The role of double-strand break-induced allelic homologous recombination in somatic plant cells

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Summary

During meiosis, homologous recombination occurs between allelic sequences. To evaluate the biological significance of such a pathway in somatic cells, we used transgenic tobacco plants with a restriction site for the rare cutting endonuclease I-*Sce*I within a negative selectable marker gene. These plants were crossed with two tobacco lines containing, in allelic position, either a deletion or an insertion within the marker gene that rendered both marker gene and restriction site inactive. After the double-strand break induction, we selected for repair events resulting in a loss of marker gene function. This loss was mostly due to deletions. We were also able to detect double strand break-induced allelic recombination in which the break was repaired by a faithful copying process from the homologue carrying the shortened transgene. The estimated frequency indicates that homologous recombination in somatic cells between allelic sites appears to occur at the same order of magnitude as between ectopic sites, and is thus far too infrequent to act as major repair pathway. As somatic changes can be transferred to the germ line, the prevalence of intrachromatid rearrangements over allelic recombination might be an indirect prerequisite for the enhanced genome plasticity postulated for plants.

Keywords: recombination, loss of heterozygosity, tobacco, genome.

Introduction

A specific plasticity has been attributed to plant genomes (McClintock, 1984). Even closely related plant species can differ quite drastically in genome size (Bennett and Leitch, 1997). In recent years, major causes of the phenomenon have been elucidated (Petrov, 2001). The spread of retroelements is one major driving force in plant genome evolution (San Miguel *et al.*, 1996). But also the repair of double-strand breaks (DSBs) via illegitimate recombination can lead – depending on the plant species – to different genomic alterations such as insertions and deletions (Gorbunova and Levy, 1997; Kirik *et al.*, 2000; Salomon and Puchta, 1998). As the germ line is defined late in plant development, genomic alterations occurring in meristematic cells can be transferred to the next generation (Walbot, 1996). However, genomic alterations would be minimized if the DSB is repaired via the use of the allelic sequence on the homologue, excluding deleterious changes from other kinds of repair. Allelic recombination occurs in a controlled fashion during meiosis (Roeder, 1997). If a similar pathway is absent, or operates only at low frequency in somatic cells, genomic changes could accumulate much more easily in a

plant population. Therefore it was important to find out if such a pathway exists at all in somatic plant cells and, if so, at what frequency DSB-induced allelic recombination takes place.

A major obstacle to address this question was the setup of an experimental situation in which allelic recombination could be analysed with the help of marker genes. As gene targeting is not feasible for flowering plants (for review see Hohn and Puchta, 1999; Mengiste and Paszkowski, 1999; Puchta, 1998a; Puchta, 2002; Vergunst and Hooykaas, 1999), it was not possible to incorporate different transgenes in allelic positions within the genome of a higher plant. We overcame the problem by crossing transgenic tobacco plants which we obtained after DSB-induced repair within a transgene (Salomon and Puchta, 1998) with the original transgenic line. Repaired and original transgenes occupied allelic positions, but only the latter carried a functional marker gene and a recognition site for I-*Sce*I. By selecting for recombination events after DSB induction, we could demonstrate that DSB-induced allelic homologous recombination indeed takes place in somatic plant cells, evaluate

the frequency of its occurrence, and estimate its potential role in plant genome evolution.

