The Plant Journal (2003) **35**, 604–612 doi: 10.1046/j.1365-313X.2003.01832.x

Different pathways of homologous recombination are used for the repair of double-strand breaks within tandemly arranged sequences in the plant genome

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Summary

Different DNA repair pathways that use homologous sequences in close proximity to genomic double-strand breaks (DSBs) result in either an internal deletion or a gene conversion. We determined the efficiency of these pathways in somatic plant cells of transgenic *Arabidopsis* lines by monitoring the restoration of the β-glucuronidase (GUS) marker gene. The transgenes contain a recognition site for the restriction endonuclease I-*Sce*I either between direct GUS repeats to detect deletion formation (DGU.US), or within the GUS gene to detect gene conversion using a nearby donor sequence in direct or inverted orientation (DU.GUS and IU.GUS). Without expression of I-*Sce*I, the frequency of homologous recombination (HR) was low and similar for all three constructs. By crossing the different lines with an I-*Sce*I expressing line, DSB repair was induced, and resulted in one to two orders of magnitude higher recombination frequency. The frequencies obtained with the DGU.US construct were about five times higher than those obtained with DU.GUS and IU.GUS, irrespective of the orientation of the donor sequence. Our results indicate that recombination associated with deletions is the most efficient pathway of homologous DSB repair in plants. However, DSB-induced gene conversion seems to be frequent enough to play a significant role in the evolution of tandemly arranged gene families like resistance genes.

Keywords: recombination, gene conversion, single-strand annealing, synthesis-dependent strand-annealing, deletion.

Introduction

Efficient repair of double-strand breaks (DSBs) is important for the survival of all organisms. In principle, DSBs can be repaired via non-homologous end-joining (NHEJ) or via homologous recombination (HR). A major question of recent research was to elucidate under what condition which pathway is preferentially used (Paques and Haber, 1999). By expression of the rare cutting restriction endonuclease I-*Sce*I we are analysing since a row of years the repair of DSBs in plants that harbour as a transgene a marker. In general, NHEJ seems to be the main mode of DNA repair in somatic plant cells (Kirik *et al.*, 2000; Salomon and Puchta, 1998; for reviews see Gorbunova and Levy, 1999; Ray and Langer, 2002). However, homologous sequences can also be used for the repair of the break. We were able to show that information from allelic (Gisler *et al.*, 2002) as well as ectopic sites (Puchta, 1999; see also Shalev and Levy, 1997 for a transposon-based approach) could be used for DSB repair at low frequencies. The fraction of HR in relation to NHEJ events increases if homologous sequences close to the break are available (intrachromosomal recombination) (Siebert and Puchta, 2002; see also Xiao and Peterson, 2000 for a transposon-based approach). However, because of the configuration of the applied recombination substrate, we were only able to monitor deletions between direct repeats in our previous study (Siebert and Puchta, 2002). Depending on the structure of the respective chromosomal locus, at least two different kinds of pathways may be used to repair the break. Whereas one pathway results (as described above) in the formation of a deletion between the repeats, the product of the other pathway is a gene-conversion event (Fishman-Lobell *et al.*, 1992). The deletion pathway is explained best by a single-strand annealing (SSA) mechanism (Bilang *et al.*, 1992; De Groot *et al.*, 1992; Puchta and

Hohn, 1991), whereas gene conversion can be explained by a synthesis-dependent strand-annealing (SDSA)-like-mechanism (Puchta, 1998; Rubin and Levy, 1997). Although several independent studies on DSB-induced intrachromosomal HR have been performed (Athma and Peterson, 1991; Chiurazzi *et al.*, 1996; Siebert and Puchta, 2002; Xiao and Peterson, 2000; Xiao *et al.*, 2000) because of differences in marker configurations, DSB induction, plant species, and detection assays, it was not possible to compare the results directly and to draw conclusions as to the efficiencies of the different pathways. Our recent results indicated that deletion formation is involved in up to one third of all repair events of a DSB when homologous sequences are close (Siebert and Puchta, 2002). The deletion-associated HR pathway can be efficiently induced by the excision of a transposon (Xiao and Peterson, 2000; Xiao *et al.*, 2000). The occurrence of multiple single long terminal repeats (LTRs) of retroelements in cereal genomes (Vicient *et al.*, 1999) indicates that this pathway might play an important role in plant genome evolution. Therefore, it was important to find out under comparable conditions to which extent the other pathway, being associated with gene conversion, can be used for the repair of DSBs by HR in somatic cells. In the current study, we now addressed this question using different repair constructs as transgenes. Our direct comparison indicates that the deletion-associated pathway is about five times more frequent than the pathway resulting in gene conversion. Nevertheless, gene conversion events can be drastically increased by DSBs and therefore may also contribute particularly to the evolution of tandemly arranged gene families, such as disease resistance genes.

