the plant journal

SEB Society for Experimental Biology

The Plant Journal (2012) **72**, 781–790 doi: 10.1111/j.1365-313X.2012.05119.x

The requirement for recombination factors differs considerably between different pathways of homologous double-strand break repair in somatic plant cells

Nadine Roth, Jacqueline Klimesch, Stefanie Dukowic-Schulze[†], Michael Pacher[‡], Anja Mannuss and Holger Puchta*
Botany II, Karlsruhe Institute of Technology, Kaiserstrasse 12, D-76131 Karlsruhe, Germany

Received 25 June 2012; revised 20 July 2012; accepted 3 August 2012; published online 1 October 2012.
*For correspondence (e-mail holger.puchta@kit.edu).
[†]Present address: Department of Horticultural Science, University of Minnesota, St Paul, MN 55108, USA.
[‡]Present address: SunGene GmbH, Correnssstrasse 3, D-06466 Gatersleben, Germany.

SUMMARY

In recent years, multiple factors involved in DNA double-strand break (DSB) repair have been characterised in *Arabidopsis thaliana*. Using homologous sequences in somatic cells, DSBs are mainly repaired by two different pathways: synthesis-dependent strand annealing (SDSA) and single-strand annealing (SSA). By applying recombination substrates in which recombination is initiated by the induction of a site-specific DSB by the homing endonuclease I-*Sce*I, we were able to characterise the involvement of different factors in both pathways. The nucleases MRE11 and COM1, both involved in DSB end processing, were not required for either SDSA or SSA in our assay system. Both SDSA and SSA were even more efficient without MRE11, in accordance with the fact that a loss of MRE11 might negatively affect the efficiency of non-homologous end joining. Loss of the classical recombinase RAD51 or its two paralogues RAD51C and XRCC3, as well as the SWI2/SNF2 remodelling factor RAD54, resulted in a drastic deficiency in SDSA but had hardly any influence on SSA, confirming that a strand exchange reaction is only required for SDSA. The helicase FANCM, which is postulated to be involved in the stabilisation of recombination intermediates, is surprisingly not only needed for SDSA but to a lesser extent also for SSA. Both SSA and SDSA were affected only weakly when the SMC6B protein, implicated in sister chromatid recombination, was absent, indicating that SSA and SDSA are in most cases intrachromatid recombination reactions.

Keywords: FANCM, MRE11, RAD51, RAD54, SMC6B, XRCC3.

INTRODUCTION

Genomic double-strand break (DSB) repair is essential for the survival of all organisms. In principle, DSBs can be repaired via two main pathways: homologous recombination (HR) and non-homologous end joining (NHEJ) (Paques and Haber, 1999). Whereas sequences for HR are linked via regions that are identical to one another, sequence information does not play a major role in the rejoining of the two DSB ends for NHEJ. The efficiency of HR and NHEJ varies between different organisms. In general, prokaryotes and yeast are more proficient for HR, whereas most higher eukaryotes use NHEJ very efficiently. Moreover, different types of mechanisms for HR as well as for NHEJ have been defined over the years. At least two different pathways of HR (Puchta, 1998; Siebert and Puchta, 2002) and, most likely, three different pathways of NHEJ exist in somatic plant cells (Charbonnel *et al.*, 2010, 2011).

Depending on genomic architecture, single-strand annealing (SSA) and synthesis-dependent strand annealing (SDSA) can be used to repair a DSB by homologous sequences (Puchta, 2005). In both pathways, single-stranded overhangs are produced via exonuclease-catalysed resection after induction of a DSB. In the case of SSA, overhangs on both ends of the break carry complementary sequences. Thus, these molecules can directly anneal to one another, and a chimeric DNA molecule is formed. If the chimeric molecule contains 3′ overhangs, the respective sequence parts will be trimmed; otherwise, single-stranded regions are filled in by DNA synthesis. Thus, all information between the formerly repeated sequences is lost (Figure 1). In the case of SDSA, one 3′ end invades a homologous double strand forming a D-loop. Repair synthesis is started using the newly paired strand as a template. After elongation, the

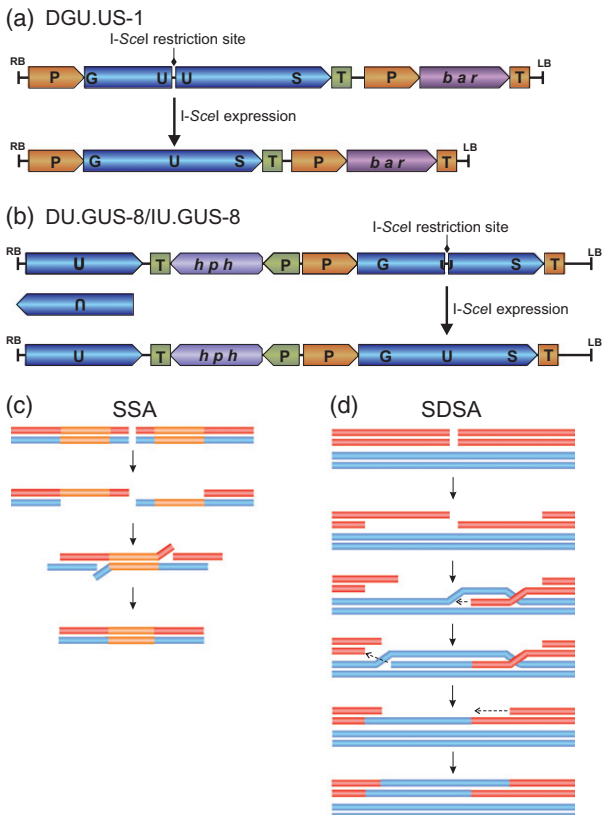

Figure 1. Models and substrates for single-strand annealing (SSA) and synthesis-dependent strand-annealing (SDSA).
Models of the SSA and the SDSA pathways of recombination are depicted in (c) (SSA) and (d) (SDSA). The homologous recombination (HR) events are determined by using the reporter lines DGU.US-1 (for SSA, a) and DU.GUS-8 and IU.GUS-8 (for SDSA, b). In DGU.US-1 the I-*Sce*I restriction site is flanked by two parts of a GUS gene harbouring an overlap of 557 bp. The 5′-GUS fragment was fused with the 35S promoter of the cauliflower mosaic virus (brown) and the 3′-GUS fragment to a nopaline synthase (NOS) terminator of *Agrobacterium tumefaciens* (green). Furthermore, the DGU.US-1 construct harbours a resistance gene for phosphinothricin resistance (*bar*) flanked by a 35S promoter and a 35S terminator (brown) (b). In the DU.GUS-8 and IU.GUS-8 line a 1087-bp GUS fragment is inserted in direct (DU.GUS) or in inverted (IU.GUS) orientation next to the right border. Next to the left border is a non-functional GUS gene with a linker sequence harbouring an I-*Sce*I recognition site. DU.GUS-8 and IU.GUS-8 also contain the hygromycin-resistance gene *hph* (hygromycin B phosphotransferase) fused with a NOS promoter (green) and a NOS terminator. The way in which the marker genes can be restored after I-*Sce*I induced HR by the respective pathway is depicted. P, promoter; T, terminator.

strand is displaced from the D-loop structure and anneals with a 3′ homologous strand that is available due to resection of the second end of the DSB. Thus, a gene conversion without loss of sequence information is the final result of the reaction (Figure 1).

The mechanisms of DSB repair are currently the focus of interest, as the development of zinc finger (Carroll, 2011) and TALE nucleases (Bogdanove and Voytas, 2011) enables the induction of DSBs almost anywhere in genomes. In plants, induction of DSBs can be used to mutate genes by NHEJ (Osakabe *et al.*, 2010; Puchta and Hohn, 2010; Zhang *et al.*, 2010) for gene targeting (Puchta *et al.*, 1996; Shukla *et al.*, 2009; Townsend *et al.*, 2009; Fauser *et al.*, 2012) or to remove unwanted sequences from the genome by NHEJ or SSA (Siebert and Puchta, 2002; Petolino *et al.*, 2010).