Results

Experimental setup

To analyse DSB-induced allelic recombination, a test line containing distinguishable DNA sequences in allelic positions is required. One of the sequences should carry a restriction site (acceptor of information), and the other should encode a selectable phenotype (donor of information). In previous experiments we analysed the rejoining of a DSB in tobacco plants that are transgenic for an I-*Sce*I site between the 35S promoter and the coding region of the negative selectable marker gene cytosine deaminase (*cod*A) (Salomon and Puchta, 1998; Stougaard, 1993; Figure 1). After *Agrobacterium*-mediated transformation of an open reading frame (ORF) encoding I-*Sce*I for induction of DSBs, plants with a non-functional *cod*A gene were isolated by selection on a 5-fluorocytosine (5-FC)-containing medium. This resulted in plant lines that contained stretches of DNA in allelic positions that either harboured a restriction site, or could be identified by a selectable phenotype. For our experiment we chose line B9, harbouring the original transgene with the functional I-*Sce*I site and a functional *cod*A
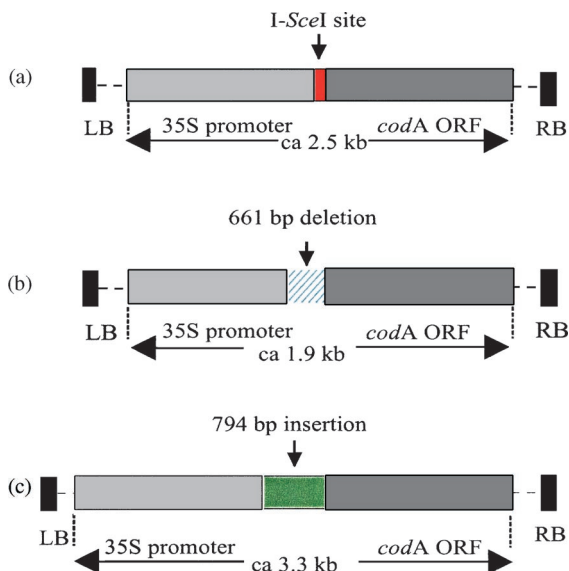
Figure 1. Scheme of *cod*A-specific transgene sequences of lines B9, B9-300 and B9-1.

B9 contains, as single copy, a functional *cod*A (cytosine deaminase) gene harbouring a functional I-*Sce*I site between promoter and ORF (a). Lines B9-300 and B9-1 were isolated from line B9 after DSB induction by I-*Sce*I and selection for a non-functional *cod*A gene. Line B9-300 carries a deletion of 661 bp (b) and B9-1 an insertion of 794 bp (c). Both alterations render the *cod*A expression cassette and I-*Sce*I site non-functional (Salomon and Puchta, 1998). RB, right border; LB, left border; bp: base pair; kb: kilobase pair.
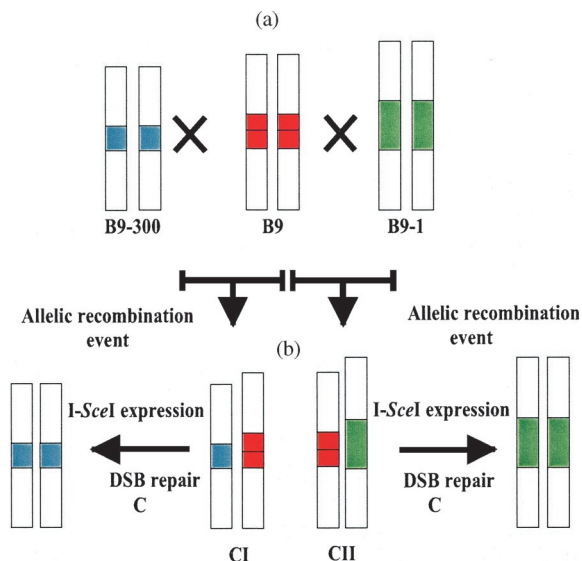
Figure 2. Strategy for detecting of allelic recombination in somatic plant cells.

Plants of line B9 (red) carrying a functional *cod*A gene and a I-*Sce*I site on both homologous chromosomes were crossed with homozygous plants of line B9-300 (blue) or line B9-1 (green) (a). Both latter lines descended from line B9 after DSB induction by I-*Sce*I and selection for a non-functional *cod*A expression cassette. Thus lines B9, B9-300 and B9-1 carry transgenes in allelic positions. The two crosses yielded uniform heterozygous offspring (lines CI and CII) that carry on one allele a functional *cod*A expression cassette with a I-*Sce*I site (b). By I-*Sce*I expression, a DSB is induced in this allele. In case of repair by allelic recombination, for line CI plants homozygous for B9-300 and for line CII plants homozygous for B9-1 should be detected (c).

gene; line B9-300, containing a 661 bp deletion within the region of the *cod*A expression cassette; and line B9-1, with a 794 bp insertion at the same position (Figure 1). The transgenes of all three lines were consistently transmitted over several generations. To create an experimental situation in which we could analyse allelic recombination, homozygous plants of both lines with modified transgenes were crossed with B9, the line carrying the original transgene (Figure 2a). The heterozygous line CI was obtained by crossing line B9 with line B9-300. Crossing line B9 with line B9-1 resulted in the heterozygous line CII (Figure 2b). This experimental setup enabled us to detect DSB-initiated allelic recombination in plants (Figure 2c).