Results

Experimental setup

For the analysis of the efficiency of homologous DSB repair in the presence of nearby homologous sequences, the binary vectors pDGU.US, pDU.GUS, and pIU.GUS were constructed (for details, see section under Experimental procedures and Figure 1). In pDGU.US, the I-*Sce*I site is flanked by two halves of a β-glucuronidase (GUS) gene harboring an overlap of 557 bp (Tinland *et al.*, 1994). In pDU.GUS and pIU.GUS, a non-functional internal fragment of the GUS gene of 1087 nucleotides is arranged in direct or inverted orientation to a GUS gene into which, in about the middle of the gene, a 34-bp-long linker with I-*Sce*I and *Xho*I

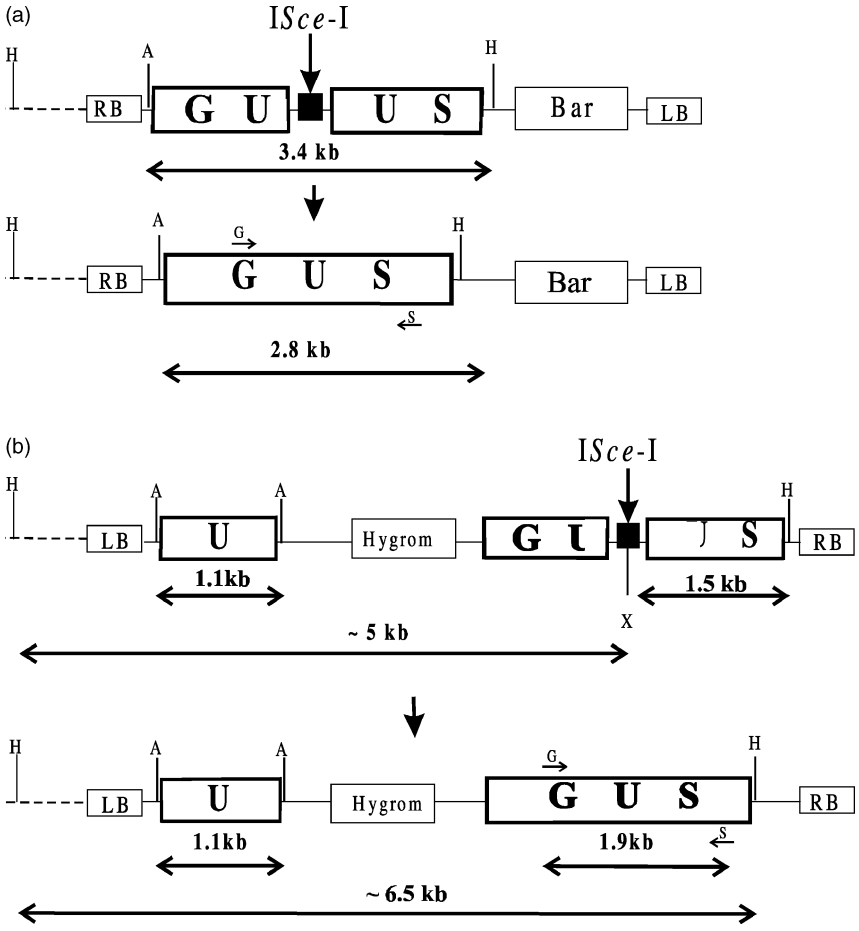

Figure 1. Schematic map of the recombination substrates used in this study.
(a) The recombination substrate DGU.US as present in the line DGU.US 1. An I-*Sce*I site is integrated between overlapping halves of a GUS gene. The T-DNA used for transformation also contains the phosphinotricin-resistance gene (Bar) as transformation marker. The predicted outcome of the HR reaction is depicted. The triangles represent the primers used for the PCR amplification of the recombined junctions. An *Acc65*I/*Hin*dIII fragment of 2.8 kb is indicative for HR. A: *Acc65*I and H: *Hin*dIII are restriction sites used for Southern blotting (see Figure 4). RB: right border; LB: left border; primers: G, S.
(b) The recombination substrate DU.GUS as present in the line DU.GUS 8. A 1.1-kb specific GUS fragment is inserted in direct (for IU.GUS in inverted) orientation to a GUS gene with a linker sequence harboring an I-*Sce*I. The T-DNA used for transformation also contains the hygromycin-resistance gene (Hygrom) as transformation marker. The predicted outcome of HR reaction is depicted. The arrows represent the primers used for the PCR amplification of the recombined junctions. A GUS-specific *Hin*dIII (H) fragment that cannot be restricted by *Xho*I (X) is indicative for HR reaction. RB: right border; LB: left border; primers: G, S.