Whereas in yeast and animals, the roles of many factors involved in HR have been characterised in detail for different recombination reactions, such questions have been sparsely addressed in plants. Some time ago, we were able to establish reporter lines that enabled us to discriminate between the SSA and SDSA pathways (Figure 1) (Orel *et al.*, 2003). The assay system depends on the restoration of a functional β-glucuronidase gene after induction of DSBs by the rare cutting homing endonuclease I-*Sce*I. After propagation of recombined plant material we were able to demonstrate by Southern blots and PCR with subsequent sequence analysis that restoration of the marker in the different assay lines was indeed due to homologous recombination. (Orel *et al.*, 2003). Using the respective markers, the frequencies of both pathways could be compared, showing that the SSA pathway appears to be approximately five times more efficient than the gene conversion pathway. Recently, we applied these lines to elucidate the role of recombination intermediate processing factors RAD5A, MUS81 and RECQ4A in SSA and SDSA (Mannuss *et al.*, 2010). Interestingly, the assay system was also used during the discovery of the involvement of small RNAs in DSB repair and for the characterisation of factors involved in the respective small RNA processing pathway (Wei *et al.*, 2012).

Here, we apply the transgenic lines to define the role of a number of factors that are putatively involved in DNA processing in the SSA and SDSA mechanisms. We tested factors involved in both DSB end processing (COM1, MRE11) and in the process of strand exchange (RAD51, RAD51C, XRCC3 and RAD54). Moreover, we also tested two factors that, according to recent results, seem to be involved in the stabilisation of certain recombination intermediates (FANCM and SMC6B).

RESULTS

Setup of the assay system

The efficiency of somatic HR pathways can be addressed by using transgenic plants harbouring different configurations of a non-functional marker gene (in our case, β-glucuronidase) that can only be restored by the respective pathway (Figure 1). According to the model for the SDSA mechanism, the orientation of the donor sequence in relation to the break has no influence on gene conversion, and the break should lie within a homologous region that can be repaired by the use of a template that is homologous to both ends of the break. The two recombination reporter lines DU.GUS-8 and IU.GUS-8 differ only by the orientation of the part of the

β-glucuronidase marker that should be used as a template for repair after induction of a DSB at the I-*Sce*I site (Orel *et al.*, 2003). Therefore, we expected to find similar recombination efficiencies in the same genetic background with both lines. However, the transgene construct in DGU.US-1 is constructed in such a way that after break induction by I-*Sce*I, restoration of the marker is only possible by annealing the two direct repeats. The I-*Sce*I expression line used in this study contained an artificial I-*Sce*I open reading frame (ORF) optimised for plant expression fused to a double 35S promoter.

The Arabidopsis plants used for the SSA and SDSA recombination assays must be heterozygous for both the reporter construct and the I-*Sce*I expressing construct in a homozygous mutant or the respective wild-type background. Depending on whether the respective mutant is fertile or sterile, different approaches to achieve this status must be taken. In the case of fertile mutants (*rad54*, *smc6b* and *fancm*), the reporter lines and an I-*Sce*I expressing line are crossed with the respective mutant lines independently. In the F_2 generation, plants that are homozygous for the I-*Sce*I expression construct or the reporter construct in the respective mutant or the corresponding wild-type background are identified by PCR and propagated. As a final step, the reporter substrates are crossed with the I-*Sce*I expressing line, either in the mutant or the corresponding wild-type background. In the next generation, all seeds are heterozygous for both the I-*Sce*I expressing construct and the reporter system. These seeds are then sown out, and recombination frequencies are determined by counting blue sectors after histochemical staining (Figure 2). If the mutant is sterile (*com1*, *mre11*, *rad51*, *rad51c*, *xrcc3*), plants that are homozygous for the different transgenes but hemizygous for the mutated genes must be produced. After crossing the hemizygous mutants in the respective homozygous marker backgrounds, the required genotypes must be identified by PCR before the recombination frequencies are determined (Figure 2).

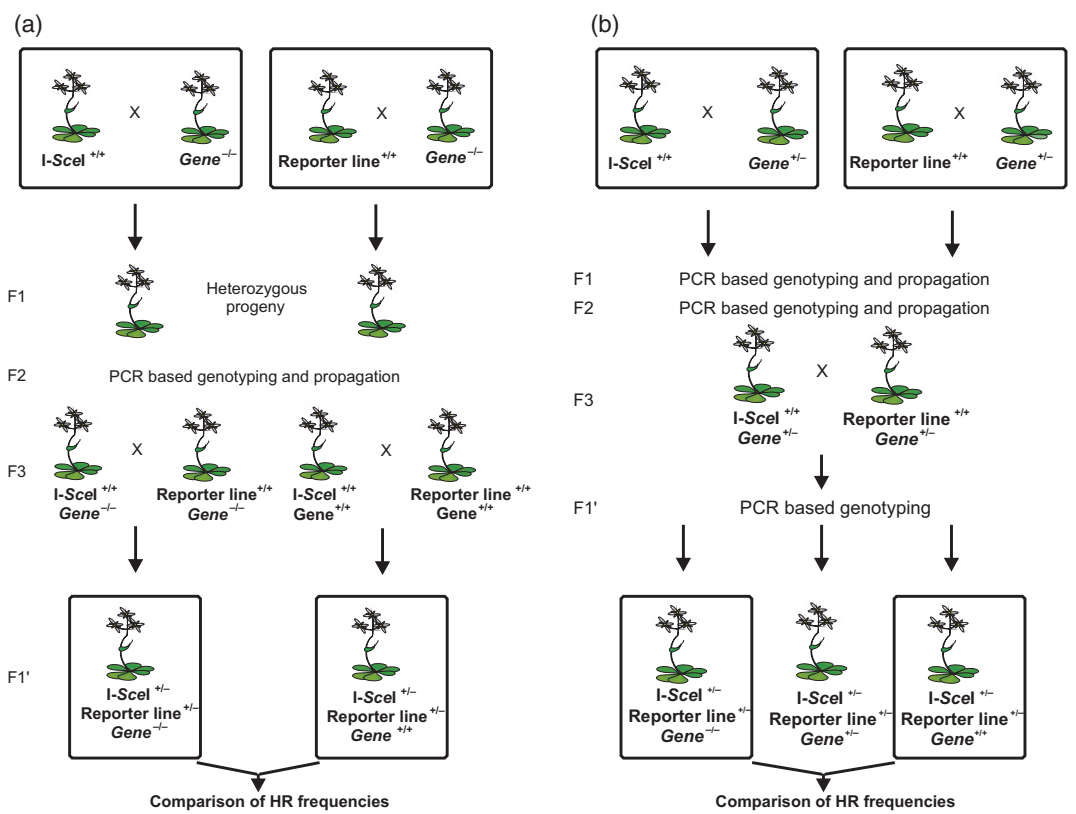

Figure 2. Crossing scheme for fertile mutant lines and sterile mutant lines.
To establish the reporter assays first the reporter lines (DGU.US, DU.GUS or IU.GUS) as well as an I-*Sce*I expression line are independently crossed with the respective homozygous mutant (a) or the segregating mutant line (in case of sterility of the mutant, b). In the second generation after crossing, all homozygous plants are identified by PCR-based genotyping and propagated. In a last step, the reporter substrate and the I-*Sce*I expressing construct are brought together by crossing the respective plants, either in the mutant or in the wild-type background. This methodology results in plants that are heterozygous for both the reporter and the I-*Sce*I expressing construct in a homozygous mutant or wild-type background (a). To assess how the loss of a gene of interest influences homologous recombination (HR), the number of repair events after the induction of a double-strand break are compared between the mutant and the corresponding wild-type plants. In case of sterility, mutant lines must be propagated in a heterozygous mutant background, and the resulting assay plants must be genotyped by PCR to identify homozygous siblings (b).

