand annealing (SSA) mechanism (Lehman *et al.*, 1994; Nicolas *et al.*, 1995; Mason *et al.*, 1996; Lieber *et al.*, 1997). In the single-strand annealing reaction two free single-stranded 3' ends interact with each other via a few complementary bases (the homologous nucleotides in the newly formed junction). This interaction is followed by removal of non-complementary nucleotides at the 3' ends so that the consecutive repair synthesis results after ligation in a double strand. Remarkably, in three out of four one-sided deletion events no homologous base pairs were detected between recombination partners at the junctions, whereas in four out of six two-sided deletion events, small homologies (≥ 2 bp) were present. This may indicate that one-sided deletions result primarily from a direct ligation pathway, whereas two-sided deletions are mainly due to a single-strand annealing-like pathway.

Deletions with small filler sequences

In eight events deletion was accompanied by the insertion of a short filler sequence (Table III). In five cases filler sequences were too small (4–19 bp) to draw valid conclusions about their origins. In one case the filler originated from the deleted region of the 35S promoter and in two others from the tobacco genome. With one exception in which the deletion comprised only the *codA* gene end, these deletions involved both ends of the break. We assume in accordance with recent results obtained with plasmid DNA (Gorbunova and Levy, 1997), that filler sequences at the genomic break sites might arise via a modified synthesis-dependent strand annealing (SDSA)

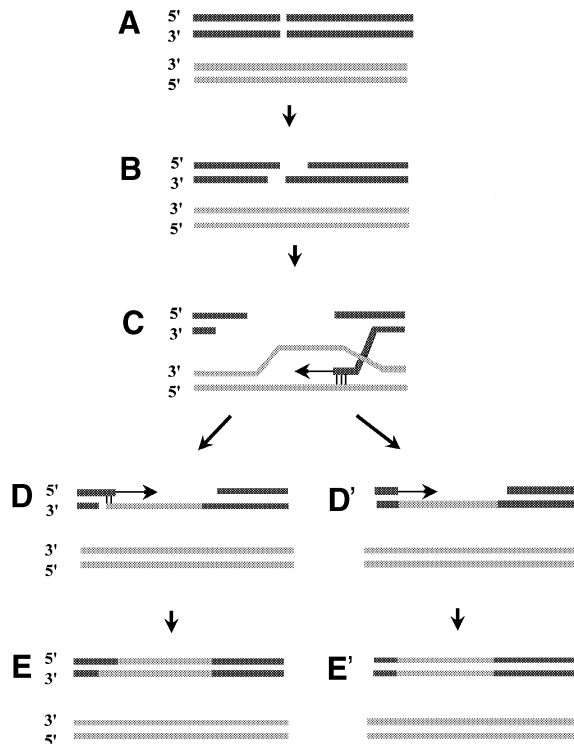


Fig. 6. A model for insertion of (genomic) sequences during the repair of genomic DSBs in somatic plant cells via illegitimate recombination based on a modified version of the SDSA model of recombination. After induction of a DSB in the acceptor molecule (A) 3'-single-strand overhangs are produced by exonuclease-catalyzed digestion (B). By forming a D-loop, a free 3'-end invades the double-stranded donor and repair synthesis starts within a short patch of homology (C). The 3'-end with the newly synthesized sequences is then used to join the break either via another short patch of homology (SSA) (D) or via ligation to a blunt end (D'). In this case no homologous sequences are involved. Finally the remaining single stranded gaps are repaired (E and E').

mechanism (Figure 6) originally proposed for homologous DSB repair in *Drosophila* (Nassif *et al.*, 1994) and recently also for homologous DSB repair in somatic plant cells (Rubin and Levy, 1997; Puchta, 1998a). The mechanism postulates that both 3'-ends of the break act independently. After pairing with a few complementary nucleotides of a donor sequence the ends are able to copy donor sequences via primer elongation into the break (Figure 6). Prerequisites for annealing and priming reactions according to the SDSA model are thus, as for the SSA model, short patches of sequence homologies between the recombination partners. With the junction containing the internal fragment of the 35S promoter, small homologies are indeed present at both ends of the filler (Figure 3). The alternative explanation that free oligonucleotides were captured by the ends of the break seems less probable, especially since such a capture could not be mimicked artificially in mammalian cells (Roth *et al.*, 1991). Moreover, as cells avoid free DNA ends, which would block cell-cycle progression, it seems unreasonable to assume that numerous 'free' nuclear DNA fragments persist for long in the nucleus.