Detection of DSB-induced loss of function of the codA marker in line CI

Besides the partly deleted *cod*A expression cassette line B9-300 also contains a co-segregating functional I-*Sce*I ORF, resulting in the expression of the I-*Sce*I restriction enzyme. After crossing line B9-300 with line B9, DSB induction could take place already within the zygotes of line CI. To screen for the loss of function of the *cod*A gene, we sowed CI seeds on a 5-FC-containing medium. In total, about 30 000 seeds

(about 20 000 from line B9 after pollination with line B9-300 and 10 000 from line B9-300 after pollination with line B9) were tested for 5-FC resistance

164 seedlings were detected that were able to grow on the 5-FC medium. By PCR analysis, in 144 cases we detected two fragments, one of the size of the locus B9-300 and the other of the size of locus B9. Thus no allelic recombination event occurred within the test locus in these lines. We sequenced 59 fragments of B9 size class and detected deletions and insertions in all cases at the I-*Sce*I site, most of which had only a few nucleotides, resembling the patterns typical for end-joining in plant cells (Gorbunova and Levy, 1997; Salomon and Puchta, 1998). However, these small changes should not have resulted in a loss of function of the *cod*A gene. It appears that (as reported in several previous studies), depending on the selection conditions, a certain number of escapes might occur with this marker gene (e.g. Gallego *et al.*, 1999; Salomon and Puchta, 1998). In our case, the escape rate was at the level of 0.5% of the examined seedlings. As the I-*Sce*I site was destroyed in all events analysed, we concluded that apparently in all, or almost all, seedlings DSBs had been induced soon after pollination. To further sustain this hypothesis, and to exclude any influence of the selection procedure, we also sequenced fragments amplified by PCR from 47 seedlings that were not grown on selection medium. In all cases, similar deletions or insertions were detected at the I-*Sce*I site, as in case of the 59 fragments obtained from seedlings under 5-FC selection.

In 20 out of the 164 plants that grew on a 5-FC medium, we were only able to amplify the B9-300 specific fragment with our PCR assay. This could be due to allelic recombination, to the loss of the chromosome, or to a deletion within the chromosome harbouring the original transgene. Using Southern analysis, in addition to the locus B9-300, we detected other transgene specific fragments in 13 cases, indicating that different, bigger deletions and insertions had occurred within the transgene locus (data not shown).

Characterization of allelic recombinants of line CI

In the remaining seven cases (A1, A2, A4, B1, B2, E42, D81) only the B9-300 specific fragment could be detected via Southern hybridization (Figure 3). This could be due to allelic recombination, or to deletions removing at least the complete original transgene locus, or to chromosome loss. The segregation behaviour of the progeny of the respective plants allowed us to distinguish between these possibilities. Allelic recombination is expected to result in a uniform progeny homozygous for the respective sequence, while large deletions, as well as chromosome loss, should result in a non-uniform segregating population of siblings.

Because four of the seven plants were sterile (a fact that might be due to major DSB-induced genome alterations),
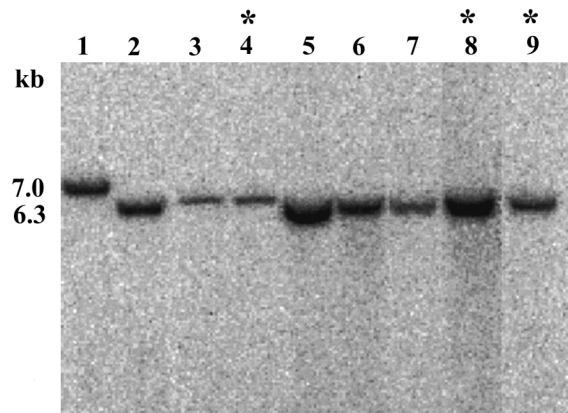
Figure 3. Southern blot with *Eco*RI-restricted DNA of recombinant plants of the cross line CI (B9 × B9-300).
The membrane was probed with a *cod*A-specific fragment which results in the original line B9 in a band of ≈7 kb (lane 1), and in line B9-300 – due to the deletion within the *cod*A gene – in a fragment of ≈6.3 kb (lane 2). Of the seven isolated recombinant plants harbouring only the 6.3 kb band (lanes 3–9), three lines (A4, D81 and E42; lanes 4, 8 and 9, all marked by asterisks) produced homozygous progeny, indicating DSB repair by allelic recombination.

