RecA-Dependent DNA Repair Results in Increased Heteroplasmy of the Arabidopsis Mitochondrial Genome[1][C][W]

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Plant mitochondria have very active DNA recombination activities that are responsible for its plastic structures and that should be involved in the repair of double-strand breaks in the mitochondrial genome. Little is still known on plant mitochondrial DNA repair, but repair by recombination is believed to be a major determinant in the rapid evolution of plant mitochondrial genomes. In flowering plants, mitochondria possess at least two eubacteria-type RecA proteins that should be core components of the mitochondrial repair mechanisms. We have performed functional analyses of the two Arabidopsis (*Arabidopsis thaliana*) mitochondrial RecAs (RECA2 and RECA3) to assess their potential roles in recombination-dependent repair. Heterologous expression in *Escherichia coli* revealed that RECA2 and RECA3 have overlapping as well as specific activities that allow them to partially complement bacterial repair pathways. *RECA2* and *RECA3* have similar patterns of expression, and mutants of either display the same molecular phenotypes of increased recombination between intermediate-size repeats, thus suggesting that they act in the same recombination pathways. However, RECA2 is essential past the seedling stage and should have additional important functions. Treatment of plants with several DNA-damaging drugs further showed that RECA3 is required for different recombination-dependent repair pathways that significantly contribute to plant fitness under stress. Replication repair of double-strand breaks results in the accumulation of crossovers that increase the heteroplasmic state of the mitochondrial DNA. It was shown that these are transmitted to the plant progeny, enhancing the potential for mitochondrial genome evolution.

Plant mitochondrial genomes are large in size and constituted by a complex and dynamic network of circular and linear, double- or single-stranded DNA molecules (Oldenburg and Bendich, 1996; Backert et al., 1997). This complex structure of the mitochondrial DNA (mtDNA) is generated by homologous recombination (HR) between repeated sequences, which are abundant in plant mitochondrial genomes (André et al., 1992). These repeated sequences are often distinguished into large pairs of repeats, involved in frequent and reversible recombination (two in the Arabidopsis [*Arabidopsis thaliana*] mtDNA of 6.5 and 4.2 kb), and intermediate-size repeats (IRs) from a few dozen to a few hundred base pairs (about 30 IRs larger than 100 bp in the Arabidopsis mtDNA). Recombination between the latter is infrequent and yields low-copy-number alternative configurations (mitotypes) of the mtDNA that coexist with the dominant mtDNA configuration (Small et al., 1989). HR, therefore, is a major determinant of the intrinsic heteroplasmic state of the mtDNA (Kmiec et al., 2006). Under relaxed control of recombination, very rapid changes may occur in the relative proportions of mtDNA variants. This phenomenon is called substoichiometric shifting (SSS; Kanazawa et al., 1994; Bellaoui et al., 1998; Janska et al., 1998). It can result in the activation or silencing of mitochondrial sequences, altering mitochondrial gene expression with deleterious consequences to the plant. SSS also contributes to the rapid evolution of the mtDNA structure and to cytoplasm-nucleus conflicts, which can result in cytoplasmic male sterility (Touzet and Budar, 2004). Therefore, rigorous control of HR is essential to maintain mtDNA stability in plants.

The mechanisms that regulate HR and the stoichiometric transmission of the alternative mitotypes to the progeny are poorly understood. Only a few factors that influence mtDNA HR and that can affect the SSS

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process have been identified so far. These include the Arabidopsis MutS-like protein MSH1 (Abdelnoor et al., 2003) and the single-stranded DNA-binding protein OSB1 (Zaegel et al., 2006). The primary effect of the inactivation of these genes is the accumulation of some products arising from recombination between IRs. In the following mutant generations, this can lead to new predominant mtDNA sequences by SSS (Zaegel et al., 2006; Arrieta-Montiel et al., 2009; Davila et al., 2011). Homologs of eubacterial RecA that are present in plant organelles (Khazi et al., 2003; Shedge et al., 2007; Odahara et al., 2009) have also been shown to modulate recombination between IRs, both in Arabidopsis and bryophytes (Shedge et al., 2007; Odahara et al., 2009). Additional genes (*WHY2* and *POL1B*) influence the outcome of recombination processes in plant mitochondria, leading, for example, to increased illegitimate microhomology-mediated recombination that results in the accumulation of chimeric molecules (Cappadocia et al., 2010; Parent et al., 2011). These examples demonstrate that multiple alternative recombination processes can be activated in plant mtDNA maintenance and that the complete set of factors involved in these processes still remain to be determined.

It has been proposed that SSS involving the amplification of IR recombination products could result from the repair of double-strand breaks (DSBs) by break-induced replication (BIR), which occurs during the replication process (Shedge et al., 2007; Davila et al., 2011). Indeed, most examples of SSS involving recombination between IRs are characterized by an asymmetrical amplification of only one of the recombinant forms, which can be explained by a BIR repair pathway. In addition, the involvement of BIR is consistent with models of the plant mtDNA replication by recombination-dependent pathways (Oldenburg and Bendich, 1996; Backert et al., 1997; Manchekar et al., 2006). A direct link between mtDNA repair, recombination, and SSS has not been formally established yet.

DNA repair via HR is known to exist in yeast mitochondria (Ling et al., 1995). In animals, mtDNA repair by HR is not believed to routinely occur, although corresponding activities have been detected (Thyagarajan et al., 1996; Kajander et al., 2001). In plant mitochondria, the present knowledge about mtDNA repair is still scarce. A short-patch base excision repair pathway and a microhomology-mediated recombination pathway have been shown (Boesch et al., 2009; Cappadocia et al., 2010). However, the important HR activities that exist in plant organelles suggest that recombination-dependent repair processes are predominant. In bacteria, RecA-type recombinases have crucial roles in HR-dependent functions, because the formation of a single-stranded DNA-RecA filament is a common intermediate in recombination pathways that require the invasion of double-stranded DNA by homologous single-stranded DNA (Cox, 2007). RecA-type proteins have been found in mitochondria and/or chloroplasts of plants and are encoded by genes inherited from the endosymbiont ancestors of mitochondria and plastids, α-proteobacteria and cyanobacteria, respectively (for phylogenetic tree, see Lin et al. [2006]). During evolution, the genes coding for a mitochondrial RecA have been lost from the entire algal lineage as well as from animals and fungi. In contrast, both mitochondrial and plastidial RecAs are present in land plants. Two *RECA* genes have been retained in the moss *Physcomitrella patens* (one for each organellar compartment [Odahara et al., 2007; Inouye et al., 2008]), and three *RECA* genes are present in Arabidopsis, named *RECA1*, *RECA2*, and *RECA3* (Shedge et al., 2007). Protein-GFP fusions showed that RECA1 and RECA3 are targeted to plastids and mitochondria, respectively, whereas RECA2 is dual targeted to both organelles. In bacteria, the RecA enzyme (together with ancillary factors) is involved in DNA recombination and repair following, for example, collapsed replication forks or DSBs (Cox, 2007). In chloroplasts, there is already extensive evidence for the role of plastid-targeted RecAs in chloroplast genome (cpDNA) repair by recombination (Cerutti et al., 1993, 1995; Kwon et al., 2010; Rowan et al., 2010). The mitochondrial RECA3 has also been shown to partially complement *Escherichia coli recA* gene deletion, enhancing survival after exposure to the DNA-damaging agent methyl methanesulfonate (Khazi et al., 2003). This suggests a role for RECA3 in mtDNA repair.

To better understand the contribution of RecA-dependent recombination and mtDNA repair to genome stability and plant survival, we performed a functional study of the *RECA2* and *RECA3* genes. We compared their abilities for heterologous functional complementation in *E. coli* and their effects on plant mtDNA recombination. We also compared the effects of genotoxins on the mtDNA and its subsequent repair between wild-type and *RECA3*-deficient plants. We found that mutants in either *RECA2* or *RECA3* displayed the same molecular phenotypes of increased recombination between IRs, suggesting that they act in the same recombination pathways. Alternative structures of the mtDNA generated by repair through recombination were found to be transmitted to the plant progeny, enhancing the potential for mtDNA evolution by SSS.

RESULTS

Flowering Plants Have Two Types of Mitochondrial RecA Proteins

Data bank searches showed that the three subclasses of RECA proteins defined in Arabidopsis (RECA1, RECA2, and RECA3; Shedge et al., 2007) are found in flowering plants, with two members of the RECA2 subclass in rice (*Oryza sativa*) and maize (*Zea mays*; Fig. 1; Supplemental Fig. S1). According to a phylogenetic analysis and targeting predictions, the plant RECA1 sequences correspond to chloroplast proteins whereas

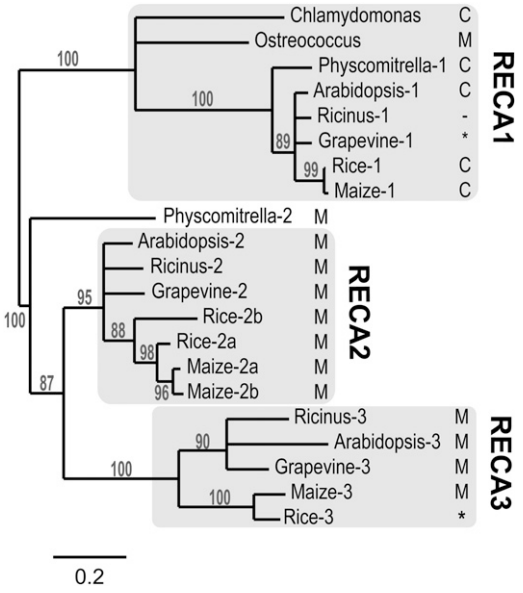

Figure 1. Conservation of two mitochondrial RecAs in flowering plants. The phylogram was constructed from an alignment of several representative plant and algal RecA protein sequences. Branches with support values smaller than 80% were collapsed. The plant subclasses RECA1, RECA2, and RECA3 are shaded. Branch lengths (scale bar) are proportional to the distance between sequences and suggest a faster evolution of RECA3 sequences as compared with RECA1 and RECA2. Subcellular targeting predicted with TargetP and Predotar is given as follows: C, Targeted to plastids according to both programs; M, targeted to mitochondria according to both programs; *, no consensus prediction; −, not determined because the N-terminal sequence was missing. Sequence accession numbers are given in Supplemental Table S1.