Table 1 Determination of recombination frequencies in different transgenic lines with and without induction of DSBs

Line	No. of seedlings	Sectors	Sectors per seedling	Enhancement
IU.GUS 7	39	40	1.03	
IU.GUS 7 × DI-*Sce*I1	29	373	12.86	12.49
IU.GUS 8	32	60	1.88	
IU.GUS 8 × DI-*Sce*I1	21	711	33.86	18.01
DU.GUS 6	37	21	0.57	
DU.GUS 6 × DI-*Sce*I1	25	381	15.24	26.7
DU.GUS 8	32	39	1.22	
DU.GUS 8 × DI-*Sce*I1	28	400	14.28	11.7
DGU.US 1	31	47	1.5	
DGU.US 1 × DI-*Sce*I1	22	2380	108.2	72.13
DGU.US 2	17	12	0.7	
DGU.US 2 × DI-*Sce*I1	14	894	63.86	91.2

sites was incorporated, rendering it non-functional. Homologous overlaps of 537 and 550 bp, interrupted by the linker, are shared by both GUS sequences. Transgenic *Arabidopsis* plants were obtained via *Agrobacterium*-mediated transformation. The plants were propagated and the progeny was screened for lines with a 3 : 1 segregation, indicating a unique genomic locus. Using Southern blotting, single-copy lines were identified, and two lines of each construct (DGU.US 1, DGU.US 2, DU.GUS 6, DU.GUS 8, IU.GUS 7, and IU.GUS 8) were used for further analysis.

The artificial I-*Sce*I open-reading frame (ORF) optimized for plant expression (Puchta *et al.*, 1993) was fused to the DMC1 promoter of *Arabidopsis* (Klimyuk and Jones, 1997). The resulting binary plasmid pDISceI was used for *Agrobacterium*-mediated plant transformation, and the progeny of the obtained transgenic lines were screened for a 3 : 1 segregation. The line pDISceI1 was used for further experiments. Although it was reported that the DMC1 promoter is preferentially expressed during meiosis (Klimyuk and Jones, 1997), data obtained in our lab indicate that efficient expression could be achieved with this construct in embryonic tissue and somatic cells of young seedlings. Expression of DMC1 has been detected before in embryonic cell culture of *Arabidopsis* (Doutriaux *et al.*, 1998).

Determination of recombination frequencies with and without DSB induction

Homozygous plants from all six transgenic lines harboring the three different recombination substrates were crossed with homozygous plants of the line pDISceI1. The progeny were grown for 2 weeks under sterile conditions and then subjected to a histochemical staining using X-Gluc to quantify recombination events. To determine the level of DNA recombination without DSB induction, seedlings of transgenic plants homozygous for the recombination substrates were stained.

The results are shown in Table 1. Without DSB induction all the lines tested revealed a similar number of sectors ranging on average from 0.57 to 1.88 per seedling. Whereas in about half of the seedlings, no recombination event could be detected, in others, one to five blue sectors could be identified. No major differences were found for the three particular substrates. Different lines harboring the same construct did not differ by more than a factor of two, indicating no major influence of the genomic locus for the lines used in this study. The means of the two lines harboring the same kind of transgene construct differed by not more than a factor of two between the three constructs. Thus, without DSB induction no clear differences between the three recombination substrates could be detected.

After crossing with the line pDISceI1, the number of blue sectors increased drastically (Figure 2). The presence of the I-*Sce*I expression cassette resulted in about 10–30 times more sectors for DU.GUS and IU.GUS and up to almost 100 times more sectors for DGU.US. As in the seedlings from the crossings, the recombination substrates are present in a hemizygous state, the rate of induction might be underestimated by a factor of two. We were able to show before (Puchta *et al.*, 1995) that the frequency spontaneous intrachromosomal recombination is reduced to about half in hemizygous in comparison to that in homozygous plants, which have been used as a control in the current experiment. For the line DGU.US 1, between 55 and 210 blue sectors, and for the line DGU.US 2, between 34 and 84 blue sectors were detected per individual seedling. Similar to the experiment without DSB induction, no major difference could be found between the lines harboring the repeat in direct or inverted orientation. Also, the distribution of numbers of recombination events between the individuals was similar for these lines. Between 6 and 22 blue sectors were detected per plant for the lines DU.GUS 6, DU.GUS 8, and IU.GUS 7. Only in the case of the line IU.GUS 8, the values were somewhat higher (between 9 and 59 blue sectors). If we compare the distribution of non-induced