Insertions of genomic sequences into DSBs: a mechanism of genome evolution

For yeast strains deficient in homologous recombination, the integration of retroelement sequences into a DSB

induced by the HO endonuclease has been demonstrated recently (Moore and Haber, 1996; Teng *et al.*, 1996). It was suggested that such insertion events might also play a role in genome evolution of higher eukaryotes (Moore and Haber, 1996). We can now demonstrate that DSB repair can indeed be associated with DNA insertions in higher eukaryotes. A broad spectrum of unique and abundant genomic sequences was found inserted into chromosomal breaks of plants. None or only a few (1–11) base pairs were concomitantly lost at the break site. All sequences were integrated in an uninterrupted way in contrast to the insertion of scrambled plasmid sequences associated with large deletions, which were detected recently in an extrachromosomal assay (Gorbunova and Levy, 1997). This is not surprising, as extrachromosomal 'naked' plasmid DNA differs in its recombination behavior from plant DNA organized in nuclear chromatin (Puchta and Meyer, 1994). In contrast to what has been proposed for yeast (Teng *et al.*, 1996) we do not favor the involvement of mRNA or cDNA intermediates in the reaction, as none of the inserted sequences contained an uninterrupted ORF over its entire length. The simplest explanation of our data would be an uninterrupted copying mechanism of ectopic chromosomal DNA. A stretch of several nucleotides is homologous between the 3'-end of the I-SceI site and the subterminal repeat (Figure 3, C15-36) as well as one end of the T92 promoter (Figure 3, B9-86). Thus, an annealing and priming reaction seems to be involved in the process and genomic insertions might occur, as already suggested for filler sequences (Gorbunova and Levy, 1997), via an SDSA mechanism (Figure 6). We assume that (under our experimental conditions) in most cases only one 3' end is actively invading a genomic donor sequence (Figure 6C). If the two 3' ends would simultaneously invade different donor sequences, a combination of different genomic sequences would result. We did not detect this type of recombination event. The linkage of the invading DNA strand with the other end of the break can be mediated by a micro-annealing step (Figure 6D) or by a simple ligation to the other end without involvement of homology (Figure 6D'). The latter was most probably the case for the insert originating from the T92 gene.

The assay system applied in this study has certain experimental restrictions as it uses staggered breaks that only partly resemble naturally occurring breaks. Moreover, it does not address the question of the frequency with which DSB repair occurs via conversion from the chromosomal homolog. Nevertheless, our data clearly demonstrate that various kinds of sequences can spread into new positions within the plant genome as a result of DSB repair. As a large fraction of the tobacco genome consists of repeated sequences, it is not surprising to find them inserted into breaks. Current models explain amplification of repetitive genomic sequences by unequal homologous recombination between repetitive sequences in tandem (Flavell, 1985) or by replicative transposition of retroelements (e.g. SanMiguel *et al.*, 1996). However, these models do not explain how transposition-inactive sequences can be transferred into new positions within the plant genome. Through DSB repair nearly any kind of genomic sequence which is available for a copying process might be inserted into new genomic positions. This phenomenon might be

of general importance for higher plants and their genome evolution. [In plants the 'germline' is only set apart late in development and alterations occurring in meristem cells can be transferred to the next generation (Walbot, 1996).] Many plants contain large genomes with large amounts of repetitive DNA. The high variability in genome size between closely related species indicates considerable plasticity. It is tempting to speculate that part of the genome size differences might be due to DSB repair. The spread of retrotransposons was postulated as a main force in enlarging plant genomes (Bennetzen and Kellogg, 1997). This genomic enlargement, however, seems to be counterbalanced, at least in part, by deletions (Petrov, 1997). DSB repair is a prominent source of deletions. Insertions in turn may partly counterbalance these deletions, as suggested by our results on frequent insertions upon DSB repair. Therefore it will be of significant interest to test the ratio of insertions versus deletions not only for tobacco with a large genome but also for plants with relatively small genomes like that of *Arabidopsis*.