RECA3 sequences code for proteins targeted to mitochondria (Fig. 1). Orthologs of Arabidopsis RECA2 are all predicted to be mitochondrial; however, they may be dual targeted to mitochondria and plastids, as shown for Arabidopsis RECA2 (Shedge et al., 2007). RECA3 proteins are characterized by the absence of a C-terminal extension that is found in all other plant and bacterial RecA proteins and that is often acidic (Supplemental Fig. S1). In *E. coli*, this C-terminal region was found to modulate the affinity of RecA to single-stranded DNA and its ability to displace prebound single-stranded DNA-binding proteins (Eggler et al., 2003). These observations suggest that RECA2 and RECA3 evolved to support distinct functions.

The Arabidopsis *RECA2* and *RECA3* Genes Present Similar Patterns of Expression

The existence of two RecA proteins in Arabidopsis mitochondria could be related to differential expression patterns during plant development. This hypothesis was tested by expressing *RECA2* and *RECA3* promoter-GUS fusions in transgenic Arabidopsis. The expression patterns of the two constructs (p*RECA2*::GUS and p*RECA3*::GUS) were found to be very similar. In young seedlings, GUS staining was visible mainly at the tip of cotyledons and of true leaves as well as at the tip of serration protrusions that could correspond to hydathodes (Fig. 2, A–E). In p*RECA3*::GUS plants, GUS staining was often seen in the root vascular tissue of budding lateral roots (Fig. 2C) but not in the root apical meristem. In flowers, expression was visible in the anthers of developing flowers and mature pollen for both p*RECA2*::GUS and p*RECA3*::GUS and in sepals for only p*RECA2*::GUS (Fig. 2, F, G, K, and M). In some flowers, *RECA2* and *RECA3* promoter activity was also observed in ovules, mostly within the central cell (Fig. 2, H–J and L). The staining was not uniform in ovules from the same silique, suggesting differential expression. Thus, *RECA2* and *RECA3* also might be expressed in the female gametophyte.

RECA2 and RECA3 Functionally Complement Bacterial RecA

To unveil possible differences between the roles of RECA2 and RECA3, we compared their ability to complement bacterial RecA in DNA repair. The coding sequences of RECA2 and RECA3 without their predicted N-terminal targeting sequences were introduced in the low-copy-number plasmid pACYC under the control of the isopropyl β-D-thiogalactopyranoside (IPTG)-inducible *lac* promoter. The resulting constructs (*PrecA2* and *PrecA3* plasmids) were transformed into the *E. coli* JM103 *recA*$^-$ strain and compared with the *recA*$^+$ strain. Recombinant protein expression was verified by western blot using an antibody directed against recombinant RECA3 that also recognizes RECA2 (Fig. 3A). The effects of RecA expression on cell survival after treatment with hydroxyurea (HU), mitomycin C (MMC), and UV-C were measured (Fig. 3). HU is an inhibitor of ribonucleotide reductase, the rate-limiting enzyme in nucleotide biosynthesis. After HU treatment, the replication fork collapses because of deoxyribonucleotide triphosphate depletion, and recombination-dependent functions are then required to reestablish replication. MMC is an alkylating agent that causes interstrand DNA cross-links (Thomasz, 1994), which induce DSB during replication. UV-C irradiation induces the formation of photoproducts like pyrimidine (6-4) pyrimidone dimers (Britt, 1999), which are repaired either directly by photolyases or by nucleotide excision repair. Some repair mechanisms following HU and MMC treatments and UV irradiation have been shown to mobilize RecA (Friedberg et al., 1995).

The expression of either RECA2 or RECA3 increased the resistance of bacteria to both HU (20- and 17-fold, respectively) and to MMC (11- and 21-fold, respectively; Fig. 3B). In UV-C-irradiated cells, *recA*$^-$ cultures transformed with *PrecA2* were able to partially survive to doses up to 4 and 8 J m^{-2}, whereas untransformed cells and those transformed with *PrecA3* were not (Fig. 3C). Thus, RECA2 and RECA3 can both partially substitute for *E. coli* RecA in the repair of DNA breaks induced by HU and MMC. RECA2 can also partially complement *E. coli* RecA in the repair of UV-C-

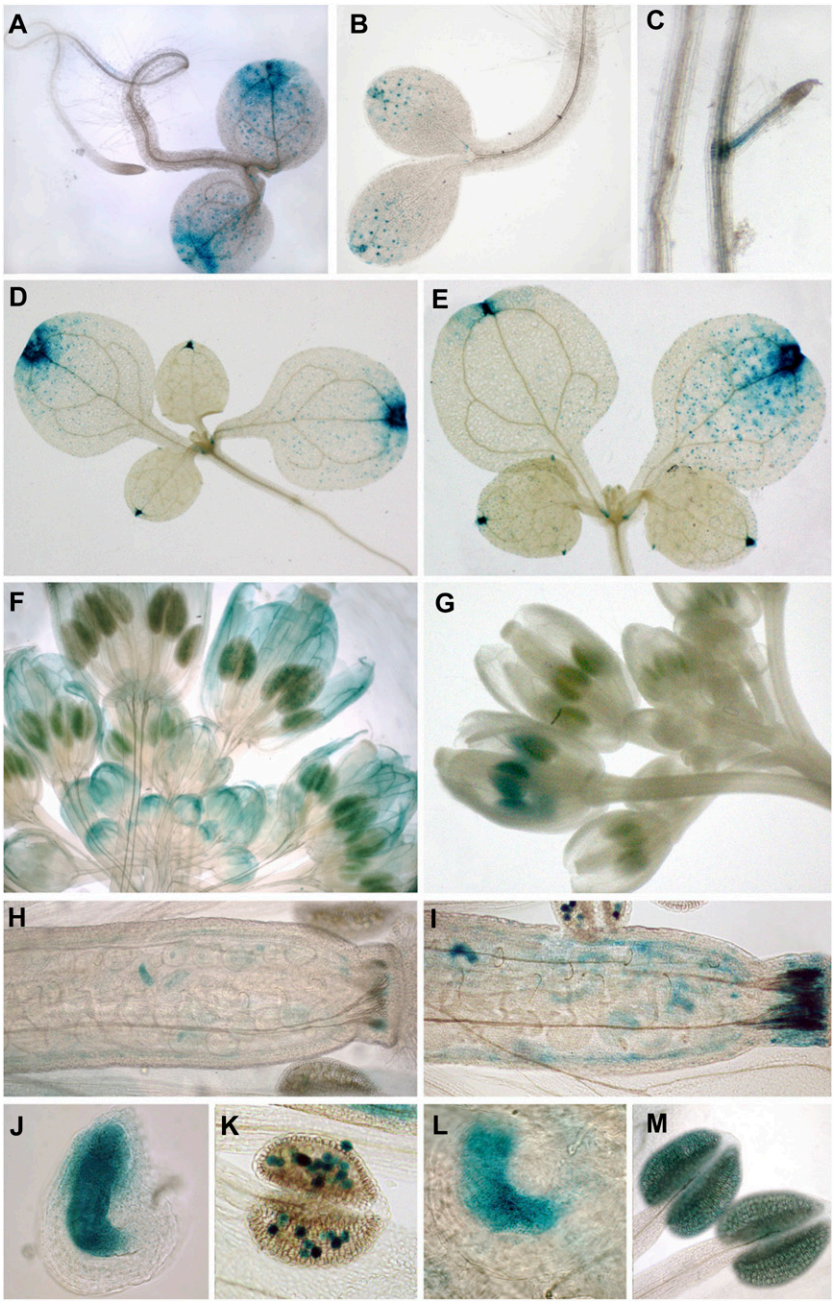

Figure 2. *RECA2* and *RECA3* have similar patterns of expression according to promoter::GUS fusion. Promoter::GUS fusion reporter plants of RECA2 (p*RECA2*::GUS; A, D, F, H, J, and K) and RECA3 (p*RECA3*::GUS; B, C, E, G, I, L, and M) were stained for GUS detection at different developmental stages. A and B, Three-day-old seedlings. C to E, Seven-day-old seedlings. F to M, Inflorescences. p*RECA2*::GUS and p*RECA3*::GUS plants showed strong GUS expression in anthers of immature flowers (F and G) and in mature pollen (K and M). GUS staining of several ovules was visible in carpels of mature flowers (H and I), mostly colocalizing with the central cell (J and L).

induced damage, whereas RECA3 cannot. This suggests that both RECA2 and RECA3 might be involved in DSB repair of plant organellar DNA, but RECA2 might have additional roles that cannot be fulfilled by RECA3.