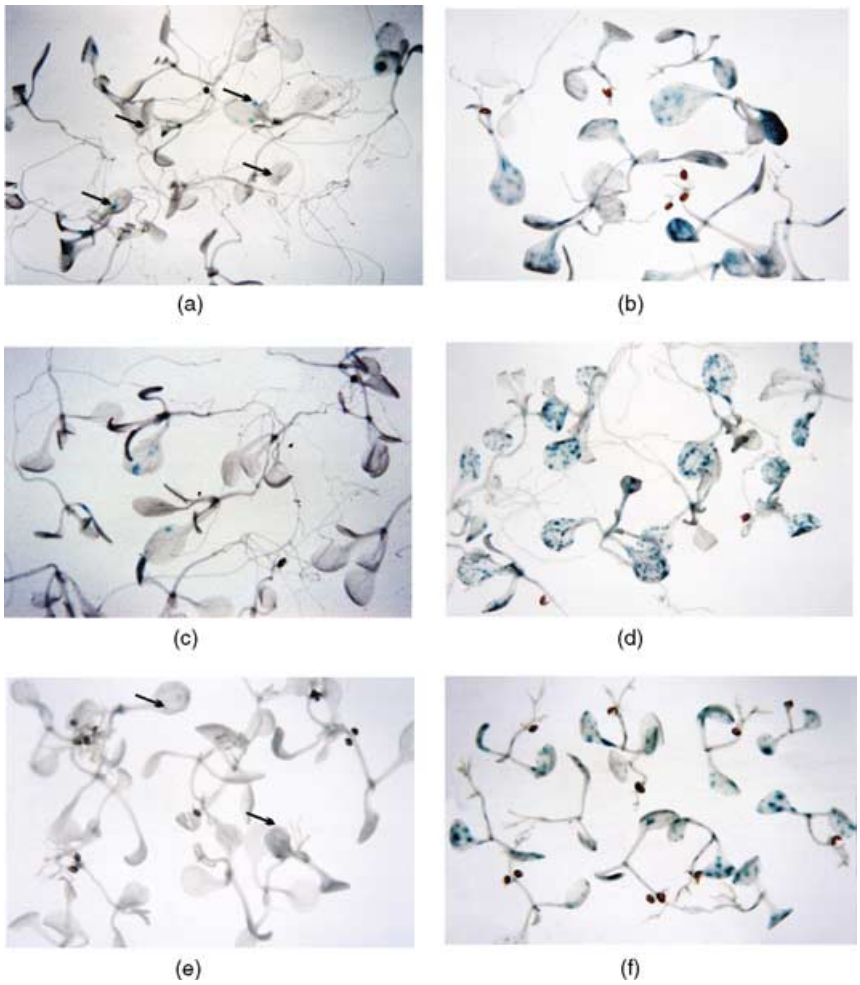

Figure 2. GUS assay with transgenic seedlings harboring a recombination substrate before and after crossing with the line expressing DI-*Sce*I1. Recombination events caused by HR that restore the marker gene, result in blue staining of the corresponding cell and its progeny. On the left – seedlings of the lines (a) IU.GUS 7, (c) DGU.US 1, and (e) DU.GUS 8, and on the right – seedlings of the same lines crossed with line DI-*Sce*I1, (b) IU.GUS 7, (d) DGU.US 1, and (f) DU.GUS 8. The strong enhancement in the number of blue sectors indicates that I-*Sce*I expression strongly induces HR.

to induced recombination events of the line IU.GUS 8 with that of DU.GUS 6, DU.GUS 8, and IU.GUS 7, the χ^2 values (1.607, 3.297, and 1.911) indicate that the differences are statistically not significant (rejection $P < 0.05$). This clearly demonstrates that the efficiency of gene conversion is not influenced by the orientation of the donor sequence in respect to the acceptor. In contrast, a comparison of IU.GUS 8 with the lines DGU.US 1 and DGU.US 2 reveals statistical significant differences ($P < 0.0005$; χ^2 values 37.777 and 23.897), whereas the two DGU.US lines do not differ significantly between themselves ($P > 0.6$, χ^2 value 0.245).

Molecular analysis of recombinants

To demonstrate that the restoration of the GUS activity is indeed caused by HR, several recombination events were molecularly characterized. A GUS assay under non-destructive conditions was performed. Tissue that was considered to descent from a recombination event because of light blue staining was cut out, transferred to growth medium containing plant hormones for callus induction, and propagated via tissue culture for further analysis. As the efficiency of regeneration with this procedure is quite low (less than 1% of the calli, Swoboda *et al.*, 1994), for this analysis, we also used progeny from seedlings of the third generation after crossing, which harbored both the recombination substrate and the I-*Sce*I expressing cassette in a homozygous state. These plants showed a similar enhancement of recombination as the parental lines. DNA was extracted from callus material. When staining indicated a recombination event early in development (bigger parts of the plants stained blue), these seedlings were transferred directly to soil. After propagation, part of the obtained plant material was stained to confirm expression of the recombined GUS gene, and from another part, DNA was extracted for Southern blot and PCR analysis. In this way, we were able to characterize recombination events from plants harboring each of the three recombination substrates. The analysis confirmed that the restoration of the marker was caused by the restoration of the GUS ORF in all cases. Southern blotting of DNA from the original line IU.GUS 8 and from the recombined line IU.GUS 8R1 (Figure 3) indicated that – as expected for a gene conversion event – no detectable changes in the size of the transgene occurred (lanes 1 and 2,

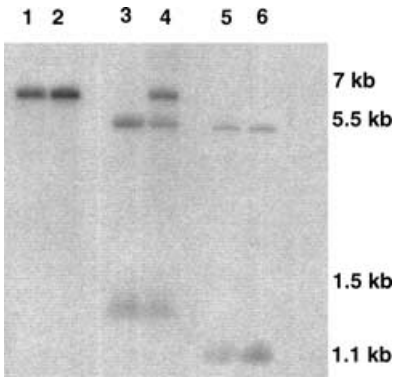

Figure 3. Southern blot with restriction-digested DNA of the plant line IU.GUS 8 and the recombinant line IU.GUS 8R1.
Lanes 1 and 2: *Hin*dIII-restricted DNA, lanes 3 and 4: *Hin*dIII- and *Xho*I-restricted DNA, and lanes 5 and 6: *Acc65*I restricted DNA. The DNA was hybridized with a GUS gene-specific probe. The 7-kb band in lane 4 indicates that the *Xho*I site was removed by gene conversion that did not change the donor sequence (compare lanes 3 and 4).

