

Two closely related RecQ helicases have antagonistic roles in homologous recombination and DNA repair in *Arabidopsis thaliana*

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Edited by Nina Fedoroff, U.S. Department of State, Washington, DC, and approved October 4, 2007 (received for review June 26, 2007)

RecQ helicases are involved in the processing of DNA structures arising during replication, recombination, and repair throughout all kingdoms of life. Mutations of different RecQ homologues are responsible for severe human diseases, such as Blooms (BLM) or Werner (WRN) syndrome. The loss of RecQ function is often accompanied by hyperrecombination caused by a lack of crossover suppression. In the *Arabidopsis* genome seven different RecQ genes are present. Two of them (*AtRECQ4A* and *4B*) arose because of a recent duplication and are still nearly 70% identical on a protein level. Knockout of these genes leads to antagonistic phenotypes: the *RECQ4A* mutant shows sensitivity to DNA-damaging agents, enhanced homologous recombination (HR) and lethality in a *mus81* background. Moreover, mutation of *RECQ4A* partially suppresses the lethal phenotype of an *AtTOP3 α* mutant, a phenomenon that had previously been demonstrated for RecQ homologues of unicellular eukaryotes only. Together, these facts strongly suggest that in plants *RECQ4A* is functionally equivalent to *SGS1* of *Saccharomyces cerevisiae* and the mammalian BLM protein. In stark contrast, mutants of the closely related *RECQ4B* are not mutagen-sensitive, not viable in a *mus81* background, and unable to suppress the induced lethality caused by loss of *TOP3 α* . Moreover, they are strongly impaired in HR. Thus, *AtRECQ4B* is specifically required to promote but not to suppress crossovers, a role in which it differs from all eukaryotic RecQ homologues known.

Blooms syndrome | crossover suppression | Holliday Junction |
Mus81 | topoisomerase

Genes coding for RecQ helicases are present in all pro- and eukaryotes investigated. The data available so far from completed genome sequences indicate that the number of RecQ genes rises from organisms with low complexity to those of higher complexity. Single RecQ genes are present in *Escherichia coli* and *Saccharomyces cerevisiae*, and five to eight RecQ genes are found in mammals and plants, respectively (1, 2). Therefore, the function of RecQ helicases seems to have adapted to the complexity of genomes present in higher eukaryotes by increasing their number. Sequence duplications were followed by subsequent differentiation of known functions or possible acquisition of new ones. In most cases knockout of RecQ genes results in a hyperrecombination phenotype in various organisms, such as bacteria, yeasts, mammals, or plants (3–6). Mutations in the *BLM*, *WRN*, and *RECQ4* genes are the cause of severe diseases, such as Blooms, Werner, and Rothmund–Thomson syndromes, respectively. Furthermore, a mutant of the mammalian *RECQ5* gene shows a synergistic increase of sister chromatid exchange in a *blm* background (7, 8).

A prominent function of several RecQ helicases is the processing of double-holliday junctions (dHJs) that occur as intermediates during replication, DNA repair, or recombination and dissolve them in a manner which prevents deleterious crossover recombination (9–11). The respective RecQ homologue (e.g., BLM, SGS1, or RQH1) acts in concert with topoisomerase 3 (TOP3) in unicellular organisms, or TOP3 α in multicellular eukaryotes (6, 12–17). As a third member of the complex, the

RMI1 protein was characterized in mammals and yeast (10, 18, 19). Moreover, in the lower eukaryotes *S. cerevisiae* and *Schizosaccharomyces pombe* deletion of their respective RecQ homologue leads to partial suppression of the severe phenotypes caused by mutation of the TOP3 gene (14, 20–22).

Several RecQ helicase mutants are synthetically lethal in a combination with mutations in the endonuclease MUS81 (9, 23, 24). This inviability of the double mutants is most probably because both proteins act in parallel pathways resolving aberrant DNA structures arising during replication. When both genes are mutated, these structures accumulate, leading to cell death (9, 23).