In all cases, mutant recombination frequencies were normalised to the respective wild types. In all cases, at least three independent recombination assays were analysed. In the following sections, the results of analyses of the different factors will be presented in an order that relates to their role in the consecutive steps of DSB repair.

COM1 and MRE11 are dispensable for efficient homologous recombination by the SDSA and the SSA pathway after site-specific DSB induction

In DSB processing in meiosis, MRE11 and COM1/SAE2 are essential to process an intermediate of the cleavage reaction consisting of SPO11 covalently linked to the 5′ termini of DNA. In Arabidopsis, the *com1* insertion mutant is sterile due to meiotic defects, indicating a conserved function. Moreover, COM1 is involved in crosslink repair in Arabidopsis (Uanschou *et al.*, 2007). Similarly, plants carrying a null allele of MRE11 are sterile (Puizina *et al.*, 2004) and sensitive to DNA-damaging agents (Bundock and Hooykaas, 2002).

As shown in Figure 3(a), in the case of COM1 we detected only a very minor reduction in the mean recombination frequency for all three different assay lines that were indeed not statistically significant. In the case of MRE11 (Figure 3b) the outcome was different, as the efficiency of recombination was enhanced in the SSA line by approximately half at a statistically significant level ($P = 0.036$). The mean was also approximately a third higher for both SDSA lines (although due to higher variation in the case of IU.GUS, the result was only statistically significant for DU.GUS). Thus, recombination apparently proceeds with higher efficiency in the *mre11* mutant than in the wild type, independent of the mechanism. These results are most likely due to the fact that MRE11 is also involved in a certain class of NHEJ events in Arabidopsis (Heacock *et al.*, 2004). As NHEJ is competing with the two HR pathways analysed in this study, in the *mre11* mutant more breaks are channelled into HR. Nevertheless, our results indicate that MRE11 plays no role in any of the two pathways tested. Thus, neither MRE11 nor COM1 are required for SSA and SDSA in somatic cells, at least if DSBs are induced by a sequence-specific endonuclease.

RAD51, RAD51C, XRCC3 and RAD54 are important for homologous recombination by the SDSA pathway but not for the SSA pathway

It has been demonstrated for yeast that proteins involved in strand exchange are required for gene conversion and crossovers. RAD51 filament stability and D-loop formation are controlled by the RAD51 paralogues (Liu *et al.*, 2011) and the SWI2/SNF2 ATPase RAD54 (Ceballos and Heyer, 2011). In our analysis, we concentrated our efforts on RAD54 RAD51 and its two paralogues XRCC3 and RAD51C. Current knowledge about the involvement of these factors in different recombination mechanisms in Arabidopsis is quite rudimentary. For RAD51 (Li *et al.*, 2004) and its paralogue XRCC3 (Bleuyard and White, 2004), it has been reported that

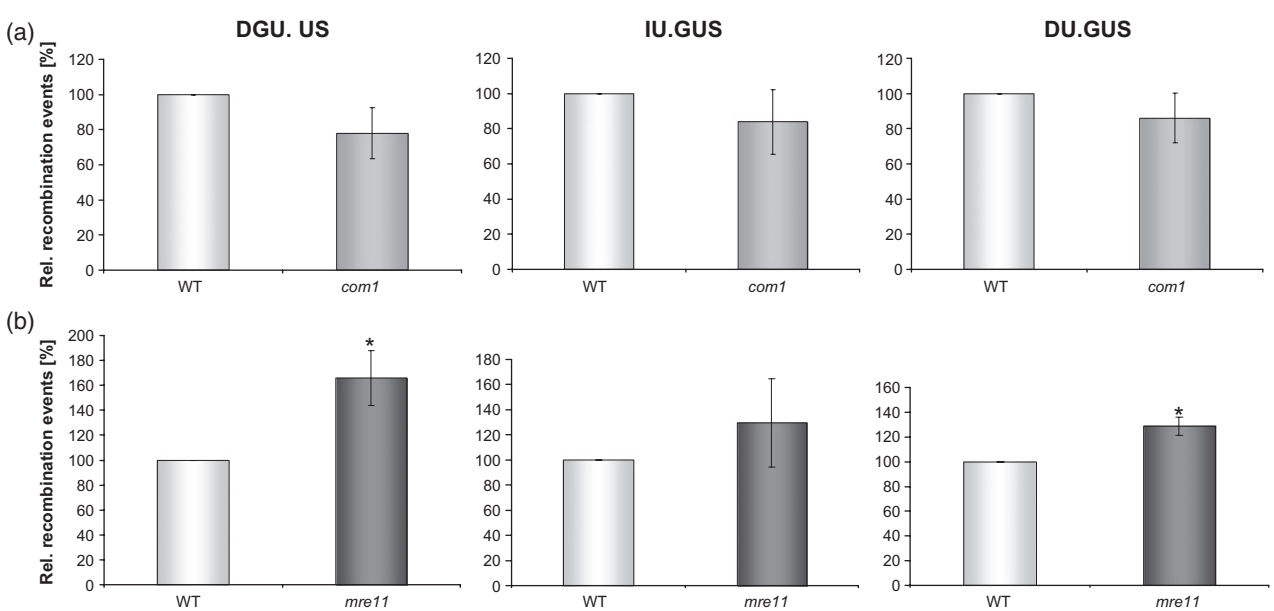

Figure 3. Recombination frequencies of *com1* and *mre11* mutant plants.
The relative recombination frequencies of the *com1-2* (a) and the *mre11-4* (b) mutant plants are presented in relation to the corresponding wild-type (WT) control plants (100%). The homologous recombination frequencies were determined using the reporter lines DGU.US-1 (SSA), IU.GUS-8 (SDSA) and DU.GUS-8 (SDSA) as depicted in Figure 1. The relative recombination is calculated as the mean value of three independent experiments. Error bars indicate the SD. Asterisks indicate *P*-values from two-tailed paired *t*-tests. (***$P < 0.001$; **$P = 0.001$–0.01; *$P = 0.01$–0.05; $P > 0.05$, no asterisk = not significant).

both mutants are sterile and sensitive to DNA-damaging agents, although no data about somatic HR have been published. Using recombination traps that could not discriminate between different recombination pathways, it has been reported previously that the loss of the paralogue RAD51C (Abe *et al.*, 2005; Bleuyard *et al.*, 2005) and the ATPase RAD54 (Osakabe *et al.*, 2006; Shaked *et al.*, 2006) results in a defect in HR and sensitivity against cross-linking agents.