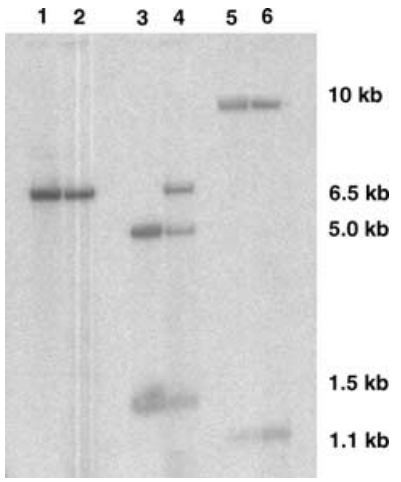

Figure 4. Southern blot with restriction-digested DNA of the plant line DU.GUS 8 and the recombinant line DU.GUS 8R1.
Lanes 1 and 2: *Hin*dIII-restricted DNA, lanes 3 and 4: *Hin*dIII- and *Xho*I-restricted DNAs, and lanes 5 and 6 *Acc65*I-restricted DNA. The blot was hybridized with a GUS gene-specific probe. The 6.5-kb band in lane 4 indicates that the *Xho*I site was removed by gene conversion that did not change the donor sequence (compare lanes 3 and 4 and see Figure 1b).

*Hin*dIII digested DNA). We also found no indication for any change within the donor sequences (lanes 5 and 6, the size of the small *Acc65*I fragment did not change between parental and recombined lines). However, the *Xho*I/*Hin*dIII digest indicated that the linker sequence was removed (lane 4), resulting in the restoration of the functional GUS gene as detected by histochemical staining. IGU.US 8R1 is homozygous for the recombination substrate, and therefore, besides the upper band in lane 4 that could not be restricted by *Xho*I, two smaller bands, corresponding to the bands of the same size in IGU.US 8, were also present. Thus, the second copy of the transgene did not undergo gene conversion. This was confirmed by PCR analysis. Using the primers G and S (the binding sites of both positioned outside of the *Acc65*I-flanked GUS donor sequence to avoid PCR artifacts; see Figure 1b), a 1.9-kb fragment was amplified. In contrast to the original plant line (IU.GUS 8), the amplified fragment could only partly be restricted by I-*Sce*I, indicating that in part of the PCR templates, the site had been removed. For further characterization, the amplified band was cloned. Restriction analysis indicated that plasmid clones containing inserts with and without I-*Sce*I site occurred with similar frequencies. Sequence analysis demonstrated that the removal of the I-*Sce*I was indeed correlated with the restoration of the functional GUS gene, whereas the original transgene sequence including the I-*Sce*I site was conserved in the other plasmids. A similar analysis was performed for a DSB-induced recombination event of the line IU.GUS 7 (IU.GUS 7R1) with an identical outcome (data not shown). We were able to regenerate plant material from three more recombination events of the line IU.GUS 8 × DI-*Sce*I1 (IU.GUS 8R2, 3 and 4), and one more of the line IU.GUS 7 × DI-*Sce*I1 (IU.GUS 7R2). In all cases, PCR followed by sequence analysis revealed that the marker gene was restored by a gene conversion event that removed the linker sequence within the GUS gene.

As the outcome of the recombination reaction with the substrate DU.GUS was expected to be identical to that of IU.GUS, corresponding analyses were performed for the line DU.GUS 8. As shown in Figure 4, Southern blotting revealed no major changes in the recombinant DU.GUS 8R1 in comparison to the parental line DU.GUS 8, as to the transgene construct beside the elimination of the inserted linker with the *Xho*I and I-*Sce*I sites. As in case of the recombinant IU.GUS 8R1, the analyzed line DU.GUS 8R1 was homozygous for the marker construct, but only one copy underwent gene conversion. This finding was further corroborated by PCR analysis as described above. Sequence analysis revealed an error-free restoration of the marker by gene conversion. We were able to regenerate two more recombination events (DU.GUS 8R2 and 3), and in all cases, restriction of the amplified PCR fragment with *Xho*I sustained the loss of the linker sequence within the GUS gene. The loss of the complete linker sequence could be confirmed by sequence analysis.