Comparing the complete genome sequences of four different plants (*Arabidopsis thaliana*, *Oryza sativa*, *Physcomitrella patens*, and *Populus trichocarpa*), all in all, eight different RecQ-like gene types can be classified, seven of which are present in *Arabidopsis* (reviewed in ref. 1). So far, only one of these genes has been functionally characterized in planta: *AtRECQ4A* (3, 25). T-DNA insertion mutants were sensitive to DNA-damaging agents and showed an enhanced frequency of homologous recombination (HR) by using an assay specific for crossover events. Furthermore, expression of *AtRECQ4A* in yeast resulted in full suppression of the *sgs1* mutant phenotype (3). Recently, we demonstrated that the *recq4A* mutant in combination with the *mus81* mutation is lethal in *Arabidopsis* as has been shown for *blm* in *Drosophila melanogaster*, *sgs1* in *S. cerevisiae*, and *rph1* in *S. pombe* (9, 23–25). Interestingly, *AtRECQ4A* is one member of a recently duplicated gene pair in *A. thaliana* (26). The second ORF, *AtRECQ4B*, is nearly 70% identical to *4A* on the amino acid level and shows most differences outside of the known domains of RecQ proteins. The aim of our present analysis was to elucidate the biological function of both genes, not only in regard to their role in HR, but also in respect to their genetic interactions with *MUS81* and *TOP3 α* .

Results

Sensitivity to Genotoxic Agents. To elucidate the biological role of *AtRECQ4B* we characterized two different T-DNA insertion lines of *AtRECQ4B* (*At1g60930*): *recq4B-1* (*GABL399C04*) and *recq4B-2* (*SALK_011357*) (27, 28). The sequences of the insertion sites were determined: the T-DNA of line 1 is inserted in the 8th intron (of a total of 24) with a genomic deletion of 51 bp in this area (Fig. 1 *B* and *D*). The T-DNA of line 2 is inserted in the 10th intron, accompanied by a genomic deletion of 20 bp (Fig. 1 *B* and *D*). Furthermore, we isolated a T-DNA insertion line (*GABL203C07*) of *AtRECQ4A* (*At1g10930*) to compare mu-

Author contributions: F.H. and H.P. designed research; F.H. and S.S. performed research and analyzed data; and F.H. and H.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0705998104/DC1.

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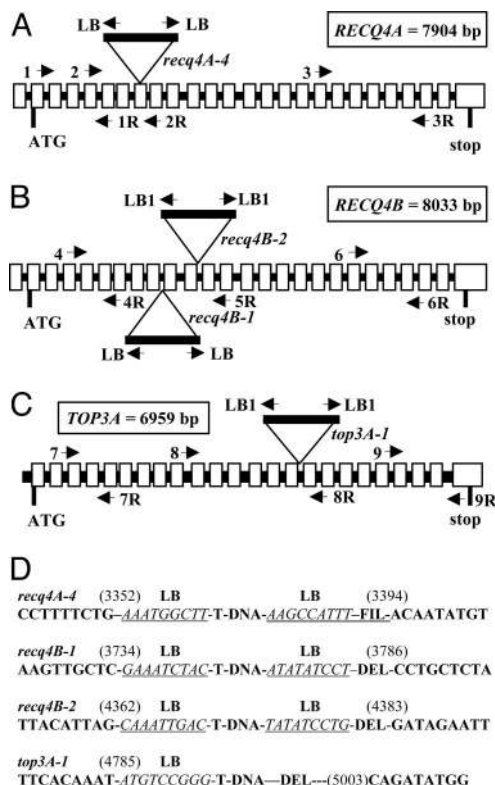


Fig. 1. Molecular analysis of the T-DNA insertion lines. (A–C) The respective location of the T-DNA insertion in lines *recq4A-4*, *recq4B-1* and *2*, and *top3 α -1* is depicted. The schematically drawn genes of *RECQ4A* and *RECQ4B* contain 25 exons (gray boxes) and 24 introns (black lines) in the coding region and one additional exon and intron in the 5' UTR, each. Both genes are interrupted in their helicase domains by the respective T-DNAs. The gene of *TOP3 α* (*At5g63920*) contains 24 exons vs. 23 introns and the T-DNA is inserted in the 15th intron. Primers used are indicated by arrows. (D) Border sequence analysis of the insertion loci. The genomic sequences adjacent to each T-DNA insertion locus were determined by PCR and are depicted. In the case of *top3 α -1* on the right side, a deletion of ≈ 200 bp occurred. The genomic sequences are shown in bold, the respective left border sequence is in italics and underlined. FIL, filler sequence; DEL, deletion.

tants of both genes in the same ecotype. The T-DNA is inserted in the 7th exon (of 25 total exons), accompanied by a deletion of 40 bp of exon 7 sequence and an insertion of 19 bp of an ectopic filler sequence (Fig. 1 *A* and *D*). To distinguish it from the lines (3) published already, we refer to this line as *Atrecq4A-4*. All three lines described above harbored a tail-to-tail insertion of T-DNA with an LB on each end (Fig. 1 *A* and *B*). By RT-PCR full-length mRNA spanning the insertion sites could not be detected in any of the mutants (see [supporting information \(SI\) Fig. 5](#) and [SI Table 1](#) for details).