To test the involvement of these factors in SSA and SDSA, we applied our assay system. In the case of RAD51, we were only able to combine the mutant background with the recombination assay lines DGU.US-1 and IU.GUS-8, but not DU.GUS-8, as the location of the DU.GUS-8 transgene and RAD51 are too close on chromosome V to combine both by crossing. Our results clearly demonstrate that loss of RAD51 has a strong influence on SDSA, resulting in a reduction of the wild-type recombination efficiency by almost 80%. In contrast, there is a minimal but statistically insignificant reduction in the case of SSA (Figure 4a). This finding demonstrates that the strand exchange protein is essential for the initiation of D-loops but not for a simple annealing reaction. Not surprisingly, almost the same result is observed for both paralogues tested, RAD51C and XRCC3. In the case of XRCC3, no decrease in efficiency can be detected in SSA. In the case of RAD51C, our experiments show a reduction of approximately a quarter, which is barely statistically significant ($P = 0.04$). However, in both mutant

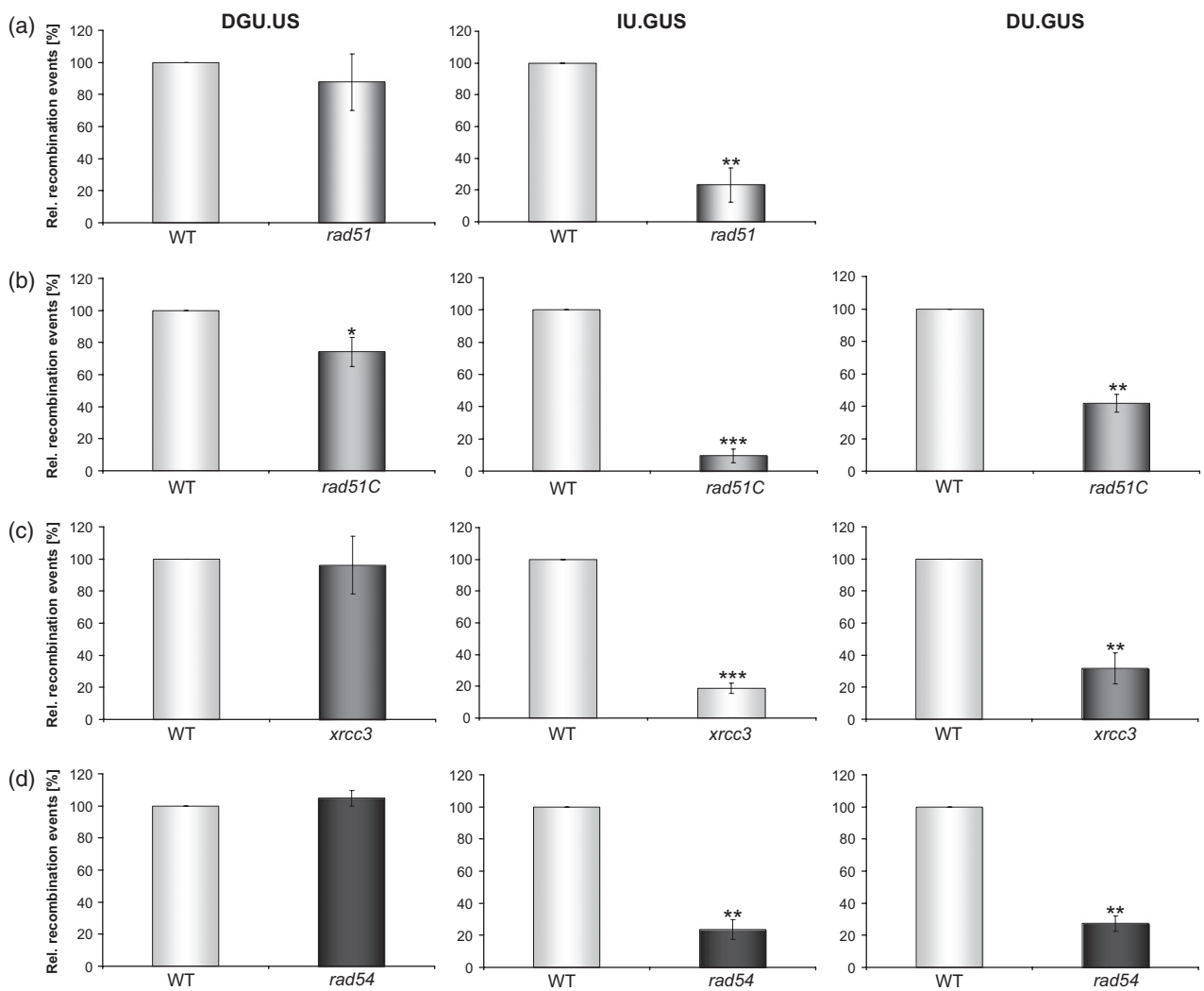

Figure 4. Recombination frequencies of *rad51*, *rad51C*, *xrcc3* and *rad54* mutant plants.
The representative diagrams show the relative recombination frequencies of the *rad51-1* (a), *rad51C-1* (b), *xrcc3* (c) and *rad54-1* (d) mutant plants presented in relation to the corresponding wild-type (WT) control plants (100%). The homologous recombination frequencies were determined using the reporter lines DGU.US-1 (SSA), IU.GUS-8 (SDSA) and DU.GUS-8 (SDSA), as depicted in Figure 1. The relative recombination is calculated as the mean value of three independent experiments. Error bars indicate the SD. Asterisks indicate *P*-values from two-tailed paired *t*-tests. (***$P < 0.001$; **$P = 0.001$–0.01; *$P = 0.01$–0.05; $P > 0.05$, no asterisk = not significant).

backgrounds, the efficiency of SDSA with both recombination traps is severely reduced (Figure 4b,c). Finally, the absence of the SWI2/SNF2 ATPase RAD54 has no influence on recombination frequency for the SSA pathway, but recombination is reduced to a quarter in comparison with the wild type in the IU.GUS-8 and in the DU.GUS-8 assay lines (Figure 4d). Thus, RAD54, as well as RAD51 and its paralogues, are extremely important for SDSA in Arabidopsis.

The role of SMC6B and FANCM in the SDSA and the SSA pathways

Furthermore, we were interested in elucidating the role of two factors that were only recently shown to be involved in HR in Arabidopsis: FANCM and SMC6B. The human hereditary disease Fanconi anaemia (FA) leads to severe symptoms, including developmental defects and breakdown of the haematopoietic system. FA is caused by single mutations in the *FANC* genes, one of which encodes for the DNA translocase FANCM (Knoll and Puchta, 2011). Recently, we were able to demonstrate that AtFANCM acts during meiosis as an anti-recombinase to suppress ectopic recombination-dependent chromosome interactions and is involved in the suppression of interference-insensitive crossovers (Knoll *et al.*, 2012). It has been postulated that yeast FANCM binds to recombination intermediates like D-loop structures and thus might control the efficiency of the reaction (Prakash *et al.*, 2009). Interestingly, AtFANCM suppresses spontaneous somatic HR via a RECQ helicase (AtRECQ4A)-independent pathway but on the other side is required for DSB-induced HR (Knoll *et al.*, 2012). It was therefore important to test how the absence of FANCM would influence SSA and SDSA. Our results (Figure 5a) indicate that gene conversion efficiency by SDSA is reduced for both recombination traps to less than half of the wild type. Surprisingly, SSA efficiency is reduced, but to a lesser extent, to approximately two-thirds of the wild type.

The SMC5/6 complex, together with cohesin, is involved in DSB repair by sister chromatid recombination during the S/G_2 phase in yeasts and mammals (De Piccoli *et al.*, 2009). Whereas in Arabidopsis the mutation of the single SMC5 homologue is non-viable, mutation of one or the other SMC6 homologue (SMC6A and SMC6B) results in fertile plants, although the double mutant is also non-viable. We were able to demonstrate that SMC6A and SMC6B are both required for efficient repair of DNA damage via HR in somatic cells (Watanabe *et al.*, 2009). However, using an assay that could not discriminate between different types of intra- and intermolecular recombination mechanisms, it was not possible to define the role of the SMC6 homologues in HR in greater detail. For the current study, we used the same allele of SMC6B that has been used previously (Watanabe *et al.*, 2009) to define the role of the protein in SSA and SDSA. Interestingly, in contrast to our previous results that showed a drastic decrease in HR with and without induction of random DSBs by bleomycin with the line DGU.US 1 (Watanabe *et al.*, 2009), our recent analysis with site-specific induction of the DSB between the overlaps showed a reduction of only approximately a fifth (Figure 5b). Both SDSA lines showed similar results, although the reduction