we were able to test only the progeny of plants E42, D81 and A4. All three lines descended from the seed batch obtained from demasculated flowers of the line B9 pollinated with line B9-300. Whereas in case of pollination of demasculated flowers of line B9-300 with line B9 an inadvertent contamination with pollen of the mother plant would have resulted in a genotype identical to an allelic recombinant, the reverse combination *per se* excludes any putative pollen contamination as the source of such a genotype.

All seeds of the three lines germinated. Thirty-two seedlings of the selfed plant E42 were tested by Southern hybridization (Figure 4a). The analysis revealed the presence of the B9-300-specific fragment in all plants. PCR and sequence analysis confirmed the nature of all 32 fragments as B9-300 specific. Thus we can conclude at the 95% confidence level ($\chi^2 = 4.57$) that the progeny of E42 carries uniformly the transgene locus (Simpson *et al.*, 1960). For 32 seedlings of the selfed plant A4, Southern hybridization gave the same results as for line E42. The respective sequence analysis revealed only B9-300-specific sequences. Similar results were obtained for line D81. Moreover, the homozygous line B9 was fertilized with pollen from the lines E42 and D81. Southern blotting of 16 seedlings of the cross B9 × E42, and 32 seedlings of the cross B9 × D81, revealed a uniform progeny carrying the B9-300 allele (Figure 4b). Thus we can conclude by a second independent experiment at the 99.5% confidence level (E42 $\chi^2 = 9.74$; D81 $\chi^2 = 18.02$) that the pollen of both recombinants tested was homogeneous for the transgene. All this strongly indicates that the isolated events are indeed due to allelic recombination.

(a)

(b)
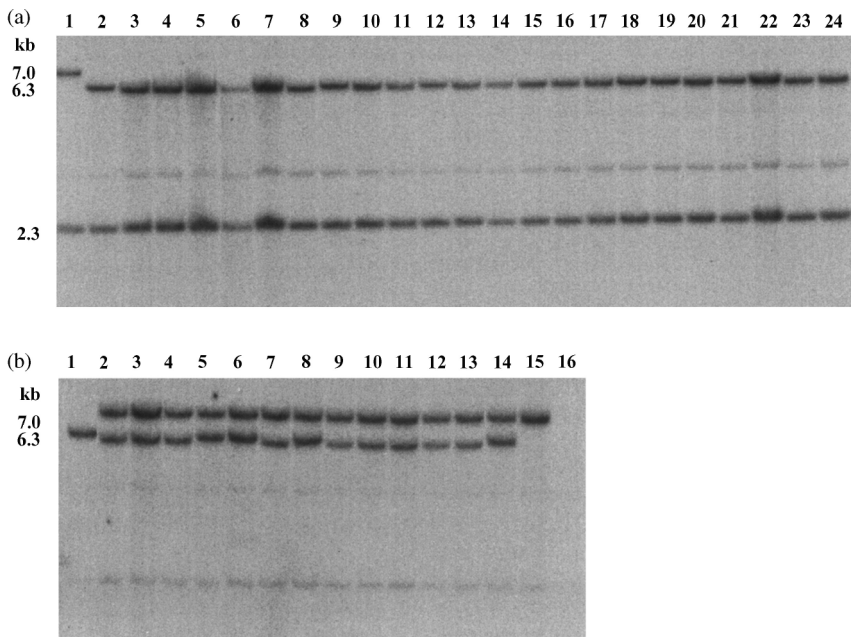
Figure 4. Southern blot of *Eco*RI-restricted DNA of progeny from the recombinant E42 (line CI).
(a) Analysis of seedlings obtained after selfing. The membrane was probed with a kanamycin-specific fragment. In lanes 1 and 2, DNA of the parental plants (B9, B9-300) was loaded. All 22 seedlings of plant E42 show a hybridization pattern identical to line B9-300 (lanes 3–24). The band of ≈2.5 kb originates from a second transgene fragment that does not contain *cod*A-specific sequences. The faint band of 3.5 kb is not transgene-specific as it is also present in wild-type SR1 tobacco (Figure 5). In total 32 seedlings were tested; all plants show the same hybridization pattern.
(b) Analysis of seedlings obtained after fertilization of demasculated flowers of the line B9 with pollen of E42. The membrane was probed with a *cod*A-specific fragment. Lane 1, line B9-300; lanes 2–14, seedlings of the cross B9 × E42; lane 15, line B9; lane 16, wild-type SR1 tobacco. The presence of a B9-300-specific allele indicates that all pollen grains of E42 harbour this allele. In all, 16 seedlings were tested with the same result. The blots demonstrate that plant E42 is homozygous for the B9-300 allele.