Loss of *RECA2* Results in Seedling Lethality

We further investigated the potential roles of RECA2 and RECA3 in mtDNA maintenance using T-DNA insertion lines (Supplemental Fig. S2). *recA3* mutants have been previously described; they are viable and indistinct from wild-type plants (Shedge et al., 2007). In contrast, inactivation of *RECA2* has been reported to be lethal (Shedge et al., 2007). We obtained seedlings homozygous for a dysfunctional *recA2* allele from T-DNA insertion lines SALK_076237 (*recA2-1*), SALK_118143 (*recA2-3*), and SALK_045420 (*recA2-4*). We found that although *recA2* mutants fail to germinate on soil, they are able to germinate in vitro in the presence of sugar. However, they do not develop roots, and their growth is arrested before the development of first true leaves (Fig. 4A). *recA2-1*, *recA2-3*, and *recA2-4* displayed the same morphological and developmental defects, indicating that these are due to *REC2* inactivation. The growth of an additional *recA* mutant line, *recA2-2* (SALK_068655), in which the T-DNA is inser-

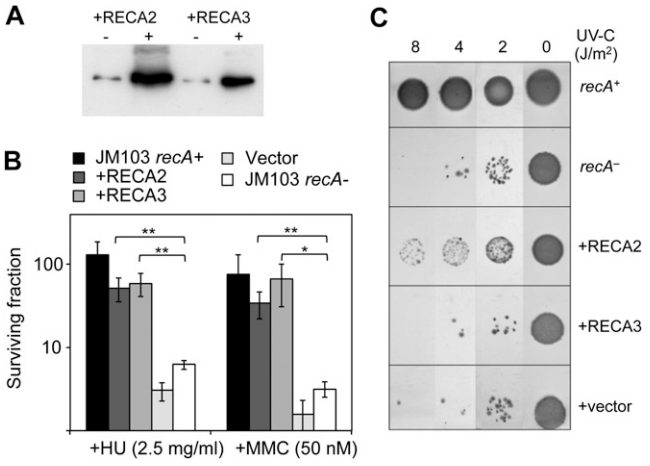

Figure 3. Partial complementation of *E. coli recA*$^-$ by Arabidopsis mitochondrial RecA proteins. *E. coli* strains JM103 *recA*$^+$ and JM103 *recA*$^-$ were used as control strains in the experiments. JM103 *recA*$^-$ was transformed with the *pACYC-lacZ* empty vector (+vector), *PrecA2* (+RECA2), or *PrecA3* (+RECA3). Recombinant RECA2 and RECA3 expression was induced with IPTG prior to treatment with genotoxins, as described in "Materials and Methods." The surviving fractions were compared between IPTG-induced and noninduced cells. A, Immunodetection of RECA2 and RECA3 in cultures before (−) and after (+) induction of expression with IPTG. B, Complementation of bacterial cultures treated with HU or MMC. Results are represented on a logarithmic scale. Asterisks indicate the statistical significance (unpaired *t* test; * $P < 0.05$, ** $P < 0.01$) of differences between cultures expressing the recombinant proteins and the untransformed control ($n = 3$). C, Complementation of bacterial RecA for repair of UV-C-induced damage. Equivalent dilutions of bacterial cells were spotted on agar plates and exposed to the indicated doses of UV-C light before growth.

ted at the end of the last exon, was indistinct from the wild type. Sequence analysis showed that the *recA2-2* allele potentially codes for a protein in which the final seven codons are changed from AVVVEAA to MCKQIDA; the amino acid sequence length remains unchanged. Given the consistent phenotypes of the three other *recA2* mutant lines, the *recA2-2* allele does not significantly affect RECA2 function.

We observed that plants heterozygous for allele *recA2-1*, *recA2-3*, or *recA2-4* produced fewer seedling-lethal and nongerminating offspring than expected for normal *recA2* allele transmission. Given the expression of *RECA2* in gametophytic tissue (Fig. 2), we analyzed the efficiency of the transmission of *recA2-1* and *recA2-3* alleles by heterozygous parents and performed reciprocal backcrosses to assess *recA2* gamete fitness. However, we found that the small reduction in *recA2-1* or *recA2-3* allele transmission was not statistically significant (Supplemental Table S2).

Both *recA2* and *recA3* Mutants Display Rearranged mtDNA by Recombination between IRs

We examined the effects of the *recA2* and *recA3* mutations on mtDNA recombination. The accumulation of crossover products that result from IR-mediated recombination was measured by quantitative PCR (qPCR) and compared between mutant and wild-type plants; primers were designed to specifically amplify a selection of repeats and their corresponding crossover products (Fig. 4B; Supplemental Table S3). Preliminary experiments were conducted to select pairs of IRs that are recombinogenically active and amenable to relative quantification of the corresponding crossover products by qPCR. We chose repeats I, L, and X (as annotated in the recently published mtDNA sequence of ecotype Columbia [Col-0]; accession no. JF729201) as suitable markers for qPCR quantification of mtDNA recombination and heteroplasmy. We observed an increase mainly in one of the crossover products of repeats I and L (sequences I-2/1 and L-2/1, respectively) in *recA3-1* and *recA3-2* compared with the wild type, indicating that RECA3 absence triggers asymmetrical recombination at these IRs. In the case of repeat X, both crossover products X-1/2 and X-2/1 accumulated (Fig. 4, C–E). In *recA2-1*, *-3*, and *-4* plants, HR activity was also increased, as indicated by the high accumulation of these mtDNA recombination products, which in *recA2-1*, *-3*, and *-4* were about 1 order of magnitude more abundant than in *recA3* plants. The relative increase in crossover products compared with the wild type reached more than 1,000-fold for I-2/1 and X-2/1 (Fig. 4, C and E). As for *recA3*, recombination was mostly asymmetrical in the case of repeats of I and L. From a comparison of cycle threshold values, we estimated that the accumulation of sequences I-2/1, L-2/1, X-1/2, and X-2/1 in *recA* mutants reached levels comparable to the parental sequences found in wild-type mtDNA. Such high levels of recombined DNA sequences have been previously observed to occur in late generations of *osb1* and *msh1* mutants and to be aggravated in *msh1 recA3* double mutants (Zaegel et al., 2006; Shedge et al., 2010; Davila et al., 2011). The moderate increase of L-2/1 in *recA* mutants compared with the wild type probably reflected the fairly high abundance of L-2/1 in wild-type mitochondria: L-2/1 was only about 30 to 40 times less abundant than the parental L-1 and L-2 sequences.

We additionally determined the relative copy numbers of several mtDNA and cpDNA genes in *recA2* and *recA3* plants by qPCR and compared them with gene copies found in wild-type plants at the same development stage (7 and 3 d after germination for *recA2* and *recA3*, respectively, to assess plants at a comparable developmental stage). At this early stage of plant growth, there is no significant endoreplication of the nuclear genome that affects the relative quantification calculations (Zoschke et al., 2007; Preuten et al., 2010). Three gene sequences of each organelle genome were quantified (Fig. 4F), and the results were standardized against nuclear sequences. The results showed that *recA3* plants have mtDNA and cpDNA copy numbers comparable to the wild type. *recA2-1*, *-3*, and *-4* similarly displayed no change in cpDNA copies, suggesting that RECA2 has no role in cpDNA replication. Replication of the mtDNA in these *recA2* lines was also

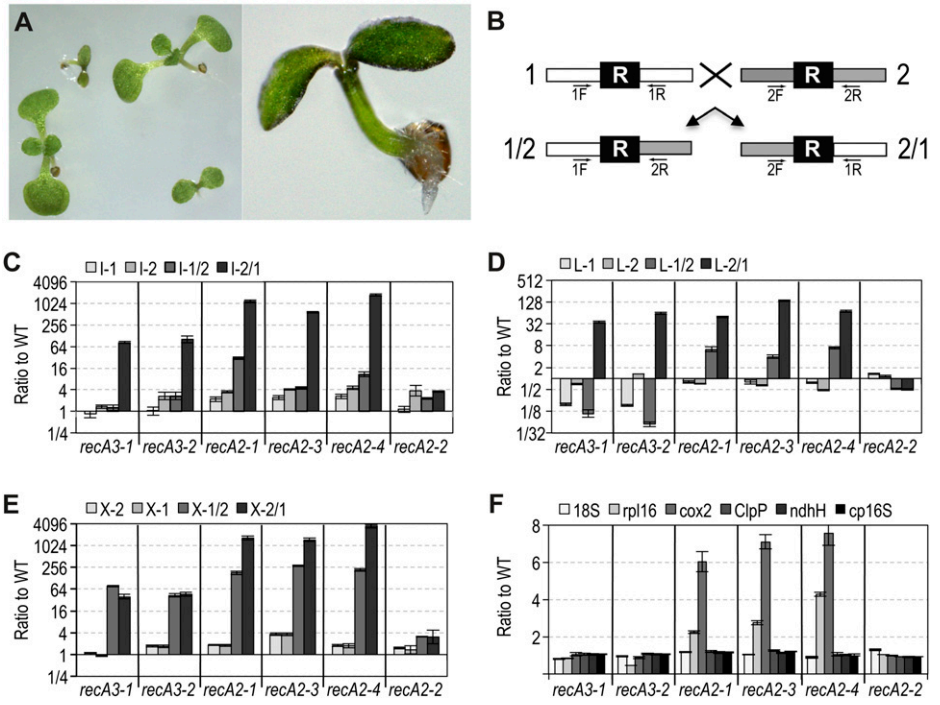

Figure 4. Mutants of *recA2* and *recA3* accumulate crossover products of IRs. A, Seedling-lethal phenotype of *recA2* homozygous plants. Left, Comparison between small seedlings corresponding to *recA2* plants and normal-growing seedlings confirmed to possess wild-type *RECA2* alleles; right, enlargement showing the *recA2* mutant phenotype of dark green unexpanded cotyledons and the absence of root elongation. B, Simplified scheme explaining the amplification of sequences 1 and 2 comprising a repeated sequence (black box R) and of the resulting crossover products 1/2 and 2/1. C to E, Relative quantification of the parental sequences and crossover products (as depicted in B) of IRs I, L, and X in *recA2* and *recA3* homozygous seedlings. Results are represented using a $\log_2$ scale. F, Relative quantification of copy numbers of mtDNA (18S, rpl16, cox2) and cpDNA (clpP, ndhH, cp16S) gene sequences on the different *recA2* and *recA3* lines. WT, Wild type. [See online article for color version of this figure.]

not arrested, because values measured for mitochondrial genes were at least as high as in the wild type. Remarkably, the *recA2-1*, *-3*, and *-4* mutants presented significantly different gene stoichiometries. While *cox2* gene copies were increased about 6-fold compared with the wild type, values for the ribosomal 18S region remained unaltered. Sharp remodeling of the mtDNA by recombination might be responsible for the differences in stoichiometry between the mtDNA gene sequences in *recA2-1*, *-3*, and *-4*. For line *recA2-2*, we found no changes in genome copy numbers or in mtDNA recombination, confirming that this line is not affected in RECA2 functions.