Formally, we cannot exclude that at least in some cases the restoration of the GUS activity in the constructs IU.GUS and DU.GUS could also have been caused by a trimming of the free DNA ends with consecutive re-ligation of the resected ends by NHEJ. Fortuitously, an intact ORF could thus be produced. However, one would expect that different kinds of deletions within the linker sequences that restore the phase of the ORFs would result in a functional GUS. As we were only able to detect the 'original' restored ORF without any linker sequences, we are convinced that all

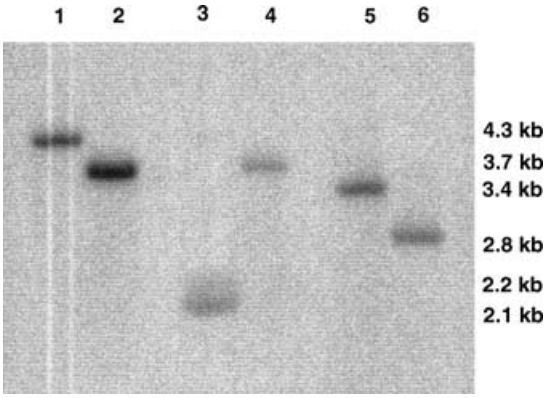

Figure 5. Southern blot with restriction digested DNA of the plant line DGU.US 1 and the recombinant line DGU.US 1R1.
Lanes 1 and 2: *Hin*dIII-restricted DNA, lanes 3 and 4: *Hin*dIII- and I-*Sce*I-restricted DNAs, and lanes 5 and 6 *Hin*dIII- and *Acc65*I-restricted DNAs. The blot was hybridized with a GUS-specific probe. The 2.8-kb band in lane 6 in comparison to the 3.4-kb band in lane 5 indicates that the GUS gene was restored by a deletion. This deletion was coupled with the elimination of the I-*Sce*I site (compare lane 3 with lane 4).

nine sequenced events, and therefore, also at least the vast majority of events detected in our study are indeed caused by gene conversion.

In case of the line DGU.US, restoration of the GUS gene was expected to result from a deletion of the interrupting sequence, including the I-*Sce*I site. Southern blotting of the recombinant line DGU.US 1R1 revealed that this was indeed the case. After the recombination event, the GUS-specific fragment (*Hin*dIII-*Acc65*I) was reduced in size from 3.4 to 2.8 kb (Figure 5, lanes 5 and 6). A similar size reduction is indicated by *Hin*dIII digest; a change of size of respective band from about 4.3 to 3.7 kb occurred (Figure 5, lanes 1 and 2). The removal of the linker is demonstrated by the fact that only the *Hin*dIII fragment of the original line, but not of the recombined line, can be restricted by I-*Sce*I (Figure 5, compare lane 3 with lane 4). PCR amplification and sequencing of products of the recombined fragment revealed that the GUS ORF was restored without any mutation by HR. In addition to the described case, six more recombination events (three from the lines DGU.US 1, DGU.US 1R 2 to 4 and three from the lines DGU.US 2, DGU.US 2R 1 to 3) could be isolated. PCR analysis of the respective lines revealed in all cases that the restoration of the marker gene was coupled with the elimination of I-*Sce*I restriction site.

Discussion

The role of HR in genomic DSB repair in plants

The main mode of repair of DSBs in plants, like in other higher eukaryotes, proceeds via NHEJ (Gorbunova and Levy, 1999; Kirik *et al.*, 2000; Salomon and Puchta, 1998). However, homologous sequences can potentially be used for repair. In several studies, we analyzed, using the rare cutting restriction endonuclease I-*Sce*I, to what extent the position of the homologous sequence in relation to the DSB determines the proportion of breaks repaired by HR. Only 1 out of about 10 000 breaks undergoes HR using homologous sequences in ectopic or allelic positions (Gisler *et al.*, 2002; Puchta, 1999; for results obtained by a transposon-based approach see Shalev and Levy, 1997). In contrast, breaks within a duplicated region can be repaired in up to one third of the cases by formation of a deletion involving homologous regions (Siebert and Puchta, 2002; for a transposon-based approach see also Xiao and Peterson, 2000; Xiao *et al.*, 2000). These differences might be as a result of the mechanisms of DSB repair acting under these experimental conditions. Whereas the SDSA model seems to be the most appropriate one to describe gene conversions between allelic or ectopic homologous sequences, the formation of a deletion caused by HR between homologous sequences in close proximity is best described by the SSA model. The SDSA model describes transfer of information from a homologous donor sequence to the break site (Gloor *et al.*, 1991) without a loss of sequences at the donor locus. The SSA model (Lin *et al.*, 1984, 1990) describes a non-conservative reaction resulting in a loss of information that is positioned between the annealing repeats. SSA-like models have also been postulated for explaining the loss of information during NHEJ (e.g. Nicolas *et al.*, 1995). Both models are depicted in Figure 6. It is tempting to speculate