T-DNA insertions

Remarkably, T-DNA was found to be inserted into the DSB in four recombination events (Table IV). In three cases almost the complete sequence of the 2.4 kb long T-DNA was integrated, in one case a fragment of 1.7 kb. In our experimental setup the cauliflower mosaic virus (CaMV) 35S promoter is driving the transient expression of the I-*SceI* gene. The same promoter is also present adjacent to the I-*SceI* site in the target locus, representing a sequence homology of 0.5 kb between the incoming T-DNA and the transgene. Using a similar setup we recently were able to show that in about one out of 125–300 transformation events such a construct recombines with the homologous sequence after DSB induction in the target locus (Puchta, 1998a). However, the four insertions analyzed here were not integrated via homologous recombination. Thus even in the vicinity of a homologous sequence T-DNA integration into a genomic break may occur more frequently via illegitimate than homologous recombination. The fact that T-DNA was inserted into the same break in the plant genome in several cases implies that (i) breaks might play a significant role in T-DNA integration and (ii) the number of breaks in a cell might be rate limiting for integration, as genomic breaks seem to be kept to a minimum. Further support for our interpretation can be found in the literature. A recent study on hamster cells reported the integration of plasmid-specific sequences carrying an I-*SceI* ORF into an I-*SceI* site in two out of 11 analyzed recombination events, although these experiments could not distinguish between one-sided homologous or illegitimate integration (Pipiras *et al.*, 1998). It was also shown that X-rays, which induce DSBs in the genome can enhance transgene integration in plant cells (Köhler *et al.*, 1989). Using cotransformation it could be demonstrated that different T-DNAs integrate into a single genomic locus (De Block and Debrouwer, 1991; De Neve *et al.*, 1997). This indicates that a surplus of extrachromosomal DNA ends is recognized by the cell as breaks to be ligated, yielding a tandem arrangement of transgenes, which is then linked to free genomic ends.

Various studies on T-DNA integration indicate that there are in principle two different classes of integration patterns

(e.g. Matsumoto *et al.*, 1990; Gheysen *et al.*, 1991; Mayerhofer *et al.*, 1991). In about two-thirds to three-quarters of the cases the right border (which is conserved in the recombinant) and the target locus contain no or only one base pair microhomology, whereas longer homologies exist between the left border (combined with truncations) and the pre-insertion site. In these events the two ends behave differently, and it has been postulated that integration occurs via single-strand annealing followed by the right border being linked to genomic sequences directly or indirectly by the VirD2 protein which is covalently bound to the 5' end of the T-strand (Tinland, 1996). In a second class of events not only the left but also the right border is partly truncated in the recombinants, and homologous nucleotides can be found in most cases also between the right border and the pre-insertion site (e.g. Matsumoto *et al.*, 1990; Mayerhofer *et al.*, 1991). This pattern of integration was found in our study. We therefore suggest that integration events of this class are generally mediated by DSB repair. T-DNA molecules are transferred as single strands to the nucleus, where some of them become double-stranded before integration (Tinland *et al.*, 1994). By interacting with break ends, a single- or double-stranded T-DNA could be copied into the genome via SDSA (Figure 6). Alternatively, a double-stranded T-DNA could also be integrated at the break by two consecutive SSA reactions or by a single-strand annealing reaction and a subsequent simple ligation step.