The unchanged or increased mtDNA copy numbers in the three other *recA2* lines indicated that the consistent phenotype of seedling lethality was not due to a general loss of the mtDNA. To test if the dramatic *recA2* phenotype was due to a strong reduction in critical mitochondrial transcripts, which would be feasible as a consequence of major mtDNA rearrangements, we conducted a global mitochondrial transcriptome analysis by quantitative reverse transcription (qRT)-PCR as described (Kühn et al., 2009). The results showed that there is no significant reduction in steady-state transcript levels for any of the genes tested (Supplemental Fig. S3). On the contrary, most genes displayed increased transcript abundances. A global increase in mitochondrial transcripts has also been described in other mutants affected in mitochondrial functions (Sung et al., 2010; Kühn et al., 2011). qRT-PCR also showed that in *recA2* there are no changes in the relative abundance of the *RECA3* transcript that could suggest a compensatory increase in RECA3 expression.

These results showed that RECA-dependent recombination is tightly regulated and that loss of either of the two mitochondrial RECAs results in an increase of the recombination activity that modifies the structure of the mtDNA. To verify if increased recombination is still observed in the absence of both RECAs, we tried to obtain *recA2 recA3* double mutants. Reciprocal crosses between heterozygous *recA2-3* and homozygous *recA3-2* were performed, and the progeny of F1 plants heterozygous for both mutations were analyzed. A reduced germination rate was observed in the F2 generation, and very few plants were obtained displaying the characteristic *recA2* growth phenotype (Supplemental Table S2). Among these, no double mutant was found in 60 plants genotyped, suggesting that a mitochondrial RECA function is essential for plant viability.

recA3 Mutant Lines Are Selectively Sensitive to Genotoxic Stress

To better understand the roles of mitochondrial RECA proteins in mtDNA repair and the relationship between repair and mtDNA heteroplasmy, we studied the effects of DNA damage on the wild type and on mutant lines. The seedling lethality of *recA2* precluded any functional study of *RECA2* in repair. Our studies, therefore, were concentrated on *recA3* mutant plants, which are phenotypically normal under standard growth conditions. We challenged Col-0 wild-type and *recA3-2* plants with several genotoxic agents that induce different types of DNA damage. Our experimental protocol (see "Materials and Methods") essentially followed established ones (Heitzeberg et al., 2004; Hartung et al., 2007). We tested MMC, ciprofloxacin (CIP), bleomycin (BLM), and hydrogen peroxide. CIP is an antibiotic that inhibits bacteria-type gyrase, which in plants is present in both mitochondria and chloroplasts (Wall et al., 2004). Oxidation lesions caused by hydrogen peroxide, such as 8-oxoG, are mainly repaired by base excision repair mechanisms (Boesch et al., 2011). MMC, CIP, and BLM induce DSBs (Kampranis and Maxwell, 1998; Menke et al., 2001; Rowan et al., 2010), but only BLM is a radiomimic chemical that directly induces breaks on DNA, whereas MMC and CIP induce DNA breaks indirectly after replication. While MMC and BLM are expected to affect the DNA in all three genomic compartments (nucleus, chloroplast, and mitochondria), CIP should only affect the organelle genomes.

As a preliminary experiment, we tested by qRT-PCR the effects of MMC and BLM on the expression of *RECA2*, *RECA3*, and several other genes known or predicted to be involved in mtDNA maintenance and recombination. In addition to *MSH1*, *OSB1*, and *WHY2*, these genes comprised *POLIA* and *POLIB* (Parent et al., 2011), Arabidopsis Twinkle and Topoisomerase I homologs (Carrie et al., 2009), and the *GYRB2* gene for the mitochondrial gyrase subunit B (Wall et al., 2004). As a control, we followed the induction of *TSO2* (At3g18780), which codes for a small subunit of ribonucleotide reductase. Its expression is induced by DSB on the nuclear genome and is under the control of the ATM signaling pathway (Roa et al., 2009). As expected, its expression was induced by BLM and MMC in our experiments (Fig. 5). However, none of the genes coding for mitochondrial mtDNA maintenance was induced by the same treatments. Thus, the perception and repair of DSBs in mitochondria are not controlled by the same pathway as the nuclear DSBs. Nevertheless, this experiment confirmed the effectiveness of our experimental conditions.

We then tested the effects of the same drugs on plant growth (Fig. 6). *recA3* plants were as sensitive as the wild type to oxidative damage induced by hydrogen peroxide (Fig. 6B), but *recA3-2* plants were significantly more sensitive to BLM, MMC, and CIP (Fig. 6, C–E). Similar results were obtained with the *recA3-3* line (Supplemental Fig. S4). We also found that *recA3-2* plants grown on plates were unable to develop normal true leaves at 0.25 μM CIP, a concentration that had little effect on the development of wild-type plants (Fig. 6, F and G). Altogether, these results showed that RECA3 is required for plant fitness in the presence of genotoxins inducing DSBs.

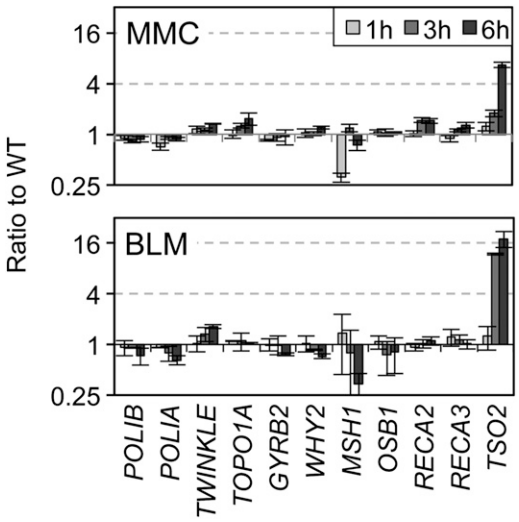

Figure 5. Expression of genes involved in mtDNA maintenance is not sensitive to treatments that induce genes involved in nuclear DNA repair. Wild-type (WT) seedlings were treated with 100 μM MMC or BLM as described in "Materials and Methods" during the times indicated. The relative expression of several genes known or predicted to be involved in mtDNA replication or recombination was compared between treated and untreated plants. Induction of the *TSO2* gene was tested as a positive control. Values are presented on a $\log_2$ scale. Genes are as follows: *POLIB*, At3g20540; *POLIA*, At1g50840; *TWINKLE*, At1g30680; *TOPO1*, At4g31210; *GYRB2*, At5g04130; *WHY2*, At1g71260; *MSH1*, At3g24320; *OSB1*, At1g47720; *RECA2*, At2g19490; *RECA3*, At3g10140; *TSO2*, At3g27060.

Repair of DNA Breaks Is Compromised in the Absence of RECA3

We showed that deficiency in *RECA3* per se does not affect mtDNA replication (Fig. 4F). Therefore, defects in mtDNA replication are not likely the cause of the increased sensitivity of *recA3* to genotoxins; probably inefficient mtDNA repair is the cause. To confirm this hypothesis, we set up a qPCR assay to test the recovery of mtDNA integrity after induction of DSBs. This assay is based upon the following rationale: the amount of DNA damage in a given template is directly proportional to its size and will cause the abortion of its PCR amplification. We focused our assay on BLM because it is the only agent that directly induces DNA breaks independently from replication, and we followed the recovery of DNA integrity after a pulse of BLM treatment. A protocol has been described previously where the amplification of sequences as big as 15 kb was compared (Hunter et al., 2010). However, in our hands,