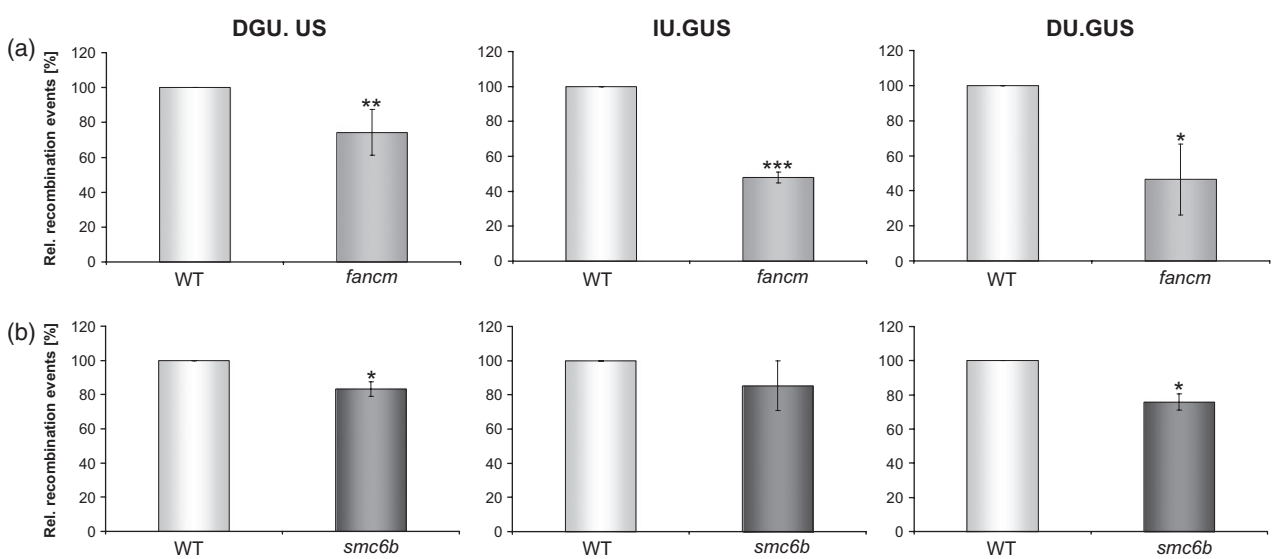

Figure 5. Recombination frequencies of *fancm* and *smc6b* mutant plants.
(a) and (b) depict the relative recombination frequencies of the *fancm-1* (a) and the *smc6B-1* (b) mutant plants, which are presented in relation to the corresponding wild-type (WT) control plants (100%). The homologous recombination frequencies were determined using the reporter lines DGU.US-1 (SSA), IU.GUS-8 (SDSA) and DU.GUS-8 (SDSA), as depicted in Figure 1. The relative recombination is calculated as the mean value of three independent experiments. Error bars indicate the SD. Asterisks indicate *P*-values from two-tailed paired *t*-tests. (***$P < 0.001$; **$P = 0.001$–0.01; *$P = 0.01$–0.05; $P > 0.05$, no asterisk = not significant).

was only statistically significant in the case of DU.GUS-8. This finding indicates that under these specific conditions, the majority of recombination reactions indeed occur intramolecularly and that sister chromatids are minimally involved.

DISCUSSION

The specificity of the assay systems for intrachromatid recombination

To detect specific recombination pathways, transgenic plant lines were used in which a reporter gene is restored after induction of a site-specific DSB with the meganuclease I-*Sce*I. One must state that both the setup of the transgene as well as the induction of a DSB at a unique position are prerequisites that the marker gene is restored – at least in the overwhelming majority of cases – by the respective mechanism, which is nicely exemplified by the DGU.US-1 line. Indeed, one can envisage different types of marker restoration mechanisms in cases where DSBs are induced by genotoxins at random positions within the transgenic marker sequence. In addition to SSA, break-induced replication (BIR) using the sister chromatid as a template or unequal sister chromatid exchange via SDSA can lead to the restoration of the marker (Watanabe *et al.*, 2009). Only the fact that the break is exclusively induced directly between the overlaps channels the reaction into the SSA pathway. This phenomenon is clearly demonstrated by the behaviour of the GU.US construct (DGU.US-1 line) in the absence of SMC6B. In cases in which DSBs are induced randomly by bleomycin, the recombination efficiency is drastically reduced by approximately half an order of magnitude (Watanabe *et al.*, 2009), indicating that at least in cells in the S or G_2 phase the sister chromatid plays a major role in HR. In contrast, only a mild defect was detected after I-*Sce*I-mediated DSB induction (a reduction of approximately a fifth), demonstrating that under these conditions intramolecular SSA, but not intermolecular sister chromatid recombination, preferentially occurs. In addition, the SDSA marker lines show only a mild reduction in HR efficiency. This finding also indicates that in this case homology close by on the same but not on the sister chromatid is the preferred matrix for repairing the DSB.

One has to keep in mind that beside the repair events that lead to the restoration of the marker most DSBs are still repaired by NHEJ under our experimental conditions. Although due to the experimental setup NHEJ repair should hardly result in a restoration of the marker gene we cannot exclude, that a small fraction of functional GUS ORFs are restored by imprecise NHEJ repair in our assays. In case of the DGU.US-1 line NHEJ would have to result in a deletion of exactly the duplicated region of 557 bp. In case of the DU/IU.GUS lines the 31-bp linker sequence harbouring the I-*Sce*I restriction site would either have to be eliminated completely or partially by imprecise NHEJ, resulting in a functional ORF of the marker.

The induction of DSBs most probably does not take place at all times and in all cells in our assay. Therefore, a minor fraction of the HR events that lead to the restoration of the marker might not be induced by an I-*Sce*I mediated DSB but by a naturally occurring DSB or by other kinds of DNA damage like stalled replication forks. As I-*Sce*I expression leads to the enhancement of recombination frequency by one to two orders of magnitude (Orel *et al.*, 2003) these events represent only a tiny fraction of all events and thus should not influence the outcome of our analysis.

The role of DSB end processing

Our findings that neither COM1 nor MRE11 are required for SSA or SDSA suggest that neither protein is needed after the production of processible DSB ends, which is the case if they are generated by homing nucleases. However, one has to keep in mind that most DSBs that arise naturally during the lifetime of an organism might have different properties. X-ray irradiation might result in breaks with complex ends that cannot be directly processed by polymerases or ligases. Moreover, in the case of covalent linkage of proteins to DNA, the DNA must be set free before it can be processed by the repair machinery. Interestingly, our results also indicate that, in the absence of MRE11, more DSBs are repaired by SSA as well as SDSA. We do not favour the hypothesis that MRE11 is a direct negative regulator of both pathways for the following reasons: as a complex, MRE11 and RAD50 form a functional nuclease (Daoudal-Cotterell *et al.*, 2002), and there are indications that the complex is also involved in NHEJ in Arabidopsis (Puizina *et al.*, 2004). Thus, a defect in NHEJ might be compensated by repairing more breaks by the use of homologous sequences. Indeed, using a conventional recombination trap that could not discriminate between pathways, it has been reported previously that HR is also enhanced in the absence of RAD50 (Gherbi *et al.*, 2001).