To analyze the mutagen sensitivity, we used a liquid medium assay and determined the weight of 3-week-old plantlets challenged by different concentrations of DNA-damaging agents. Whereas all mutants were as resistant as the wild type to mitomycin C and bleomycin (data not shown), *Atrecq4A-4* was sensitive to methylmethanesulfonate (MMS) (3), and *cis*-platin (Fig. 2; [SI Table 2](#)). We could not detect any mutagen sensitivity at all in both *Atrecq4B* lines (Fig. 2). Additionally, we crossed *recq4A-4* and *recq4B-2* to characterize the phenotype of the double mutant. *Recq4A-4/4B-2* exhibited a mutagen-sensitive phenotype that was indistinguishable from that of the single *recq4A-4* mutant (Fig. 2). This demonstrates that *AtRECQ4B*, in contrast to *AtRECQ4A*, is not required for the repair of DNA damage induced by the applied mutagens.

Homologous Recombination. As a hallmark, mutants of eukaryotic RecQ helicases, such as WRN, BLM, or SGS1, show enhanced sister chromatid exchange (SCE) frequencies (6, 29–33). SCEs are a specific type of HR events based on crossovers between sister chromatids. To analyze the role of *RECQ4A* and *RECQ4B* in HR, we used the two different recombination substrates, 651 (34) and IC9C (35). Both lines harbor a transgene with nonfunctional overlapping parts of a β -glucuronidase gene (*GUS*) in an inverted but different spatial orientation (Fig. 3). Restoration of the marker is possible by intra- and interchromosomal recombination in the 651 line (36), whereas in the case of IC9C interchromosomal recombination by using the sister chromatid or the homologous chromosome is required (35). It is important to note that, in the case of line 651, a crossover is necessary to restore the function of the β -glucuronidase marker, whereas, in the case of IC9C, besides crossover (CO), gene conversion can also restore the marker. Each recombination event is represented visually as a blue sector on the plant. We determined the HR frequencies with and without induction by bleomycin. Whereas the application of bleomycin induces genomic double-strand breaks (DSBs), spontaneous recombination might be initiated by replication-associated DNA intermediates (like collapsed replication forks) that differ from classical DSBs. The HR frequencies of the reporter lines 651 (as described in ref. 3) and IC9C were enhanced in the *recq4A-4* background (3- to 7-fold, respectively; Fig. 3). However, after induction of DSBs, such an enhancement in comparison with the wild type could not be detected anymore.

In contrast, the HR level was significantly reduced in the *recq4B* lines in both substrate backgrounds with and without induction by using bleomycin (2- to 5-fold in line 651 and 2- to 4-fold in IC9C, respectively; Fig. 3). The double mutant *recq4A-4/recq4B-2* showed an intermediate phenotype of HR for both substrate lines with and without bleomycin treatment (Fig. 3; [SI Table 3](#)). This intermediate phenotype indicates that *RECQ4A* and *4B* are involved in different recombination pathways. HR, which is enhanced because of the loss of the *RECQ4A* gene, is clearly reduced in the double mutant but not to the same extent as in the single *recq4B* lines. This can be taken as a hint that a second HR pathway, independent of *RECQ4B* and possibly promoted by one of the other plant RecQ helicases, might be enhanced by the loss of *RECQ4A* and is responsible for the remaining events.

Genetic Interactions with *AtMUS81* and *AtTOP3 α* . To detect a possible genetic interaction between *AtRECQ4B* and *AtMUS81* we crossed *Atrecq4B-2* with *Atmus81-1*. In stark contrast to *recq4A-4*, the *recq4B-2/mus81-1* double mutant developed like the wild type, was fertile and exhibited the same mutagen-sensitive phenotype as the *mus81* single mutant (data not shown) (25). Thus, it is obvious that *RECQ4B* performs a role not only different from *RECQ4A* in DNA repair and recombination, but also in respect to the resolution of aberrant replication intermediates.