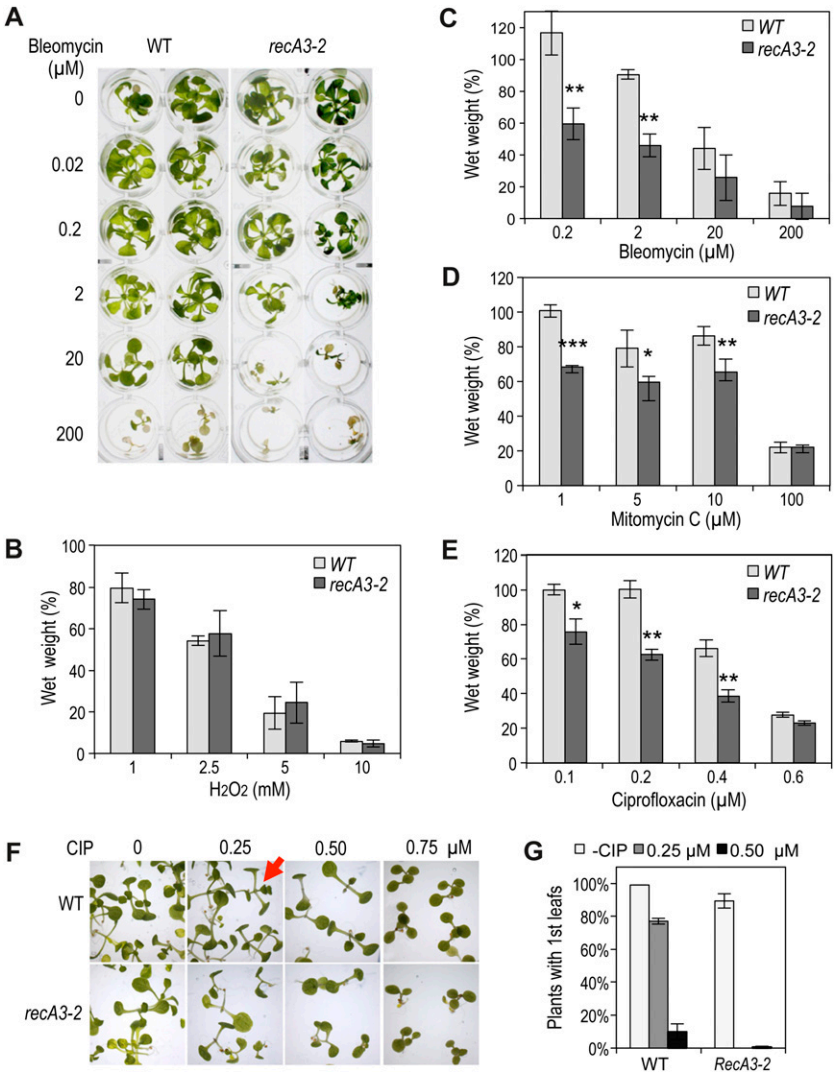

Figure 6. The *recA3* mutant is sensitive to genotoxic treatments. A, Image from a typical assay showing the enhanced sensitivity of *recA3-2* seedlings to BLM. B to E, Sensitivities of wild-type (WT) and *recA3-2* plants to different genotoxic treatments. Wet weights of seedlings grown under genotoxic conditions as described in "Materials and Methods" were compared with those of the untreated controls. Results were obtained from at least three independent experiments; SD values are indicated. Asterisks show the statistical significance (unpaired *t* test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) of differences between wild-type and *recA3* plants. F, Increased sensitivity of *recA3* plants to CIP. Plants were grown on agar plates containing increased concentrations of CIP. *recA3* plants did not develop normal first true leaves (arrow) at 0.25 μM CIP, which had little effect on wild-type seedlings. G, Percentage of plants developing first true leaves at 0.25 and 0.5 μM CIP. Error bars represent SD of two independent experiments. [See online article for color version of this figure.]

long-range PCR amplification of such big Arabidopsis mtDNA fragments was too inefficient to allow accurate and reproducible quantitative comparisons. Therefore, we adapted the protocol to the amplification of moderately long nuclear and mitochondrial DNA sequences (ranging in size between 513 and 680 bp): two nuclear and three mitochondrial sequences were quantified, and the results were standardized against two small (60 and 92 bp) nuclear DNA fragments that should be less affected by BLM treatment because of their smaller size (Hunter et al., 2010). In wild-type plants, the recovery of both nuclear DNA and mtDNA was found to be virtually complete 30 min after BLM treatment (Fig. 7). In the *recA3-2* mutant, the recovery of nuclear DNA was comparable, reflecting that RECA3 does not affect the repair of nuclear sequences. In contrast, the recovery of mtDNA was not complete, even 60 min after BLM treatment (Fig. 7). Thus, efficient repair of breaks in the mtDNA depends on RECA3, which can explain the reduced fitness of *recA3* plants under genotoxic growth conditions.

RECA3-Dependent Repair of DNA Breaks Induces Rearrangements of the mtDNA

HR-dependent repair can result in crossovers if homologous but distinct donor sequences are used to guide the repair of broken DNA. These crossovers can be reciprocal or nonreciprocal, depending on the HR mechanism involved (Aguilera and Gómez-González, 2008; Hastings et al., 2009; Maréchal and Brisson, 2010). We measured the increase in crossover products resulting from HR between IRs under genotoxic treatments (Fig. 8A). Crossovers involving repeats I, L, and X were analyzed as described above (Fig. 4). The growth of plants in the presence of BLM, which induces DSBs in somatic tissues, had negligible effects on the accumulation of crossover products, both on wild-type and *recA3-2* plants (Fig. 8A). On BLM-treated wild-type plants, the highest increases observed in crossover products were only 1.6- and 2.2-fold for products X-1/2 and X-2/1, respectively. This suggests that the effects induced by BLM on the mtDNA are

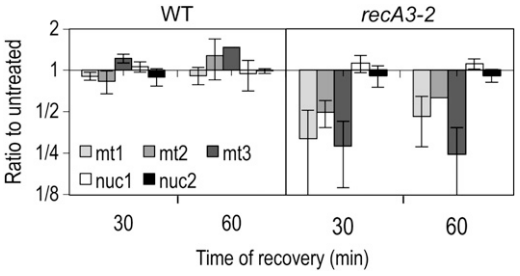

Figure 7. RECA3 is required for the efficient repair of breaks induced by BLM on the mtDNA. Wild-type (WT) and *recA3-2* seedlings were treated with BLM and allowed to recover for 30 or 60 min as described in "Materials and Methods." Total DNA was extracted and used for qPCR analysis of nuclear and mtDNA recovery. The relative amplification efficiencies of large amplicons (ranging in size between 513 and 660 bp) of nuclear (nuc1 and nuc2) and mitochondrial (mt1, mt2, and mt3) origin were compared between nontreated and treated seedlings. The results were normalized against two small amplicons arbitrarily considered 100% DNA integrity. Values are means of three independent experiments and are represented on a $\log_2$ scale.

repaired by faithful mechanisms that do not favor crossovers. However, wild-type plants grown in the presence of MMC or CIP exhibited an important accumulation of crossover products (Fig. 8A). Recombination appeared to be reciprocal for repeats I and X, with the accumulation of both reciprocal recombination products (I-1/2, I-2/1 and X-1/2, X-2/1). Asymmetrical recombination was seen for repeat L, with an increase solely in product L-1/2 in MMC-treated plants (41-fold) and CIP-treated plants (102-fold; Fig. 8A). In contrast, *recA3* seedlings exposed to genotoxic treatments showed no significant increase in the accumulation of recombination products when compared with untreated plants. This indicates that RECA3 is required for HR-dependent mtDNA repair pathways activated upon mtDNA damage and proceeding via IR-mediated recombination. Crossovers, which arise from the activity of these repair pathways, led to a concomitant increase in mtDNA heteroplasmy.

We verified by semiquantitative PCR that after CIP treatment and induction of HR-mediated mtDNA repair in wild-type plants, crossovers were increased for several other pairs of IRs in addition to those tested by qPCR (Fig. 8B). In some cases, crossover products were already abundant in untreated plants and no obvious effect of the CIP treatment could be detected (repeat pairs C and Q). On the other hand, for certain repeats, crossover products were virtually absent in untreated plants but their proliferation was clearly induced by the replicative repair of lesions induced by CIP (repeats P, T, and U). Recombination induced by CIP was reciprocal (repeats J, P, I, K, X, T, and V) or asymmetrical (repeats A, L, R, and U). The same analysis conducted on BLM-treated plants was mostly negative (data not shown), confirming the results obtained by qPCR for repeats I, L, and X.

Different mtDNA Rearrangements Are Seen in Mutants Affected in Recombination Surveillance as Compared with Plants under Genotoxic Stress

Recombination involving IRs has been associated with SSS of the Arabidopsis mtDNA in mutants *osb1* and *msh1* (Zaegel et al., 2006; Shedge et al., 2007; Arrieta-Montiel et al., 2009). In the case of *msh1*, it has been described that SSS mostly manifests as asymmetrical amplification of only one of the possible crossover products, which was proposed to result from BIR processes of DNA repair (Shedge et al., 2007; Arrieta-Montiel et al., 2009; Davila et al., 2011). Crossover products of IR-mediated recombination also accumulated to high levels in both *recA2* and *recA3*, but while for some repeats this event was asymmetrical (repeats

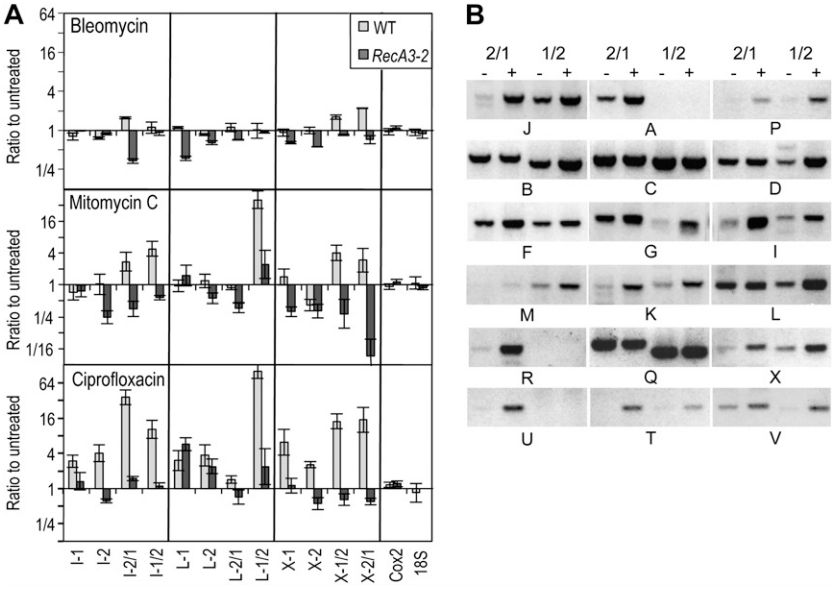

Figure 8. Repair of DSBs results in the accumulation of sequences deriving from recombination between IRs. A, Effects of different genotoxins on the accumulation of crossover recombination products. Wild-type (WT) and *recA3-2* seedlings treated for 7 d with 1 μM BLM, 75 μM MMC, or 0.75 μM CIP were analyzed by qPCR for the accumulation of crossover products from repeats I, L, and X, as described in Figure 4. qPCR results were normalized against the mitochondrial *cox2* and *rrn18* gene sequences. Results are represented on a $\log_2$ scale. B, Wild-type plants treated with 0.75 μM CIP (+) and the untreated control (−) were tested by semiquantitative PCR for the accumulation of crossover products (2/1 and 1/2) resulting from the recombination of pairs of perfect and imperfect repeats with sizes ranging between 175 and 694 (for primers and coordinates on the mtDNA, see Supplemental Table S3).