The importance of multiple factors for processing recombination intermediates in SDSA

We showed that the recombinase RAD51 and its paralogues RAD51C and XRCC3 are of great importance for SDSA but not for SSA, which can be easily explained, as RAD51 filament formation is required for invasion of a single strand into a double-stranded region. This phenomenon supports our finding that the Arabidopsis *BRCA2* double mutant, which is deficient in RAD51 filament formation, also has a tremendous defect in HR (Seeliger *et al.*, 2012). We propose that, similar to yeast, the paralogues are needed for the stabilisation of recombination intermediates associated with the RAD51 filament (Liu *et al.*, 2011). As in mammals, three more paralogues exist in addition to RAD51C and XRCC3, namely RAD51B, RAD51D and XRCC2 (Bleuyard *et al.*, 2005; Osakabe *et al.*, 2005). The proteins are found in at least two

distinct complexes in human cells. One complex contains RAD51B, RAD51C, RAD51D and XRCC2, whereas the other complex consists of RAD51C with XRCC3 (Masson *et al.*, 2001). As we detected a mild but significant reduction in the SSA efficiency in the case of RAD51C deficiency, it will be interesting to test whether the loss of any of the other three paralogues also has some minor influence on SSA in addition to a strong defect in SDSA.

It is interesting to compare the current results with data we obtained using the same assay system to define the role of factors involved in the processing of DNA recombination intermediates (Mannuss *et al.*, 2010), namely the SWI2/SNF2 ATPase RAD5A (Chen *et al.*, 2008), the endonuclease MUS81 (Hartung *et al.*, 2006) and the helicase RECQ4A (Hartung *et al.*, 2007). For IU.GUS, the recombination frequencies were reduced by half in the *rad5A* mutant background compared with the wild-type control. In contrast, no significant difference was observed between the mutants and the wild-type controls with the DGU.US reporter system. Thus, both SWI2/SNF2 ATPases RAD5A and RAD54 are involved in gene conversion, but not in the SSA pathway of HR in somatic plant cells. Because loss of RAD54 reduced SDSA more dramatically, its role seems to be more prominent.

In contrast, a slight reduction of the HR efficiency in the SSA pathway was observed in both the *recq4A* and the *mus81* mutants (Mannuss *et al.*, 2010). The reduction was less than a third, arguing that other factors might have played more prominent roles or that they were able to substitute for the nuclease or for the helicase. Interestingly, a much stronger effect was observed with the SDSA substrate, in which the HR efficiency was reduced to less than half for both single mutants. This observation is reminiscent of FANCM in this study. Indeed, FANCM and RECQ4A - a member of the RTR complex (Hartung *et al.*, 2008) - both are involved in suppressing replication-associated HR in two independent pathways in Arabidopsis (Knoll *et al.*, 2012). Therefore, one is tempted to speculate that both helicases might be involved in the processing of a subset of slightly different recombination intermediates that might arise within the SDSA pathway. Additionally, RECQ4A and MUS81 might be involved in the processing of different classes of recombination intermediates, as the double mutant is non-viable but can be rescued by knocking out RAD51C, which, as demonstrated in this study, is involved in SDSA (Hartung *et al.*, 2006; Mannuss *et al.*, 2010). Thus, FANCM, RECQ4A and MUS81 could all be involved in alternative means of processing SDSA-dependent recombination intermediates that arise after strand exchange.

With the current work, we were able to identify a group of factors that play a major role in SDSA. These findings are a clear indication that the pathway is quite complex and requires a larger number of proteins that are involved in the processing of different types of intermediates during different steps of the reaction. Formally, we cannot exclude the fact that the β-glucuronidase gene in IU.GUS-8 and DU.GUS-8 can also be restored by the classical double strand break repair (DSBR) mechanism (Szostak *et al.*, 1983). However, this mechanism plays no significant role in DSB repair in somatic plant cells as the initiation of recombination homology to one end of the break is sufficient (Puchta, 1998). The DSBR model postulates that homology on both ends of the break is required. Recent findings in yeast also indicate that the repair by the DSBR mechanism is only a minor pathway of homologous DSB repair in mitotic cells (Bzymek *et al.*, 2010). Single-strand annealing seems to be much simpler, and the required functions might be present redundantly in plant cells. This might also be the reason why we could not until now identify a factor whose loss would severely influence SSA.

EXPERIMENTAL PROCEDURES

Strains

The T-DNA insertion lines of *COM1* (At3g52115), *MRE11* (At5g54260), *XRCC3* (At5g57450), *RAD54* (At3g19210), *RAD51C* (At2g45280), *FANCM* (At1g35530) and *SMC6B* (At5g61460) were all obtained from the SALK collection (Alonso *et al.*, 2003). The mutant alleles *com1-2* (Uanschou *et al.*, 2007), *xrcc3* (Bleuyard and White, 2004), *rad51C-1* (Bleuyard *et al.*, 2005), *rad54-1* (Osakabe *et al.*, 2006), *fancm-1* (Knoll *et al.*, 2012) and *smc6B-1* (Watanabe *et al.*, 2009) were previously described. The GABI T-DNA insertion line GABI_134A01 (*rad51-1*) of *RAD51* (At5g20850) was described before (Li *et al.*, 2004) and provided by Bernd Reis. The *mre11-4* allele has its T-DNA insertion in exon 17 and is sterile, a strong indication of a loss-of-function allele.

The I-*Sce*I expression line was produced by cloning the artificial I-*Sce*I ORF optimised for plant expression (Puchta *et al.*, 1993) fused to a double 35S promoter and an octopine terminator in the plasmid PZP221 (Hajdukiewicz *et al.*, 1994). The resulting vector was transferred into *Agrobacterium tumefaciens*, and the resulting strains were used to transform Arabidopsis utilising the floral dip method (Clough and Bent, 1998). By segregation analysis, we could define different I-*Sce*I expressing lines that harbour the I-*Sce*I expression cassette at each single genomic locus. In the following Southern blot analysis, we selected for lines that carry only one copy of the I-*Sce*I construct at a defined locus. For the recombination assays in this study, we used the I-*Sce*I expressing line I-*Sce*I-8. The I-*Sce*I-8 line contains the I-*Sce*I cassette on chromosome I at the position 4125836 with a deletion of 1182 bp and an insertion of 47 bp at the left border of the T-DNA and 152 bp at the right border. The reporter lines were previously described (Orel *et al.*, 2003). Additionally, we characterised the IU.GUS-8, DU.GUS-8 and DGU.US-1 lines by Southern blot analysis and gained evidence that these each carry a single copy of the recombination trap at one locus. Furthermore, we determined the integration site of the reporter cassettes via site finder PCR (Tan *et al.*, 2005). In the DGU.US-1 line, the reporter cassette is inserted on chromosome III at the position 7386798 with a deletion of 10 bp and an insertion of 9 bp at the right border of the T-DNA and 4 bp at the left border of the T-DNA. The DU.GUS-8 line harbours the reporter cassette on chromosome V at the position 7081288 with a duplication of 6 bp at the 5′ and 3′ side of the T-DNA and an insertion of 25 bp at the right border of the T-DNA and 25 bp at the left border of the T-DNA. The reporter cassette in the IU.GUS line is inserted on chromosome I at the position 27372237 with a deletion of 40 bp and an insertion of 6 bp at the right side of the

T-DNA border and an insertion of 10 bp at the left border. Furthermore, for the recombination assays, the respective wild-type plants were always produced simultaneously and taken from the same crossing and segregation as the mutant of interest with the same marker line backgrounds. All T-DNA mutant lines, the I-*Sce*I expression line and the GUS reporter lines are in the Columbia-0 background.

Plant handling and growth conditions

Arabidopsis seeds were sterilised in 6% sodium hypochlorite for 5 min and rinsed several times with sterile water. Plants were grown in chambers at 22°C (CU-36L4, Percival Scientific, http://www.percival-scientific.com/) under white light (16-h light/8-h dark) and a dark phase at 20°C. For assays, sterilised seeds were spread onto solid germination media (GM)-agar [4.9 g L^{-1} Murashige and Skoog micro- and macro-elements, including vitamins and MES buffer (Duchefa, http://www.duchefa.com/), 10 g L^{-1} sucrose, pH 5.7, and 8 g L^{-1} plant-agar (Duchefa)].