Because an intimate interaction of specific RecQ helicases with *TOP3* or *TOP3 α* has been demonstrated in several eukaryotes, we tested whether *RECQ4A* and/or *4B* participate in such an interaction as well. For our study we used an *Attop3 α -1* mutant (SALK_139357, *At5g63920*) that harbors a T-DNA insertion in its 15th intron leading to a knockout of mRNA expression spanning the insertion site, whereas the mRNA level in front and behind the T-DNA remained unchanged (see [SI Fig. 5](#)). One border sequence of the T-DNA (the left border) was present, whereas the right border, together with 218 bp of the gene, were deleted in the mutant (Fig. 1 *C* and *D*). *Attop3 α -1* was severely impaired in growth and barely germinated on growth medium or in soil; the mutants had deformed cotyledons and were not able to form roots at all. In Fig. 4*A* the development of a *top3 α -1* seedling on an agar plate is shown in comparison with

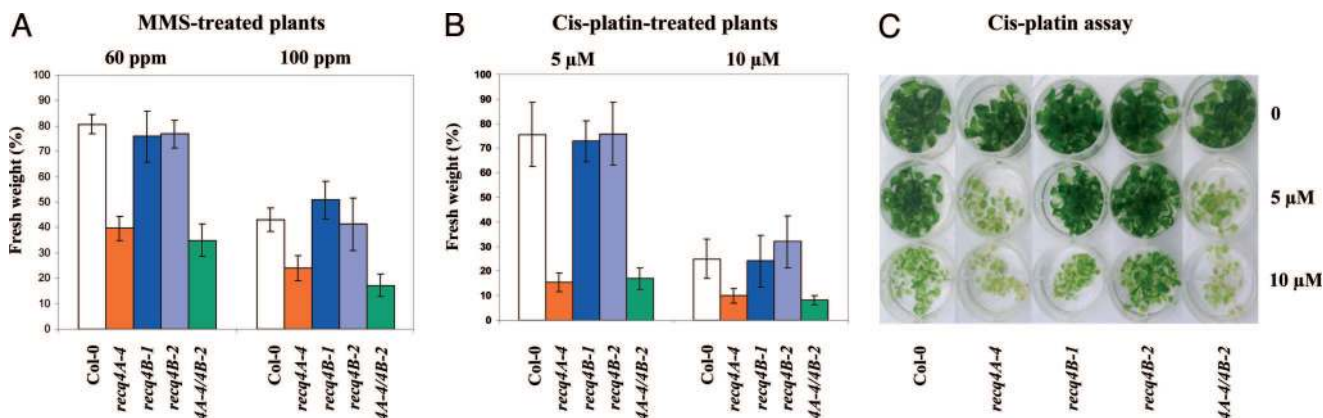


Fig. 2. Mutagen sensitivity assays with MMS or *cis*-platin. (A and B) Fresh weight of 10 plantlets at each mutagen concentration was determined and put into relation to the fresh weight of the untreated plantlets of the same line. The percentages were calculated from the relation of fresh weight at a given mutagen concentration to the fresh weight without mutagens. Each assay was performed at least four times as described, and the mean values including standard deviations are depicted (the detailed data are given in [SI Table 2](#)). *recq4A-4* and the double mutant *recq4A-4/4B-2* are sensitive to MMS and *cis*-platin, whereas both *recq4B* lines behave like the wild type. (C) A six-well assay plate showing a typical *cis*-platin assay revealing the enhanced sensitivity of *recq4A-4* to this cross-linking agent. ppm, parts per million.

a heterozygous *top3α-1* seedling. Yet, after 14 days only malformed cotyledons can be seen, whereas heterozygous seedlings had already developed four genuine leaves. The *top3α-1* seedlings never reached larger sizes and slowly died. Seedlings

heterozygous for the insertion developed like the wild type, showing no growth impairment and full fertility ([Fig. 4A Upper](#)). Thus, the mutant exhibited a phenotype similar to the dramatic *top3α* phenotypes reported for other eukaryotes. Lethality was