I and L; Fig. 4), other repeats showed an amplification of both reciprocal products (i.e. repeat X).

Remarkably, the products of IR-mediated recombination that accumulate in wild-type plants after the repair of DNA breaks induced by MMC or CIP are not the same as those accumulating as a consequence of the inactivation of *MSH1*, *OSB1*, or *RECA2* and *RECA3* in the recombination mutants. In the mutant background, we observed an asymmetrical accumulation of I-2/1, whereas in MMC- and CIP-treated plants, we found an accumulation of both I-1/2 and I-2/1. Most strikingly, while mutants showed an asymmetrical accumulation of mainly L-2/1, MMC- and CIP-treated plants displayed a sharp increase in L-1/2 but not in L-2/1. This suggests that the mechanisms of recombination mobilized for repair may not be entirely the same as the recombination events prevailing in recombination mutants.

Not all IRs are 100% identical; therefore, it is possible to determine which parental sequence has been used as template for mismatch copy correction of the heteroduplex by sequencing the crossover products amplified by PCR. If the invading donor strand is the one used as template, as has been proposed (Davila et al., 2011), then different mechanisms of recombination involving a different invading strand should result in crossover products with different sequences. We sequenced crossover products that accumulate in the *recA3* and *recA2* backgrounds as well as in plants in which mtDNA repair is induced after CIP treatment. For this analysis, we selected repeats I, R, and X (repeats L are identical). Comparisons of polymorphisms and insertions/deletions found in crossover products of these repeats showed that the DNA strands used as templates for copy correction were the same in the mutants as in CIP-treated wild-type plants (Supplemental Fig. S5). However, we found different outcomes according to the repeats analyzed. For repeats I, a different parental sequence is used as template for each one of the crossover products. For repeats X, a single parental sequence is used for both reciprocal crossover products. And both parental sequences are used as templates in recombination, leading to the single crossover product R-2/1 issued from the recombination of repeats R (Supplemental Fig. S5). Thus, it is possible that some repeats are mobilized by a single recombination pathway whereas recombination between others can result from multiple alternative pathways. This might depend on the position of the repeats in the mtDNA and the polarity of the replication fork progression.

Rearranged mtDNA Sequences Resulting from Recombination-Dependent Repair Can Be Transmitted to the Progeny

As shown above, HR repair of DSBs can generate mtDNA variants by crossovers involving IRs. To test if these sequences are transmitted, we analyzed the progeny of CIP-treated plants. Wild-type seedlings grown in the presence of 0.5 μM CIP that developed normal first leaves (10% of germinated seedlings; Fig. 6) were transferred onto nonselective medium for recovery (Fig. 9A). These plants developed extremely distorted leaves, similar to phenotypes observed in *osb1* mutants, which accumulate recombined mtDNA (Zaegel et al., 2006). After 3 weeks, individual plants (C1–C12) were transferred to soil and grown for seeds. Five-week-old plants still showed phenotypes of leaf distortion and variegation (Fig. 9A), but plants were fertile and their progeny grew indistinguishable from control plants. qPCR analysis confirmed that after CIP treatment, plants accumulated high levels of recombination product L-1/2 (about 45-fold). After 3 weeks of recovery, however, the amount of the same sequence in samples extracted from third true leaves was lowered, with a maximum of only a 9-fold increase in some plants as compared with untreated plants (Fig. 9B). This could be because recombination between the direct repeats L, which gives rise to L-1/2, resulted in the deletion of gene-bearing sequences located between the two repeats on the mtDNA. Subsequently during cell division, selection for intact, functional mtDNA could have led to the loss of recombined mtDNA molecules. Among the progeny of recovered plants (Fig. 9B), we observed either no apparent transmission of recombined mtDNA (progeny of plants C2 and C4) or, on the contrary, the segregation of plants retaining recombined mtDNA, with an up to 9-fold overrepresentation of sequence L-1/2 (progeny of plants C1 and C3). In contrast, an analysis of untreated control plants showed no significant variation in the copy number of L-1/2 under normal growth conditions. In conclusion, recombined mtDNA generated by repair mechanisms can be transmitted to the plant progeny and ultimately contribute to the evolution of the mtDNA structure.

DISCUSSION

Living organisms evolved multiple repair pathways to deal with the detrimental effects of DNA damage (for review, see Britt, 1999; Friedberg, 2003; Puchta, 2005; Jackson and Bartek, 2009). Of all types of DNA damage, among the most severe are DSBs, which can arise from exposure to chemical agents, oxidation, ionizing radiation, or when replication forks run into single-strand breaks. Repair of DSBs is essential for genome stability because they can lead to collapsed replication forks, gene loss, and cell death (Pâques and Haber, 1999). Little is known about mtDNA repair pathways in plants, although a short-patch base excision repair pathway and a microhomology-mediated BIR pathway have been shown to exist (Boesch et al., 2009; Cappadocia et al., 2010). The latter is an error-prone pathway of postreplicative DSB repair that can account for the complex rearrangements often observed in plant mitochondrial genomes (Woloszynska, 2010). However, faithful repair of DSBs requires HR-

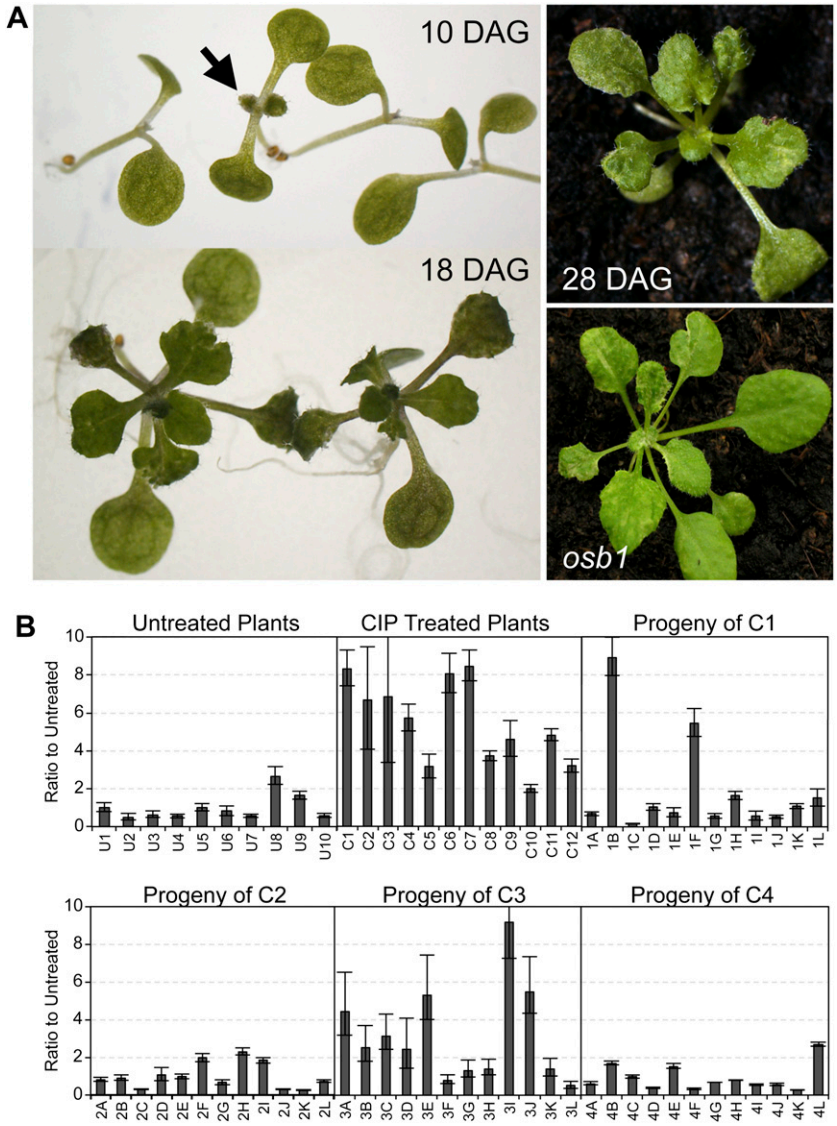

Figure 9. Products of HR-dependent repair of mtDNA breaks can be transmitted to the plant progeny. Wild-type plants were grown for 10 d at a sublethal concentration of CIP (0.5 μM), and the few plants able to develop normal first leaves (arrow) were recovered on genotoxin-free medium before transfer to soil. The presence of crossover product L-2/1 that accumulates after RECA3-dependent repair was quantified in individual plants and in their progeny. A, Phenotypes at 10, 18, and 28 d after germination (DAG) of recovered plants. Phenotypes of distorted leaves presenting variegated areas are similar to those found in *osb1* mutants. B, Relative quantification of recombination product L-1/2 in individual untreated control plants (plants U1–U10), recovered CIP-treated plants (C1–C12), and the progeny of plants C1, C2, C3, and C4. The results were compared with plant U1.

dependent pathways involving recombinases such as the bacteria-type RecAs found in plant organelles.