Molecular characterization of DSB repair events in line CII

To further address the question of how frequently allelic recombination occurs in comparison to other genomic changes, we performed experiments with line CII. In this line the allele B9-1 carries an insertion of 794 bp within the original transgene (Figure 1). As line B9-1 did not carry a functional I-*Sce*I ORF, in these experiments we had to inoculate the seedlings, obtained from the cross with B9, with *Agrobacterium* to induce the DSB as described before (Puchta, 1999b). In total, 395 seedlings were inoculated and 21 5-FC resistant calluses were isolated. In a control experiment, 1034 seedlings were incubated without inoculation on 5-FC medium. Whereas no calluses could be detected at a similar time point when calluses arose in the inoculated

seedlings, after prolonged incubation few small calluses could be detected. PCR analysis indicated the presence of both the B9 and B9-1 alleles, demonstrating that these calluses escaped the selection. Due to the different selection procedures (seeds in the case of line CI versus inoculated seedlings), the frequency of escapes was apparently lower in the experiments performed with line CII. The result of the control experiments clearly indicated that no detectable recombination events occurred within the transgene without DSB induction.

From the 21 calluses obtained after I-*Sce*I expression, one plant each was regenerated and DNA was prepared from leaf tissue. Southern analysis revealed in 18 cases a deletion within the *cod*A gene (Figure 5). In one case a longer insertion had occurred, and in the two remaining cases we
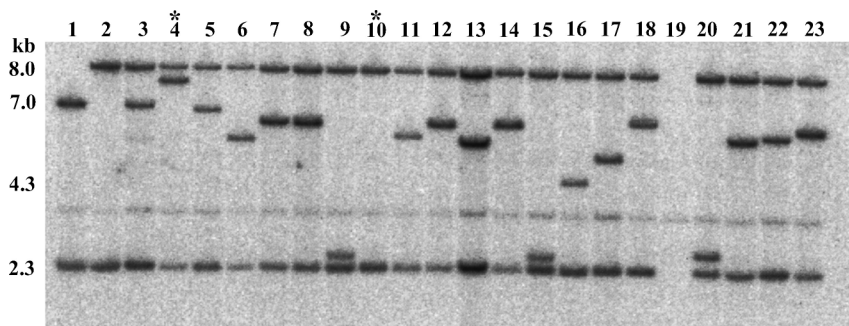
Figure 5. Southern blot of *Eco*RI-restricted DNA from 5-FC-resistant plants obtained after DSB induction from line CII.
The membrane was probed with a kanamycin-specific fragment. In the first two lanes, parent lines B9 (band of ≈7 kb) and B9-1 (band of ≈7.8 kb) are loaded; in the third lane, line CII obtained by crossing the two parental lines. These fragments represent the *cod*A-specific sequences in the transgene (Figure 3). In 18 out of 20 lines (lanes 5–9, 11–18 and 20–23) a deletion within the B9 *cod*A-specific fragment occurred. In one line there is a high probability that an insertion into the B9-specific transgene occurred (III/1, lane 4). Line III/10 has a the same pattern as line B9-1 (lane 10). The band of ≈2.5 kb originates from a second transgene fragment that does not contain *cod*A-specific sequences. The faint band of 3.5 kb is not transgene-specific as it is also present in wild-type SR1 tobacco (lane 19).

detected a loss of heterozygosity for the transgene locus (II4.1, data not shown; III10, Figure 5). A test of the progeny of lines II4.1 and III10 by Southern hybridization revealed that in about a quarter of seedlings of the selfed progeny, no B9-1 specific fragment was present, revealing the hemizygous state of the transgene in both recombinants. This indicates that, in these cases, the loss of heterozygosity for the transgene locus was not due to allelic recombination, but most probably was due to a larger deletion or the loss of the chromosome harbouring the transgene.