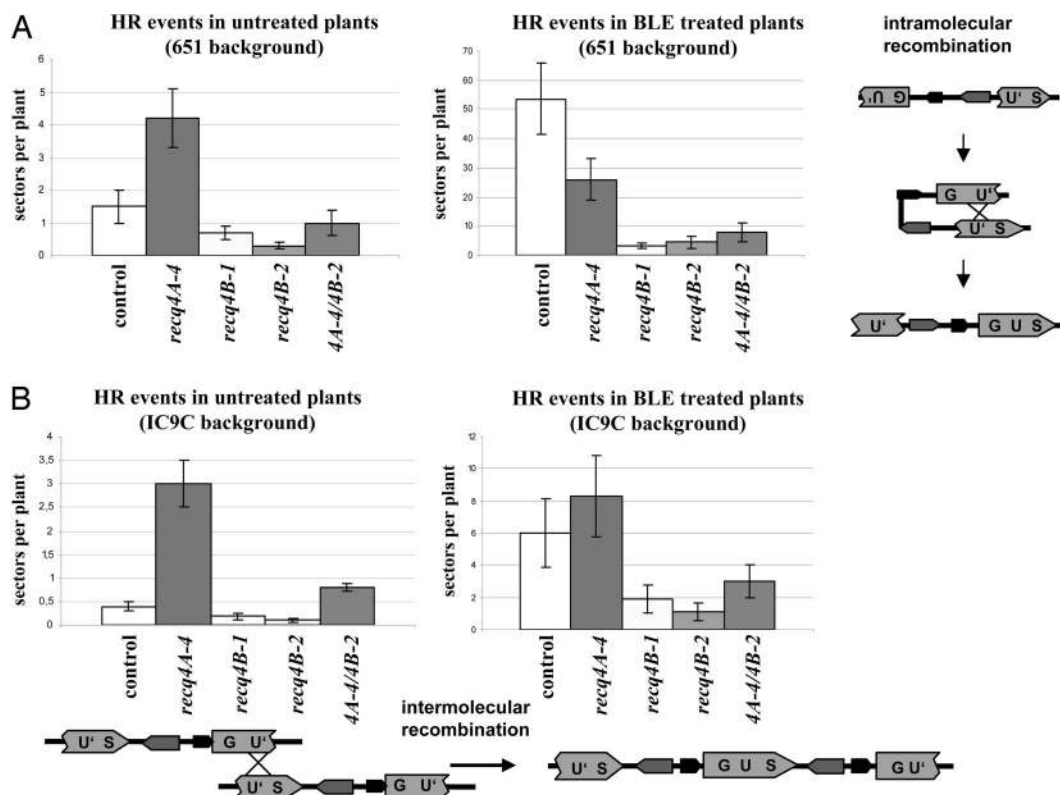


Fig. 3. Recombination frequencies of untreated and bleomycin-treated *recq4A* and *4B* insertion lines. (A) Mean value of at least five independent CO assays with use of the 651 background line that is capable of restoring the functional GUS gene either by intra- or intermolecular recombination (only the intramolecular mechanism of CO is shown schematically right of the diagram). *recq4A-4* shows a 3-fold enhanced CO basic level and is poorly induced by bleomycin, whereas both *recq4B* lines have a 2- to 5-fold reduced basic level. The double mutant exhibits an intermediate phenotype. (B) Mean value of at least five independent HR assays with use of the IC9C background line (the mechanism of HR is restricted to an intermolecular reaction as shown schematically below the diagram). *recq4A-4* shows a 7-fold enhanced HR level and is poorly induced by bleomycin, whereas both *recq4B* lines possess a 2- to 4-fold reduced basic level. The basic and induced HR levels of the double mutant *recq4A-4/4B-2* are intermediate compared with the single lines. Because of the differing number of blue sectors per plant, the scales of the x axes vary. In the IC9C substrate line, much less HR occurs because only HR events using the sister chromatid or homologue as template lead to the restoration of the marker. The detailed data including the HR induction factor are given in [SI Table 3](#).