Overlapping and Distinct Roles of RECA2 and RECA3 in Arabidopsis Mitochondria

The presence of two types of RecA in mitochondria of flowering plants raises the question of whether these proteins evolved to carry out distinct functions in mtDNA maintenance. Divergent molecular roles of RECA2 and RECA3 are supported by (1) the different capacities of recombinant RECA2 and RECA3 to complement RecA-deficient *E. coli* (Fig. 3), (2) the highly overlapping *RECA2* and *RECA3* expression patterns (Fig. 2), and (3) the different effects on plant development that we observed for *recA2* and *recA3* mutations. RECA2 but not RECA3 function is essential for plant development beyond the seedling stage. As we were unable to obtain *recA2 recA3* double mutant seedlings, RECA2 and RECA3 functions in mitochondria may in part be redundant.

Disruption of *RECA2* had no effect on mtDNA replication but induced major reshuffling of the mtDNA by recombination, which exceeded the mtDNA rearrangements seen in *recA3*. Major mtDNA reshuffling, which almost certainly modifies the mitochondrial transcriptome, is probably the cause of the severe *recA2* phenotype. Although a qRT-PCR analysis of the mitochondrial transcriptome revealed no changes in mitochondrial mRNA and rRNA levels in *recA2*, detrimental transcript alterations are feasible that remained undetected. For example, specific mtDNA rearrangement may have resulted in splicing defects or the high expression of toxic chimeric sequences.

Because RECA2 is dual targeted to mitochondria and chloroplasts (Shedge et al., 2007), it could be argued that essential functions of RECA2 relate to its role in the photosynthetic organelle. However, two

lines of evidence suggest that RECA2 disruption does not deleteriously affect chloroplast function at the seedling stage: (1) *recA2* seedlings were dark green and showed no apparent deficiency in photosynthesis, and (2) the *recA2* mutation had no apparent effect on cpDNA copy number and stoichiometry. It is possible that RECA2 function in chloroplasts is important at a later developmental stage, which, due to the early arrest of *recA2* plants, we have been unable to explore.

RECA2 and RECA3 are both functional RecA proteins, as attested by the complementation of bacterial RecA. Interestingly, RECA2 but not RECA3 is able to partially complement bacterial RecA deficiency under UV-C irradiation, indicating that RECA2 performs additional roles that cannot be executed by RECA3. This could be because RECA2 but not RECA3 can functionally interact with other bacterial factors required for the repair of UV-C-induced lesions. Distinct molecular roles of RECA2 and RECA3 may in part be related to the absence of a C-terminal extension in RECA3 that is found in all other RecAs. In *E. coli*, the C-terminal domain was shown to modulate the ability of RecA to displace competing single-stranded DNA-binding proteins and to affect the pH dependence of RecA in the DNA strand-exchange reaction (Eggler et al., 2003; Lusetti et al., 2003). It is possible that in plant mitochondria, RECA2 and RECA3 interact in the formation of RecA-single-stranded DNA filaments. RECA3, which is not essential for plant viability, for instance, could act as a recombination mediator in the context of other factors involved in recombination. In agreement with suggested complementary roles of RECA2 and RECA3, deficiency in either results in reduced rejection of heterologous sequences in recombination involving IRs.

RECA3 Is Involved in Multiple Pathways of HR-Dependent Repair

mtDNA repair by HR-dependent processes has been proposed to drive the evolution of the mtDNA by recombination (Zaegel et al., 2006; Arrieta-Montiel et al., 2009; Maréchal and Brisson, 2010) and to be responsible for the copy correction of mismatches in heteroduplexes generated during recombination, thus contributing to the low nucleotide substitution rates of plant mitochondrial genomes (Davila et al., 2011). HR-dependent repair of DNA breaks can follow different pathways (for review, see Aguilera and Gómez-González, 2008; Hastings et al., 2009; Maréchal and Brisson, 2010). Current models of mtDNA repair propose BIR as the major pathway involved in the repair of DSBs and are mainly based on comparisons of the mtDNA structure between different Arabidopsis accessions or between wild-type plants and mutants affected in recombination (Shedge et al., 2007; Davila et al., 2011). However, these comparisons do not take in account that the structure of the mtDNA passed through the sieve of mtDNA segregation, which can result in the rejection of recombination intermediates that are either not replicated or that negatively affect mitochondrial gene expression. Therefore, models of HR pathways involved in mtDNA repair also have to be built in agreement with the outcomes observed under genotoxic treatments.

Our functional analysis of plant growth in the presence of different genotoxins was limited to RECA3-deficient plants as compared with wild-type plants, because the seedling lethality of *recA2* precluded any study beyond germination. Despite this limitation, the results obtained confirmed the existence of multiple pathways of HR-dependent repair in plant mitochondria and the involvement of RECA3 in these processes. Most importantly, the hypersensitivity of *recA3* plants to several genotoxic treatments further showed that RecA-dependent mtDNA repair significantly contributes to plant fitness under genotoxic stress. The effect of CIP on the growth of *recA3* plants is similar to the effects described for *polIb* plants, deficient in the organellar DNA polymerase proposed to have a preferential role in recombination-dependent repair (Parent et al., 2011). Contrary to genes involved in nuclear DNA repair, which are activated by pathways that perceive DSBs on the nuclear genome, we found no evidence for a similar transcriptional control of the expression of *RECA2*, *RECA3*, and other genes involved in mtDNA metabolism. This also contrasts with reports of an induction of the expression of the chloroplast RecA by genotoxic treatments in *Chlamydomonas*, *Physcomitrella*, and flowering plants (Cerutti et al., 1993; Nakazato et al., 2003; Inouye et al., 2008). Further work will be required to understand how plant mitochondria perceive DNA damage.

Several general pathways of DSB repair by recombination have been described and constitute a framework for a hypothesis to explain our experimental results (Aguilera and Gómez-González, 2008; Hastings et al., 2009; Maréchal and Brisson, 2010). Two-ended DSBs, such as the ones induced by BLM in somatic tissues, are apparently repaired by faithful repair pathways that avoid the accumulation of crossover products. This pathway is RECA3 dependent, because the recovery of mtDNA integrity is retarded in *recA3*. According to existing models, this pathway could either be the synthesis-dependent strand annealing or other Holliday junction resolution mechanisms that avoid crossovers. One-ended DSB resulting from collapsed replication forks (as provoked by CIP or by interstrand cross-links induced by MMC) should mostly be repaired by the BIR pathway that leads to nonreciprocal crossovers. Indeed, CIP and MMC treatments result in increased IR-mediated recombination, indicating involvement of the BIR pathway. It also requires RECA3, because the increase in crossover products is lost in the *recA3* background. According to the repeats analyzed, recombination is either asymmetrical or reciprocal, with accumulation of only one or both reciprocal recombination crossover products. In the context of the BIR pathway, the latter can result from independent repair events involving replication

forks progressing in opposite directions. However, the absence of information on the origins of replication in the Arabidopsis mtDNA does not permit us to draw further conclusions.

Although there is apparently a certain degree of redundancy in RECA2 and RECA3 functions, it is puzzling and counterintuitive that loss of a RecA results in increased recombination between IRs. It is possible that loss of either RECA2 or RECA3 does not abolish RecA-mediated recombination, maintained by the remaining one of the two. It would then be a correct balance in their expression that would allow their tight control on unwanted recombination events, leading to rejection of heteroduplexes that do not share extensive homology. A possible alternative explanation is that a reduction in the tightly controlled RecA-dependent recombination relaxes RecA-independent recombination pathways more tolerant to heteroduplexes. In bacteria, RecA-independent recombination is also augmented on the *recA*$^-$ background (Dutra et al., 2007). We were unable to test if IR-mediated recombination can be RecA independent, as we failed to retrieve *recA2 recA3* double mutants. But in *Physcomitrella*, disruption of the gene coding for the single mitochondrial RecA (ortholog of REC2) results in developmental and molecular phenotypes that mirror the ones observed in Arabidopsis: reduced cell growth, no reduction in mtDNA copy number, but increased recombination between short repeated sequences and accumulation of the resulting crossover products (Odahara et al., 2009). Therefore, a RecA-independent HR activity had to be postulated in *Physcomitrella* mitochondria. Our results about recombination involving direct repeat L also showed that the mechanisms of recombination that lead to the accumulation of crossover products in *recA2* and *recA3* can be different from the ones active in repair after the induction of DSBs (MMC and CIP treatments). Repeat L is found in the 3′ region of atp9 and upstream of cox3, and asymmetrical recombination involving this repeat in *recA2* and *recA3* leads to the accumulation of crossover product L-2/1. The same occurs in the mutants of surveillance genes *OSB1* and *MSH1*. But in plants treated with MMC or CIP, RECA3-dependent replicative repair only yields crossover product L-1/2. Therefore, certain events of recombination activated in these mutants are not the result of normal repair by recombination but more likely result from inefficient RecA-dependent recombination in the mutant background, together with a concomitant increase in RecA-independent recombination processes.