Discussion

Double-strand breaks have to be eliminated before genomes can be replicated. Therefore the repair of DSBs is critical for the survival of all organisms. Generally, DSBs can be repaired via two different pathways, either via homologous or via illegitimate recombination (also known as non-homologous end joining). In higher eukaryotes, illegitimate recombination appears to be the main mode of DSB repair (Sargent *et al.*, 1997). Using transposable elements or highly specific restriction endonucleases for induction of breaks at specific loci within eukaryotic genomes (for general reviews on endonuclease-induced repair, see Jasin, 2000; Paques and Haber, 1999) it has been possible to characterize DSB-induced homologous recombination in somatic plant cells (for review see Gorbunova and Levy, 1999). In previous studies, besides enhancing integration of T-DNAs at homologous loci (Puchta, 1998b; Puchta, 2002; Puchta *et al.*, 1996; Reiss *et al.*, 2000), repair of a DSB with homologous sequences close to the break (Athma and Peterson, 1991; Chiurazzi *et al.*, 1996; Siebert and Puchta, 2002; Xiao and Peterson, 2000) or at an ectopic position in the genome (Puchta, 1999a; Shalev and Levy, 1997) were analysed. However, the question of whether a DSB can also be repaired by homologous recombination using transgenic allelic sequences on the homologous chromosome has not been analysed to date. This is mainly because gene targeting is not well established in plants (for review see Hohn and Puchta, 1999; Mengiste and Paszkowski, 1999; Puchta, 1998a; Vergunst and Hooykaas, 1999) and therefore – in contrast to mammalian (Moynahan and Jasin, 1997) and yeast cells (Hiraoka *et al.*, 2000) – allelic positions in plant genomes cannot be manipulated directly. To overcome this limitation we have developed a strategy in which the original locus carrying the negative selectable marker *cod*A (Stougaard, 1993) was rendered non-functional by the induction of a DSB-repair reaction (Salomon and Puchta, 1998). Plants that were homozygous for the modified sequence were then crossed with plants homozygous for the original transgene. Thus we were able to obtain plant lines with two different alleles at the transgene locus on homologous chromosomes. One allele contained a I-*Sce*I site within the *cod*A gene, and the other either a

deletion or an insertion within the region of the *cod*A expression cassette that rendered both I-*Sce*I site and *cod*A gene non-functional. This approach is similar to that of Dooner and Martinez-Ferrez (1997), in which natural occurring alleles of the *bz* gene of maize were combined. One of the alleles carried an active transposable element. However, the germinal excision of the element did not stimulate meiotic recombination, a fact that might be due to the programmed induction of DSBs at a specific time point during meiosis. In our heteroallelic tobacco plants we could – after DSB induction by I-*Sce*I expression in somatic cells – select for cells resistant to 5-FC in which the *cod*A gene was destroyed. We were able to prove for somatic plant cells that a pathway exists by which DSBs are repaired by using the allelic sequences of the homologue. Due to the fact that the plant line B9-300 permanently expressed the functional restriction endonuclease I-*Sce*I, after simple crossing we could obtain an estimate of how frequently allelic recombination is used for DSB repair in somatic plant cells. As all 106 PCR fragments of the B9 size class (59 from 5-FC-resistant and 47 from randomly selected seedlings) contained sequence alteration within the I-*Sce*I site, we conclude that, in at least 95% of the plantlets, a DSB was induced after crossing. Thus DSB repair in line CI probably occurred directly in the zygote stage or shortly thereafter. Therefore each single plantlet should represent one DSB repair event. As we analysed 3×10^4 plantlets and obtained three allelic recombinants, we can estimate a frequency of allelic recombination of about 10^{-4} under our experimental conditions.