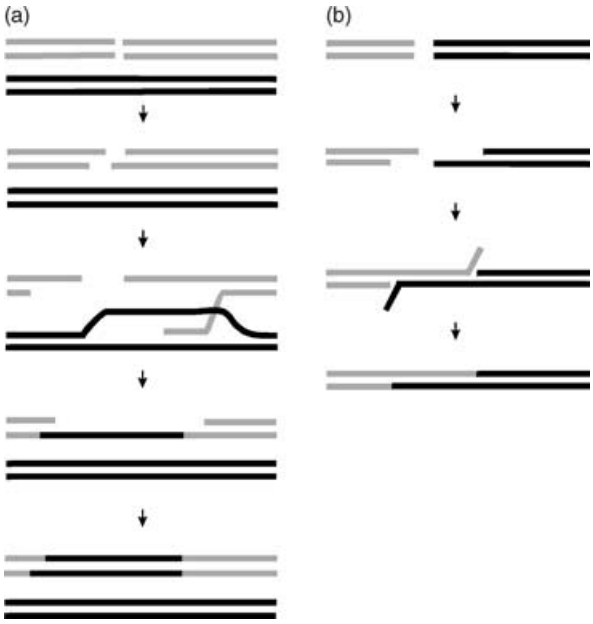

Figure 6. Models for the repair of DSBs by homologous sequences.
On the right, the SSA mechanism, and on the left, the SDSA mechanism are depicted.

that the enzyme machinery involved in both mechanisms differs in at least some of the factors. For the formation of a D-loop structure and the switch of the template during DNA synthesis, probably more factors are required than are required for a simple annealing reaction. According to the SDSA model, the orientation of the donor sequence to the break has no influence on gene conversion. As expected, both with and without DSB induction, no difference was found as to the frequency of GUS gene restoration for both orientations of the constructs detecting gene conversion events (DU.GUS; IU.GUS). Without DSB induction, the frequency of HR found with the DGU.US line was similar to that observed for DU.GUS and IU.GUS. The picture changes when we look at the frequencies of DSB-induced marker gene restoration. Here, the SSA pathway seems to be more efficient (approximately fivefold) than the gene conversion pathway. A possible explanation for this phenomenon would be that the kind of lesion leading to the spontaneous HR events might differ from the DSB induced by I-*Sce*I. One might speculate that spontaneous events are linked to replication. A prominent recombination reaction linked to replication is sister chromatid exchange (SCE). Indeed, besides the recombination mechanisms discussed above (SSA and SDSA), the GUS gene could be restored by crossing-over, using information from the sister chromatid. This applies for all three different recombination substrates used in this study. By crossing the transgenic lines produced in this study with insertion mutants of *Arabidopsis* deficient in expressing individual members of the repair and replication machinery (for a recent review on mutants see Hays, 2002), it might be possible to differentiate between these reactions by identifying the factors involved.

Although our results clearly show that classical GC events are less efficient than SSA events under similar conditions, one has to keep in mind that according to the SDSA pathway, besides classical conversion events, other products might arise too. Earlier work of our group demonstrated that DSBs can also be repaired by a combination of HR and NHEJ (Puchta, 1998, 1999; Puchta *et al.*, 1996). However, with the assay system applied in this study, such events cannot be detected as only repair of both ends of the break via HR results in a functional marker. Our previous results indicated that repair of one and that of both ends of a break by HR occur at about the same frequency (Puchta, 1998), so that the SDSA-like pathway of DSB repair seems to be in any case less efficient than the SSA-like pathway in plants.

If we take all our previous results into account and compare them with the results obtained in this study, we are able to obtain a detailed picture on how DSBs are repaired in somatic plant cells. The most efficient way to repair a break is by NHEJ. If homologous sequences are available close to the break, the repair can take place in up to a third of the cases by the SSA pathway (Siebert and Puchta, 2002). If gene conversion is five times less efficient than deletion formation, as it was demonstrated in this study, then one can expect that in about 1 out of 15 breaks, repair could also proceed via the SDSA pathway. This is two to three orders of magnitude more efficient than a gene conversion event that uses homology from an allelic (Gisler *et al.*, 2002) or ectopic site (Puchta, 1999). Thus, DSB-induced gene conversion between members of tandemly arranged gene families might well play an important role in plant genome evolution. Various resistance genes are organized in tandem arrays in plant genomes, and gene conversion events between the various members have been demonstrated by sequence analysis (e.g. Parniske and Jones, 1999; Parniske *et al.*, 1997). Our results indicate that such events might not necessarily be caused by meiotic recombination but may – at least partially – also occur during vegetative growth. It has been shown before that somatic changes in meristems can be transferred to the 'germ line' in plants (for discussion see Puchta and Hohn, 1996). Indeed, pathogen attack is inducing intrachromosomal recombination between tandemly repeated sequences (Lucht *et al.*, 2002). Moreover, certain sites in plant genomes might be less stable for repeated sequences than others. This is documented by finding that certain transgene sequences are deleted from the genome at high rates (Zubko *et al.*, 2000), a phenomenon that has been postulated to take place because of DSB induction (Puchta, 2000). It is tempting to speculate that the various gene conversion events, detected in tandemly arranged resistance gene clusters, might be correlated with higher rates of transient breaks in these regions of the plant genome (Ramakrishna *et al.*, 2002).