For propagation and crossings, plants were grown in a greenhouse under constant 22°C with a light phase of 16 h and a dark phase of 8 h.

Analysis of recombination

Generally, recombination frequencies were measured in the F_1 generation after crossing plants harbouring the respective reporter construct and the I-*Sce*I expressing construct in a homozygous mutant or the segregated wild-type background.

We first crossed both reporter lines and an I-*Sce*I expression line independently from one another with the respective mutant lines. For crossing, we removed sepals, petals and stamens to access the gynoecium. Pollination was performed by tapping mature stamens from the father plant to the stigma. Seeds from crosses were propagated through the F_1 and F_2 generations. In the F_2 generation, plants homozygous for the transgenes and the respective mutant or the corresponding wild-type background were identified by PCR with primers for the respective loci. As a final step, the reporter substrates were crossed with the I-*Sce*I expressing line either in the mutant or the corresponding wild-type background. The seeds of these crossings were then sown out on Petri dishes containing solid GM medium supplemented with the antibiotics phosphinotricin (PPT) and hygromycin for the different reporter lines to exclude plants that are self-fertilised from the mother line (which was always the I-*Sce*I expressing line). The DGU.US-1 line carries a PPT resistance marker, and the IU.GUS-8 and DU.GUS-8 lines carry a hygromycin resistance marker. After 2 weeks, plantlets were histochemically stained in an X-Gluc staining solution as described (Swoboda *et al.*, 1994) for 2 days at 37°C. Destaining of leaf pigments with 70% ethanol overnight at 60°C facilitated the following analysis of recombination events by counting blue sectors under a binocular.

For sterile mutants, we first crossed heterozygous mutants into the reporter and I-*Sce*I expressing lines. Then, genotyped F_2 progeny, heterozygous for the mutation and homozygous for either the reporter or the I-*Sce*I transgene, were crossed to obtain a segregating population with the desired genotype in the next generation. For analysis of the recombination efficiency, 130 seeds per line were spread onto GM media supplemented with the antibiotics as mentioned above. After 2 weeks of growth, the roots of the plantlets were removed and used for DNA isolation and subsequent PCR analysis. Each single seedling was placed into a single well of a 24-well plate and marked individually. Staining was performed by filling each well with 1.5 ml of staining solution. After 2 days, the plantlets were destained with 70% ethanol. Correlation of the PCR results obtained with the roots removed before staining enabled us to identify plants homozygous for the respective mutant or wild-type background. Plantlets with identical genetic backgrounds were then pooled, and the number of recombination events was determined per seedling using a binocular.

For every line, between 35 and 45 plants per assay were analysed. The results were obtained from at least three independent experiments. Error bars indicate the standard deviation between these three experiments. The values of the mutant lines are presented in relation to the frequency of the corresponding wild-type control plants (100%). *P*-values were calculated from two-tailed, paired *t*-tests between the wild type and the corresponding mutant line. Significant differences, defined as having $P < 0.001$ (***), $P = 0.001$–0.01 (**), $P = 0.01$–0.05 (*) and $P > 0.05$ (no asterisk = not significant) are indicated by asterisks above the corresponding bars.

ACKNOWLEDGEMENTS

The authors wish to thank Maren Scheidle and Mandy Meier for technical assistance and Manfred Focke for reading the manuscript. The work was funded by the ERC Advanced Grant 'COMREC' and the DFG grant Pu 137-8.

REFERENCES

Abe, K., Osakabe, K., Nakayama, S., Endo, M., Tagiri, A., Todoriki, S., Ichikawa, H. and Toki, S. (2005) Arabidopsis RAD51C gene is important for homologous recombination in meiosis and mitosis. *Plant Physiol.* **139**, 896–908.

Alonso, J.M., Stepanova, A.N., Leisse, T.J. *et al.* (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. *Science*, **301**, 653–657.

Bleuyard, J.Y. and White, C.I. (2004) The Arabidopsis homologue of Xrcc3 plays an essential role in meiosis. *EMBO J.* **23**, 439–449.

Bleuyard, J.Y., Gallego, M.E., Savigny, F. and White, C.I. (2005) Differing requirements for the Arabidopsis Rad51 paralogs in meiosis and DNA repair. *Plant J.* **41**, 533–545.

Bogdanove, A.J. and Voytas, D.F. (2011) TAL effectors: customizable proteins for DNA targeting. *Science*, **333**, 1843–1846.

Bundock, P. and Hooykaas, P. (2002) Severe developmental defects, hypersensitivity to DNA-damaging agents, and lengthened telomeres in Arabidopsis MRE11 mutants. *Plant Cell*, **14**, 2451–2462.

Bzymek, M., Thayer, N.H., Oh, S.D., Kleckner, N. and Hunter, N. (2010) Double Holliday junctions are intermediates of DNA break repair. *Nature*, **464**, 937–941.

Carroll, D. (2011) Genome engineering with zinc-finger nucleases. *Genetics*, **188**, 773–782.

Ceballos, S.J. and Heyer, W.D. (2011) Functions of the Snf2/Swi2 family Rad54 motor protein in homologous recombination. *Biochim. Biophys. Acta*, **1809**, 509–523.

Charbonnel, C., Gallego, M.E. and White, C.I. (2010) Xrcc1-dependent and Ku-dependent DNA double-strand break repair kinetics in Arabidopsis plants. *Plant J.* **64**, 280–290.

Charbonnel, C., Allain, E., Gallego, M.E. and White, C.I. (2011) Kinetic analysis of DNA double-strand break repair pathways in Arabidopsis. *DNA Repair (Amst)*, **10**, 611–619.

Chen, I.P., Mannuss, A., Orel, N., Heitzeberg, F. and Puchta, H. (2008) A homolog of ScRAD5 is involved in DNA repair and homologous recombination in Arabidopsis. *Plant Physiol.* **146**, 1786–1796.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J.* **16**, 735–743.

Daoudal-Cotterell, S., Gallego, M.E. and White, C.I. (2002) The plant Rad50-Mre11 protein complex. *FEBS Lett.* **516**, 164–166.

De Piccoli, G., Torres-Rosell, J. and Aragon, L. (2009) The unnamed complex: what do we know about Smc5-Smc6? *Chromosome Res.* **17**, 251–263.

Fauser, F., Roth, N., Pacher, M., Ilg, G., Sanchez-Fernandez, R., Biesgen, C. and Puchta, H. (2012) In planta gene targeting. *Proc. Natl Acad. Sci. USA*, **109**, 7535–7540.

Gherbi, H., Gallego, M.E., Jalut, N., Lucht, J.M., Hohn, B. and White, C.I. (2001) Homologous recombination in planta is stimulated in the absence of Rad50. *EMBO Rep.* **2**, 287–291.

Hajdukiewicz, P., Svab, Z. and Maliga, P. (1994) The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. *Plant Mol. Biol.* **25**, 989–994.

Hartung, F., Suer, S., Bergmann, T. and Puchta, H. (2006) The role of AtMUS81 in DNA repair and its genetic interaction with the helicase AtRecQ4A. *Nucleic Acids Res.* **34**, 4438–4448.

Hartung, F., Suer, S. and Puchta, H. (2007) Two closely related RecQ helicases have antagonistic roles in homologous recombination and DNA repair in Arabidopsis thaliana. *Proc. Natl Acad. Sci. USA*, **104**, 18836–18841.

Hartung, F., Suer, S., Knoll, A., Wurz-Wildersinn, R. and Puchta, H. (2008) Topoisomerase 3alpha and RMI1 suppress somatic crossovers and are essential for resolution of meiotic recombination intermediates in Arabidopsis thaliana. *PLoS Genet.* **4**, e1000285.

Heacock, M., Spangler, E., Riha, K., Puizina, J. and Shippen, D.E. (2004) Molecular analysis of telomere fusions in Arabidopsis: multiple pathways for chromosome end-joining. *EMBO J.* **23**, 2304–2313.