Without postulating the existence of an additional recombinase in plant mitochondria, mechanisms of RecA-independent HR could be those described in bacteria, which are replication slippage and single-strand annealing (Bzymek and Lovett, 2001; Hastings et al., 2009). In the case of direct repeats L, which are far apart in the mtDNA (61 kb), replication slippage is unlikely to be the reason for crossovers. But complementary single-stranded DNAs that result from the processing of DSBs can promote crossovers by single-strand annealing, in the same or between different mtDNA molecules. On the other hand, RecA-dependent repair by BIR can lead to the reciprocal crossover product, depending on the polarity of the replication fork progression on the mtDNA (Supplemental Fig. S6).

mtDNA Repair Can Result in Increased Heteroplasmy

We have shown that replicative repair, as induced by MMC or CIP, results in increased heteroplasmy of the mtDNA through IR-mediated recombination. From an evolutionary point of view, the heteroplasmy of plant mtDNA is an important reservoir of genetic variability that is available when required for the rapid evolution of the mtDNA (Shedge et al., 2010; Woloszynska, 2010). In the *osb1* and *msh1* mutants, it has been shown that the increased heteroplasmy observed in first-generation mutants can lead, in later mutant generations, to new major configurations of the mtDNA by SSS (Zaegel et al., 2006; Davila et al., 2011). Therefore, an increase in mtDNA heteroplasmy by repair can, in the long run, influence mtDNA evolution, provided that crossover products are transmitted. Indeed, our analysis of plants that recovered from massive mtDNA damage after CIP treatment showed that crossover products resulting from RECA3-dependent repair can be transmitted to the progeny, at some levels comparable to those found in the recovered parental plants. Thus, RecA-dependent repair has a dual effect on the mtDNA: maintaining the integrity of the organellar genome that is important for plant fitness after stress, but also favoring the amplification of genome configurations that could be advantageous in the adaptation of plants to environmental changes.

MATERIALS AND METHODS

Phylogenetic Analysis

Bacterial and plant RecA sequences were identified in the databases by BLASTP and TBLASTN analyses with bacterial and Arabidopsis (*Arabidopsis thaliana*) RecA sequences as queries. An alignment was constructed with ClustalW implemented in the Macvector package using the GonneT matrix. Phylogenetic trees were built with PhyML online software (www.phylogeny.fr; Dereeper et al., 2008). Subcellular targeting was predicted with the TargetP and Predotar algorithms (http://urgi.versailles.inra.fr/predotar/predotar.html and http://www.cbs.dtu.dk/services/TargetP; Small et al., 2004; Emanuelsson et al., 2007).

Escherichia coli Complementation Assays

The *E. coli* XL1-Blue strain was used as the host for routine cloning and strain JM103 (*recA*$^+$ and *recA*$^-$) was used for the complementation analyses. Complementation constructs were prepared as follows. The expression cassette of vector *pUCAP* (nucleotides 1,932–2,601) was cloned into the *Bam*HI site of the low-copy-number plasmid *pACYC184* (Chang and Cohen, 1978) to obtain vector *pACYClacz*. The RECA2 and RECA3 cDNA sequences without predicted targeting sequences (codons 33-431 and 29-389, respectively; Supplemental Fig. S1) were cloned into the *Bam*HI and *Sac*I sites of *pACYClacz*, respectively. For complementation tests, 10 mL of Luria-Bertani medium plus 1% (w/v) Glc was

inoculated with 50 μL of overnight culture and grown at 37°C for 3 h. IPTG (0.5 mM) was added, and growth was allowed to continue for 2 h. Induction of the recombinant proteins was monitored with a polyclonal antibody directed against RECA3 that also recognizes RECA2. For UV-C assays, appropriate dilutions of cells were spread on Luria-Bertani plates in triplicate and exposed to different UV-C doses before incubation overnight at 37°C. For HU and MMC assays, bacteria were harvested, resuspended in 10 mM $MgSO_4$, and treated with the genotoxic agents for 30 min at 37°C. Appropriate dilutions were spread on Luria-Bertani plates in triplicate and incubated overnight at 37°C. The colony-forming units were counted, and the surviving fraction was calculated using the method described by Pang et al. (1992). The values presented are means from three independent experiments. Recombinant RECA3 (codon 29-389) was produced in *E. coli* using expression vector pBAD/thio-TOPO (Invitrogen), purified in denaturing conditions in the presence of 7 M urea, and used to raise a rabbit antiserum. Western blots (antiserum at 1:5,000 dilution) showed that both RECA2 and RECA3 expressed in *E. coli* are recognized by the antiserum and expressed at equivalent levels.

Characterization of Arabidopsis Insertion Mutants

T-DNA insertion mutant lines (*recA2-1* [SALK_076237], *recA2-2* [SALK_068655], *recA2-3* [SALK_118143], *recA2-4* [SALK_045420], *recA3-1* [SAIL_252_C06], *recA3-2* [SALK_146388], and *recA3-3* [GK-288B01]), all in the Col-0 background, were obtained from the Nottingham Arabidopsis Stock Centre. Seeds were stratified at 4°C for 3 d, and plants were grown on soil or on MS255 medium (Duchefa) agar plates supplemented with 0.5% (w/v) Suc, at 22°C, under a 16-h-light/8-h-dark photoperiod. Genotyping was performed by PCR using gene- and T-DNA-specific primers. Insertion sites were confirmed by sequencing PCR products with the T-DNA left border-specific primer. Genomic DNA was extracted by the cetyltrimethylammonium bromide method (Murray and Thompson, 1980).

Mutagen Assays on Arabidopsis Plants

Seedlings were grown for 7 d on agar plates in nonselective medium, to avoid possible undesired germination or developmental effects, until the development of first true leaves. They were then transferred onto 12-well plates containing MS255 liquid medium, and the next day appropriate concentrations of genotoxic agents were added. The seedlings were further incubated for 7 d before analysis. Wet weights were determined and plotted against mutagen concentrations. The values presented are means from at least three independent experiments. The determination of mutagen effects on mtDNA copy number and recombination was performed for plantlets treated with 75 μM MMC, 1 μM BLM, or 0.75 μM CIP, as described above (five seedlings per well). Values correspond to the means from three independent experiments. Statistical significance (nontreated versus treated and mutant versus wild type) was assessed using unpaired *t* tests and Prism software (GraphPad). To monitor DNA recovery after BLM treatment, seedlings were transferred into 2 mL of liquid MS255 plus 10 μM BLM (15 seedlings per well). After 30 min of treatment with agitation, medium was removed and plantlets were washed three times with fresh medium without mutagen. Plantlets were allowed to recover and harvested at the indicated times. Total DNA was extracted, and qPCR analysis was as described below.

qPCR Analysis

qPCR experiments were performed in a LightCycler480 (Roche) in a total volume of 6 μL containing 0.5 μM of each specific primer and 1× LightCycler480 SYBR Green I Master Mix (Roche). Primer pairs were designed using the Universal Probe Library Assay Design Program (Roche). The thermocycling program was as follows: a 7-min denaturing step at 95°C followed by 45 cycles of 10 s at 95°C, 15 s at 60°C, and 15 to 40 s at 72°C. The second derivative maximum method was used to determine cycle threshold values, and qPCR efficiencies for each primer pair were determined from DNA serial dilution curves. For quantification of mtDNA copy numbers, qPCR results were normalized against the *ACT1* (At2G37620) and *UBQ10* (At4G05320) nuclear genes. To measure DNA recovery after BLM treatment, results obtained for nuclear and mitochondrial medium-size amplicons (513–660 bp) were normalized against the amplification of small nuclear sequences (60 and 92 bp) arbitrarily considered as 100% DNA integrity (corresponding to single-copy nuclear genes *ACT1* and *UBQ10* [At5G26710 and At1G25350, respectively]). mt1, mt2, and mt3 correspond to three mitochondrial unique sequences, coordinates 6,065:6,578, 345,127:345,725, and 36,296:36,955 in the published Col-0 mtDNA sequence. nuc1 and nuc2 correspond to genes At5g26710 (*GLUR5*) and At1g25350 (*GLNR5*), respectively. The results correspond to means of three independent biological replicates, and for each one three technical replicates were performed. The accumulation of mtDNA recombination products was assessed using primers flanking each repeat as depicted in Figure 4C. The mitochondrial *COX2* (AtMG00160) and *18S rRNA* (AtMG01390) single-copy sequences were used for normalization. Transcriptome analysis of mtDNA expression by qRT-PCR was performed as described (Kühn et al., 2011). Primer sequences are given in Supplemental Table S3.

Promoter-GUS Fusion Analysis

The 5′ upstream regions of *RECA2* (1,301 bp, comprising the 3′ upstream region and 206 bp of the first exon) and of *RECA3* (1,850 bp, comprising 79 bp of the first exon) were cloned in the binary vector pMDC162 upstream of the GUS gene. Transgenic Arabidopsis plants were produced, and tissues from promoter-GUS fusion plants from six independent lines were stained with 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (Biosynth) and observed on transmission and stereo microscopes.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Alignment of representative bacterial and plant RecA sequences.

Supplemental Figure S2. Structure of the Arabidopsis *RECA2* and *RECA3* genes.

Supplemental Figure S3. Steady-state mitochondrial transcript levels in *recA2* mutants.

Supplemental Figure S4. Sensitivity to genotoxins of two independent *recA3* lines.

Supplemental Figure S5. Copy correction in crossover products.

Supplemental Figure S6. Possible models of recombination involving repeat L.

Supplemental Table S1. Accessions of sequences used in phylogenetic analysis.

Supplemental Table S2. Segregation analysis.

Supplemental Table S3. Primer sequences.

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