We obtained three recombinants, A4, E42 and D81, which had a uniform progeny with respect to the transgene locus as demonstrated by Southern blotting (Figure 4a). Furthermore, pollination of demasculated flowers of a control line (in our case B9) with pollen from the recombinants E42 and D81 further sustained the finding that the progeny was uniform. These results can most easily be explained by the presence of an identical B9-300 specific sequence on both alleles due to allelic DSB repair. However, formally we cannot exclude that the uniform progeny could be due to exclusion of germ cells harbouring a DSB-induced chromosome loss or a big deletion including the transgene. However, we have indirect evidence that the loss of the chromosome carrying the transgene B9 does not *per se* exclude transmission to the germ line. In a different set of experiments we induced DSBs in seedlings homozygous for the B9 transgene and, in addition to other recombinants, we were able to obtain a plant line whose progeny after selfing had no B9-1-specific signal in about a quarter of cases (B.G. and H.P., unpublished results). The loss of the transgene-specific fragment was correlated by flow cytometry with a reduction of genome size by about one chromosome (A. Meister, B.G. and H.P., unpublished result). We are convinced that, in this case, this is due to the loss of the

chromosome with the transgene, indicating the germinal transferability of such a mutation due to the amphitetraploid nature of the tobacco. Therefore in lines A4, E42 and D81 the loss of the B-9-specific allele is indeed due to allelic homologous recombination.

The frequency obtained for allelic recombination in this study is similar to that for ectopic recombination obtained after DSB repair in tobacco by two different studies (Puchta, 1999a; Shalev and Levy, 1997). Although both reports were based on different approaches to inducing the break – either transiently or by a stably transformed transposon – the frequency was estimated to be around 10^{-4}. Due to the small numbers involved, and the different constructs with different marker genes used in these and the current experiments, comparisons can only be crude estimates. However, the results presented in the current study clearly show that the frequency of allelic recombination is low in somatic plant cells, and that the pathway does not play a major role as in meiosis (Roeder, 1997). Other kinds of rearrangements resulting in a loss of function of the marker gene occur more frequently; for line CI we isolated 17 other loss-of-function events, and the 21 loss-of-function events of line CII were not due to allelic recombination.

In the current experiment, we were not able to estimate directly the frequency of spontaneous allelic recombination. In previous experiments using *Agrobacterium*-mediated inoculation of tobacco seedlings, we estimated that about 10 DSBs are induced per seedling – a DSB repair event takes place in no more than one per 1000 cells, if we assume that a seedling consists of at least 10 000 cells at this stage (Puchta, 1999a). Applying these data to the experiments performed with line CII, we can estimate that about 4000 DSBs were induced. As no allelic recombination event was detected, the frequency of DSB-induced allelic recombination should be lower than 2.5×10^{-4}, a figure in accordance with the 1×10^{-4} estimated for DSB-induced allelic recombination of line CI. The results obtained for lines CI and CII clearly show that other DSB repair events, leading to a loss of function of the marker gene, occur more frequently than allelic recombination. Our control experiment, with over 1000 uninoculated seedlings of the line CII, indicates that the frequency of spontaneous allelic recombination should be lower than the inverse of the number of cells involved in the experiment ($<10^{-7}$). We were not able to perform a similar control experiment with line CI, which – if performed as the original experiment – would have required 10 million seeds. However, we believe there is no reason to assume that CI behaves differently from CII, either in the control experiment or after I-*Sce*I expression. For ectopic recombination, different kinds of DSB induction (transient expression of I-*Sce*I versus stable integration of an active transposon within the marker construct) did not reveal major differences in the estimation of the respective frequencies in

tobacco seedlings (Puchta, 1999a; Shalev and Levy, 1997). Thus it seems that DSBs increase allelic recombination in plants by at least three orders of magnitude.

In somatic plant cells, DSB repair occurs mainly by illegitimate recombination. This process can lead to major genomic alterations such as deletions and insertions (Salomon and Puchta, 1998). These can be transferred to the germline, and thus contribute to the genetic variability of a plant population. Alternatively, a DSB could be repaired using homologous sequences in the genome as a donor. These sequences are provided either by the sister chromatids in G2 of the cell cycle (Johnson and Jasin, 2000); by the allelic sequences of homologous chromosomes; or by ectopic homologous regions. Although homologous recombination may result in new combinations of genetic information, the restriction to homologies is drastically limiting the spectrum of possible changes in comparison with illegitimate recombination. In plants, homologous recombination occurs frequently between allelic chromosomes during meiosis in a controlled way. In contrast, the absence of such an efficient pathway in somatic cells appears to be a prerequisite for the fixation of major changes in the genome of somatic cells. As somatic changes can be transferred to the germ line, this fact might, in the long run, be an important factor responsible for the enhanced plasticity postulated for plant genomes.