Our results might also be interesting for practical application in plant biotechnology: as gene targeting is still not a feasible technique for plants (Puchta, 1998b) the establishment of site-specific integration systems is of considerable interest. Sophisticated techniques based either on the Cre-*lox* system (Albert *et al.*, 1995; Vergunst and Hooykaas, 1998; Vergunst *et al.*, 1998) or on DSB-induced homologous recombination (Puchta *et al.*, 1996) have been established. Inducing DSBs at desired positions in the plant genome may suffice to obtain site-specific integration of transgenes at these sites in numbers which allow easy identification of the appropriate recombinants via PCR using a combination of locus and insert-specific oligonucleotides.

Materials and methods

DNA constructs

All plasmids were propagated in *Escherichia coli* strain DH 5 α . The plasmid pNE3 (Stougaard, 1993) carrying a *codA* ORF under the control of plant expression signals was digested with *XhoI* and religated with a *SalI* fragment derived from two complementary oligo nucleotides with the recognition site of the restriction endonuclease I-*SceI* 5'-pCCGGGTCGACGGTACCTATTACCCTGTTATCCCTAG-GATCCGTCGACCCG-3', resulting in the plasmid pNE3I. The binary vector pBin19 (Bevan, 1984) was digested with *EcoRI* and *HindIII*, and ligated with the *codA*-containing *EcoRI/HindIII* fragment of pNE3I resulting in pBNE3I (see Figure 1). The *EcoRI*-linearized plasmid pNE3I was cloned into the binary vector pCIB200 (Rothstein *et al.*, 1987), resulting in the binary vector pCNE3I. Both binary vectors carry, besides the functional *codA* gene with a I-*SceI* site between promoter and ORF, a kanamycin gene as transformation marker in an identical arrangement as depicted for pBNE3I in Figure 1. The binary vectors were transferred into *Agrobacterium tumefaciens* via electroporation (voltage 2500 V, capacity 25 μ F, shunt 201 Ω and 5 ms pulse; EasyjecT Plus system, EquiBio, UK).

The I-*SceI* expression vector pCISceI (Puchta *et al.*, 1996) contains a synthetic I-*SceI* ORF under the control of the cauliflower 35S promoter (Puchta *et al.*, 1993) between T-DNA borders.

Plant transformation and transient expression

In a first series of experiments *Nicotiana tabacum* L. cv. Petite Havana line SR1 plants were transformed with the *Agrobacterium* strains harboring either the binary vector pBNE3I or pCNE3I. Vacuum infiltration of tobacco seedlings with *Agrobacterium* was performed as described (Puchta, 1998c). The seedlings were placed for 3 days on MS plates [0.3 % Gelrite (Merck, USA) without hormones] and transferred afterwards onto MS plates containing hormones [0.5 µg α-naphthalen acetic acid (NAA) and 5 µg 6-benzyl amino purine (BAP) per ml], 100 µg kanamycin sulfate, 200 µg vancomycin (Duchefa, Netherlands) and 500 µg cefotaxime (Duchefa, Netherlands) per ml. For callus induction the infiltrated seedlings were transferred to a growth chamber (24°C; 16 h light/day). Every week the transformed plantlets were transferred onto fresh media. When the calli formed shoots they were transferred to boxes (Magenta, Chicago, USA) containing MS medium without hormones for plant regeneration. After root development the plants were placed in a greenhouse and allowed to grow to maturity. Segregation of the selfed transformants was tested by germination of the seeds on MS medium supplemented with 300 µg kanamycin sulfate and 500 µg 5-FC per ml.

In a second series of experiments the transgenic lines B9, C15, C19 and C25 were inoculated with an *Agrobacterium* strain harboring the binary pCISceI as described above. Double selection using 100 µg 5-FC (Sigma, USA) and 100 µg kanamycin sulfate per ml medium was applied. After 8 weeks the 5-FC concentration was increased to 200 µg/ml medium. In most cases surviving calli were cut into two pieces after an additional 6 weeks. One piece was used for shoot regeneration and the other was used for DNA isolation.

Plant DNA extraction

DNA extraction from leaf tissues and calli was carried out as described in Fulton *et al.* (1995) using 1–2 g of plant tissue.