Experimental procedures

DNA constructs

For construction of the recombination substrate pDGU.US, the binary plasmid pGU.C.US.B (Siebert and Puchta, 2002) was used. The plasmid contains on its T-DNA two halves of the GUS gene with an overlap of 557 bp (Tinland *et al.*, 1994). Between the GUS sequences, a cytosine deaminase gene flanked by I-*Sce*I-sites is inserted. By an I-*Sce*I digest, the gene was eliminated from the vector, and after re-ligation, the plasmid pDGU.US was obtained by harboring a unique I-*Sce*I site between the GUS sequences (Figure 1a).

For construction of the recombination substrates pDU.GUS and pIU.GUS, a specific polylinker with the sequence 5′-CTCGAGATTACCCTGTTATCCCTAGTCGAC-3′ was cloned into *Msc*I-digested plasmid pGUS23 DNA (Puchta and Hohn, 1991). The resulting plasmid pGUS23I carried I-*Sce*I restriction site inside the nonfunctional GUS gene. The *Sal*I site within the linker was removed by cutting the plasmid and consecutive filling of the restriction site by Klenow enzyme and re-ligation. The obtained plasmid pGUS23I + was cut by *Acc65*I, treated with Klenow enzyme for filling in, and religated. Via PCR, using the oligonucleotides 5′-GCCGTCTAGAGGAGTCAAAGATTCAAATAGAGGACC-3′ and 5′-GCCGAAGCTTATTCCGATCTAGTAACATAGATAGCACC-3′ from the obtained plasmid pGUS23I + A, a fragment containing GUS

gene interrupted by I-*Sce*I and *Xho*I sites was isolated and cloned into *Xba*I and *Hin*dIII sites of plasmid pCH (Tinland *et al.*, 1994), resulting in the plasmid pCH23I+. Part of the GUS gene was amplified from pGUS23, using the oligonucleotides 5′-GCCGGGTACCGCGGCCGCCGCGAAAACTGTGGAATTGATCAGCG-3′ and 5′-GCCGGGTACCGCTCTTTAATCGCCTGTAAGTGCG-3′; the fragment was inserted into *Acc65*I site of pCH23I+. Clones with the GUS sequences in direct (pDU.GUS) or inverted orientation (pIU.GUS) were obtained (Figure 1b).

The I-*Sce*I expression vector pDISceI was constructed by cloning the promoter region of the AtDMC1 gene (Klimyuk and Jones, 1997), as *Hin*dIII fragment, into the binary pZP221 (Hajdukiewicz *et al.*, 1994). By digesting p35SISceI (Puchta *et al.*, 1993) with *Acc65*I, a fragment carrying the I-*Sce*I ORF and a terminator were obtained and recloned into the *Acc65*I site to obtain a functional expression cassette.

Production of transgenic plants

The binary plasmids were transferred into *Agrobacterium tumefaciens* via electroporation. The resulting strains were used to transform 5-week-old *Arabidopsis thaliana* plants of the accession Columbia-0 by vacuum infiltration.

Plants resistant to the selection applied (pDISceI: gentamycin, pDGU.US: phosphinotricin, pDU.GUS and pIU.GUS: hygromycin) were checked for a 3 : 1 segregation in the next generation to obtain lines in which the transgene was only inserted into a single locus. Southern blotting analysis revealed that the lines IU.GUS 7, IU.GUS 8, DU.GUS 6, DU.GUS 8, DGU.US 1, and DGU.US 2 all contain one copy of recombination substrate in their genomes, and the line pDISce1, which was used in this study for expression of I-*Sce*I, contained 2 copies of the T-DNA at the single locus.

Plant DNA extraction and Southern analysis

DNA extraction from leaf tissues and calli was performed, as described by Salomon and Puchta (1998). Southern blotting using the hybridization membrane 'Hybond N' (Amersham, Little Chalfont, UK) was performed, as described by Salomon and Puchta (1998). The DNA probes were labeled using a random priming labeling kit (Megaprime DNA labeling system RPN1607, Amersham, Little Chalfont, UK) and [α-^{32}P]dCTP (Amersham, Little Chalfont, UK).

PCR and sequence analysis

Genomic DNA was analyzed via PCR, using the primers **G** 5′-GCGTTAATTAATTCGAGCTCGGTAGCAATTTCGAGGC-3′ and **S** 5′-CGGAAGCTTCTCAGACTAAGCAGGTGACGAACG-3′. The PCR reactions and the direct sequencing of the amplification products were performed, as described by Hartung and Puchta (2000).

β-Glucuronidase assay

β-Glucuronidase assays were performed, as described by Swoboda *et al.* (1994). Statistical comparisons between lines on the effect of DSB induction on the number of recombination events were carried out by a χ^2 goodness-of-fit test, as described by Simpson *et al.* (1960). For all lines, the number of induced seedlings was taken as reference, and distribution of the controls were calculated accordingly.

Acknowledgements

We would like to thank Ingo Schubert for useful criticism on the manuscript and Christa Fricke for excellent technical help. The study was funded by the grant Pu137/3 of the Deutsche Forschungsgemeinschaft.

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