Knoll, A. and Puchta, H. (2011) The role of DNA helicases and their interaction partners in genome stability and meiotic recombination in plants. *J. Exp. Bot.* **62**, 1565–1579.

Knoll, A., Higgins, J.D., Seeliger, K., Reha, S.J., Dangel, N.J., Bauknecht, M., Schropfer, S., Franklin, F.C. and Puchta, H. (2012) The Fanconi Anemia Ortholog FANCM Ensures Ordered Homologous Recombination in Both Somatic and Meiotic Cells in Arabidopsis. *Plant Cell*, **24**, 1448–1464.

Li, W., Chen, C., Markmann-Mulisch, U., Timofejeva, L., Schmelzer, E., Ma, H. and Reiss, B. (2004) The Arabidopsis AtRAD51 gene is dispensable for vegetative development but required for meiosis. *Proc. Natl Acad. Sci. USA*, **101**, 10596–10601.

Liu, J., Renault, L., Veaute, X., Fabre, F., Stahlberg, H. and Heyer, W.D. (2011) Rad51 paralogues Rad55-Rad57 balance the antirecombinase Srs2 in Rad51 filament formation. *Nature* **479**, 245–248.

Mannuss, A., Dukowic-Schulze, S., Suer, S., Hartung, F., Pacher, M. and Puchta, H. (2010) RAD5A, RECQ4A, and MUS81 have specific functions in homologous recombination and define different pathways of DNA repair in Arabidopsis thaliana. *Plant Cell* **22**, 3318–3330.

Masson, J.Y., Tarsounas, M.C., Stasiak, A.Z., Stasiak, A., Shah, R., McIlwraith, M.J., Benson, F.E. and West, S.C. (2001) Identification and purification of two distinct complexes containing the five RAD51 paralogs. *Genes Dev.* **15**, 3296–3307.

Orel, N., Kyryk, A. and Puchta, H. (2003) Different pathways of homologous recombination are used for the repair of double-strand breaks within tandemly arranged sequences in the plant genome. *Plant J.* **35**, 604–612.

Osakabe, K., Abe, K., Yamanouchi, H. *et al.* (2005) Arabidopsis Rad51B is important for double-strand DNA breaks repair in somatic cells. *Plant Mol. Biol.* **57**, 819–833.

Osakabe, K., Abe, K., Yoshioka, T., Osakabe, Y., Todoriki, S., Ichikawa, H., Hohn, B. and Toki, S. (2006) Isolation and characterization of the RAD54 gene from Arabidopsis thaliana. *Plant J.* **48**, 827–842.

Osakabe, K., Osakabe, Y. and Toki, S. (2010) Site-directed mutagenesis in Arabidopsis using custom-designed zinc finger nucleases. *Proc. Natl Acad. Sci. USA*, **107**, 12034–12039.

Paques, F. and Haber, J.E. (1999) Multiple pathways of recombination induced by double-strand breaks in Saccharomyces cerevisiae. *Microbiol. Mol. Biol. Rev.* **63**, 349–404.

Petolino, J.F., Worden, A., Curlee, K., Connell, J., Strange Moynahan, T.L., Larsen, C. and Russell, S. (2010) Zinc finger nuclease-mediated transgene deletion. *Plant Mol. Biol.* **73**, 617–628.

Prakash, R., Satory, D., Dray, E. *et al.* (2009) Yeast Mph1 helicase dissociates Rad51-made D-loops: implications for crossover control in mitotic recombination. *Genes Dev.* **23**, 67–79.

Puchta, H. (1998) Repair of genomic double-strand breaks in somatic plant cells by one-sided invasion of homologous sequences. *Plant J.* **13**, 331–340.

Puchta, H. (2005) The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. *J. Exp. Bot.* **56**, 1–14.

Puchta, H. and Hohn, B. (2010) Breaking news: plants mutate right on target. *Proc. Natl Acad. Sci. USA*, **107**, 11657–11658.

Puchta, H., Dujon, B. and Hohn, B. (1993) Homologous recombination in plant cells is enhanced by *in vivo* induction of double strand breaks into DNA by a site-specific endonuclease. *Nucleic Acids Res.* **21**, 5034–5040.

Puchta, H., Dujon, B. and Hohn, B. (1996) Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination. *Proc. Natl Acad. Sci. USA*, **93**, 5055–5060.

Puizina, J., Siroky, J., Mokros, P., Schweizer, D. and Riha, K. (2004) Mre11 deficiency in Arabidopsis is associated with chromosomal instability in somatic cells and Spo11-dependent genome fragmentation during meiosis. *Plant Cell*, **16**, 1968–1978.

Seeliger, K., Dukowic-Schulze, S., Wurz-Wildersinn, R., Pacher, M. and Puchta, H. (2012) BRCA2 is a mediator of RAD51- and DMC1-facilitated homologous recombination in Arabidopsis thaliana. *New Phytol.* **193**, 364–375.

Shaked, H., Avivi-Ragolsky, N. and Levy, A.A. (2006) Involvement of the Arabidopsis SWI2/SNF2 chromatin remodeling gene family in DNA damage response and recombination. *Genetics*, **173**, 985–994.

Shukla, V.K., Doyon, Y., Miller, J.C. *et al.* (2009) Precise genome modification in the crop species Zea mays using zinc-finger nucleases. *Nature*, **459**, 437–441.

Siebert, R. and Puchta, H. (2002) Efficient repair of genomic double-strand breaks by homologous recombination between directly repeated sequences in the plant genome. *Plant Cell*, **14**, 1121–1131.

Swoboda, P., Gal, S., Hohn, B. and Puchta, H. (1994) Intrachromosomal homologous recombination in whole plants. *EMBO J.* **13**, 484–489.

Szostak, J.W., Orr-Weaver, T.L., Rothstein, R.J. and Stahl, F.W. (1983) The double-strand-break repair model for recombination. *Cell*, **33**, 25–35.

Tan, G., Gao, Y., Shi, M., Zhang, X., He, S., Chen, Z. and An, C. (2005) Site-Finding-PCR: a simple and efficient PCR method for chromosome walking. *Nucleic Acids Res.* **33**, e122.

Townsend, J.A., Wright, D.A., Winfrey, R.J., Fu, F., Maeder, M.L., Joung, J.K. and Voytas, D.F. (2009) High-frequency modification of plant genes using engineered zinc-finger nucleases. *Nature*, **459**, 442–445.

Uanschou, C., Siwiec, T., Pedrosa-Harand, A., Kerzendorfer, C., Sanchez-Moran, E., Novatchkova, M., Akimcheva, S., Woglar, A., Klein, F. and Schlogelhofer, P. (2007) A novel plant gene essential for meiosis is related to the human CtIP and the yeast COM1/SAE2 gene. *EMBO J.* **26**, 5061–5070.

Watanabe, K., Pacher, M., Dukowic, S., Schubert, V., Puchta, H. and Schubert, I. (2009) The structural maintenance of chromosomes 5/6 complex promotes sister chromatid alignment and homologous recombination after DNA damage in Arabidopsis thaliana. *Plant Cell*, **21**, 2688–2699.

Wei, W., Ba, Z., Gao, M., Wu, Y., Ma, Y., Amiard, S., White, C.I., Rendtlew Danielsen, J.M., Yang, Y.G. and Qi, Y. (2012) A role for small RNAs in DNA double-strand break repair. *Cell*, **149**, 101–112.

Zhang, F., Maeder, M.L., Unger-Wallace, E. *et al.* (2010) High frequency targeted mutagenesis in Arabidopsis thaliana using zinc finger nucleases. *Proc. Natl Acad. Sci. USA*, **107**, 12028–12033.