Experimental procedures

Segregation analysis and plant crosses

Transgenic *Nicotiana tabacum* L. cv. Petite Havana lines were used in the experiments. To perform segregation analysis, seeds of the transgenic lines B9, B9-300 and B9-1 were sown onto MS medium containing 300 mg l^{-1} kanamycin and 300 mg l^{-1} 5-fluorocytosine (5-FC), respectively. To evaluate the results of the segregation analysis, quality criteria of statistics were applied as described (Simpson *et al.*, 1960).

For crossing, anthers of closed green tobacco flowers were removed with tweezers. About 2 days later, at the beginning of rose colouring of these castrated flowers, paternal pollen was transferred onto the stigma. The pollinated flowers were packed into cellophane paper bags (Zellglas, Flachbeutel 325P, 60 × 300, Baumann Saatzuchtbetrieb, Waldenburg), and seeds were harvested after 6–8 weeks from mature seed capsules.

Plant transformation

Tobacco plantlets of line CII were inoculated with an *Agrobacterium* strain harbouring the binary vector pCI-*Sce*I. Vacuum infiltration of tobacco seedlings with *Agrobacterium* was performed as described (Puchta, 1999b). Inoculated as well as uninoculated seedlings were placed onto MS medium plates containing hormones (0.5 mg l^{-1} naphthalene acetic acid; 5 mg l^{-1} 6-benzyl amino purine) for 3 days, and afterwards onto MS medium plates containing hormones (0.5 mg l^{-1} naphthalene acetic acid; 5 mg l^{-1} 6-benzyl amino purine) as well as 300 mg l^{-1} Ticarcillin (Duchefa Haarlem, The Netherlands) and 300 mg l^{-1} 5-FC (during the first

11 days, 150 mg l⁻¹). For callus induction, the infiltrated seedlings were placed in a growth chamber (24°C, 16 h light per day). Every week the transformed plantlets were transferred onto fresh media. From the calluses, shoots arose which were planted into boxes (Magenta, Chicago, IL, USA) containing MS medium for root induction and plant regeneration.

DNA extraction

DNA extraction was carried out from leaf tissue of 2–3-week-old seedlings. The extraction was performed according to Fulton *et al.* (1995). Plant tissue (0.5–1 g) was frozen in liquid nitrogen, ground and transferred to a 3 ml micropreparation solution.

Southern analysis

Southern blotting was performed using the hybridization membrane Hybond-N+ (Amersham, Little Chalfont, UK). DNA fragments were labelled using a random priming labelling kit/Megaprime DNA labelling system RPN 1607, Amersham and [α-³²P]dCTP (Amersham). For identification of plants with different DSB repair events, either the *cod*A or the kanamycin-resistance ORFs were used as hybridization probe. The plant DNA was digested with *Eco*RI. Hybridization was performed at 65°C. After hybridization, the membrane was analysed using the Amersham Phosphor imaging system (Phosphor imager BAS 2000, Imaging Plates).

PCR and sequence analysis

Genomic DNA was analysed by PCR using the primers S10, S11 and A4 (Salomon and Puchta, 1998). To test the I-*Sce*I recognition site, the restriction endonuclease I-*Sce*I (Boehringer Mannheim, Tuzing, Germany) was used. The amplified PCR products were cloned into the pGEM-T Easy vector, System I (Promega, Madison, WI, USA) according to the manufacturer's instructions. Sequence analysis was performed with the automatic DNA-sequencer AFL-Express (Pharmacia, Uppsala, Sweden). Standard M13-20 Forward and M13 Reverse primers were used for the sequencing reaction. Using primers S10 and A4, sequence analysis was performed with the ABI PRISM DNA sequencer (Perkin Elmer, Wellesley, USA).

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