Southern analysis

Southern blotting using the hybridization membrane 'Hybond N' (Amersham, UK) was performed as described (Swoboda *et al.*, 1994). The respective DNA fragments were labeled using a random priming labeling kit (Megaprime DNA labeling system RPN1607, Amersham, UK) and [α -³²P]dATP (Amersham, UK). For identification of plants with single copy integration of pCNE3I the *codA* coding region was used as hybridization probe. The plant DNA was digested with *Hind*III and *Hind*III/*Sce*I. To determine the abundance of the inserted genomic sequences in the tobacco genome, specific probes were generated by PCR (see below). Wild-type tobacco and mice DNA (as negative control) was digested with *Hind*III or *Eco*RI (probe B9-44). Hybridizations were performed at 65°C.

PCR and sequence analysis

Genomic DNA was analyzed by PCR using the primers S1 5'-pGGCTCT-AGAGCTTAACAGCAGTTGC-3' and A1 5'-pTTGACTCTAGAG-GATCAACG-3' in a first amplification and the nested primer pair S11 5'-pGCGGGAAGCTTGAAGCTCGCCGTGAAGACTGG-3' and A4-5'-pCGCTCTAGAGCCCTCTTCGCTGGTAAC-3' in a second amplification. Two microliters of the reaction volume from the first amplification were used as the template for the second amplification. In the first amplification the PCR reactions were performed by using SAWADY Long PCR Kit (peqlab, Erlangen, Germany), in the second amplification *bio*Taq DNA polymerase (*biomaster*, Köln, Germany) was used. The PCR reactions were performed according to the polymerase manufacturer's recommendation in a Gene Amp PCR-System 2400 (Perkin Elmer, USA). The following program was used for the first amplification: 5 min denaturation at 94°C, 40 cycles of 20 s at 93°C, 35 s at 55°C and 2.5 min at 68°C, and a final step of 7 min at 68°C. For the second amplification the cycles were modified to 5 min denaturation at 94°C, 20 cycles of 20 s at 92°C, 35 s at 58°C and 2 min at 68°C, and a final step of 7 min at 68°C. In case no PCR amplification could be detected, primers S10 5'-pGCGGGAAGCTTTCAGCTGACGCGTACACAACA-3' and A10 5'-pGCGTCTAGACATTTTCAGCCGGCAGGATA-3' were used instead of S11 and A4 for the second amplification. The amplification products were cloned into pCR 2.1-Topo vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, USA) and propagated in TOP10 One Shot Cells (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. When the cloned PCR fragments were too long for a one step sequence analysis, subclonings were performed. Restricted fragments were cloned into pBluescript SK (Stratagene, La Jolla, USA). Sequence analysis was performed with the automatic DNA-sequencer AFL-Express

(Pharmacia, Uppsala, Sweden). Standard M13-20Forward, M13Reverse, T3 and T7 primers were used for the sequencing reaction.

For synthesis of hybridization probes clone B9-1 was amplified with primers 5'-pCTAAAGAGAAGATACAAATG-3' and 5'-pGAGCAGG-AAGACAATCAGTT-3', clone B9-44 with 5'-pAATTTTGTACCCCT-TTTTC-3' and 5'-pATTTTCTAGAAATCTCT-3', clone B9-82 with 5'-pATTAGGGGACGTTTGGGTTGA-3' and 5'-pTCTTCTTGGCT-GCAGTCTCCTTC-3', clone C15-36 with 5'-PTTTTATATTTCAACA-ACA-3' and 5'-pAATTTTCTTTAGTCAG-3', clone C19-1 with 5'-pTTACTTGGATTAAGAT-3' and 5'-pAGATCGTATATGAA-ACAAA-3', clone B9-62 with 5'-pCAATTACAATTACATTACA-3' and 5'-pGCGTTATTCGACATCTC-3' and clone B9-46 with 5'-pATGTCGGAGGTATTTATT-3' and 5'-pACTCTGTATACTCCA-GGATG-3'.

DDBJ/EMBL/GenBank accession numbers

The sequences of the genomic insertions are deposited under the numbers AF061073 to AF061078.

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