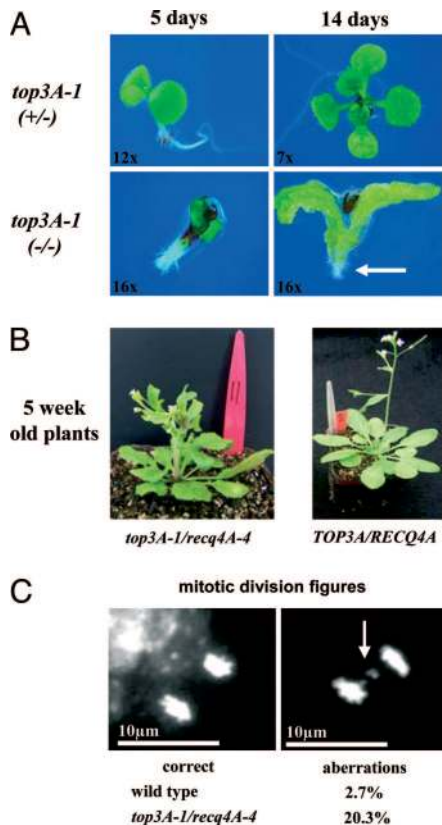


Fig. 4. Phenotype of the *top3 α -1* mutant and the double mutant *top3 α -1 recq4A-4*. (A) Typical example of a homozygous *top3 α -1* seedling on agar plates shown at days 5 and 14 after germination. In comparison, a normally growing heterozygous seedling is shown above. The homozygous mutant developed neither a root (white arrow) nor genuine leaves, and even the cotyledons were malformed. (B) Typical example of a viable, double homozygous mutant plant (*top3 α -1/recq4A-4*) in soil at 5 weeks of age. The plant shows adnated organs, demonstrates slower and dwarfed growth, and is finally sterile. (C) Examples of accurate (Left) and aberrant (Right) mitotic divisions in the double mutant (chromosome fragments are indicated by an arrow). In total, 256 mitotic figures of the control plant (Col-0) and 237 of the *top3 α -1/recq4A-4* were analyzed, detecting 7 vs. 48 aberrant mitotic divisions, respectively.

observed in mice and pleiotropic growth defects in *Caenorhabditis elegans* *TOP3 α* mutants (12, 13).

Crossing the heterozygous *top3 α -1* plants with *recq4A-4* or *recq4B-2* resulted in strikingly different homozygous double mutants. Whereas the double mutant *recq4B-2/top3 α -1* clearly reproduced the lethal *top3 α -1* phenotype (data not shown), the *recq4A-4/top3 α -1* double mutant was viable and proliferated, demonstrating that *recq4A* possesses in *Arabidopsis* a suppressor function similar to the one of *sgs1/rph1* in yeast. However, the mutation was not able to fully suppress the *top3 α* phenotype, as the *recq4A-4/top3 α -1* plants show several abnormalities, such as adnated organs and dwarfism (Fig. 4B Left) that finally result in sterility. Furthermore, the double mutant showed a very high level of mitotic distortions as determined by DAPI staining (Fig. 4C). In total, 256 mitotic divisions of a Col-0 wild-type plant and 237 of a *top3A-1/recq4A-4* double mutant were analyzed. Col-0 possessed a level of 2.7% mitotic aberrations (7 of 256), whereas the double mutant exhibited 20.3% (48 of 237). We assume that these mitotic aberrations led to the observed growth defects of the *top3A-1/recq4A-4* plants.

Discussion

AtRECQ4A: A Functional Homologue of SGS1 and BLM. Seven different RecQ-like genes are present in the *Arabidopsis* genome and the

question arises about which of the RecQs can be classified as the respective orthologues of human RecQs or SGS1 in yeast. One important aspect in the functional classification of RecQ homologues is whether their mutations induce defects in genome stability. The T-DNA mutant line *recq4A-4* was sensitive to MMS and *cis*-platin and showed an enhanced HR level in both assay systems tested. This finding is in accordance with a role of RECQ4A in homology-based repair of DNA damage and can be found in RecQ mutants of other organisms, too (5, 29). Because *cis*-platin induces mostly *intrastrand* cross-links (CLs) and MMC induces mainly *interstrand* CLs, the lack of sensitivity to MMC might be taken as an indication that RECQ4A is involved specifically in repair events in which only one of the two DNA strands is blocked. This is in line with the “chicken foot” model, which has been put forward to explain the role of RecQ helicases during replicational repair (37). The fact that, in the absence of AtRECQ4A, the increase in HR was higher with the recombination substrate IC9C, which specifically detects interchromosomal and interchromatid recombination, is reminiscent of BLM cells that have enhanced numbers of SCEs (6, 29, 30, 32, 38), although we cannot formally exclude that the difference from line 651 is due to a position effect. We could not address SCE directly, because, due to its small chromosome sizes, SCE analysis in *Arabidopsis* was not set up. Interestingly, HR frequencies are not enhanced in the *recq4A-4* background after treatment with bleomycin, indicating that RECQ4A is mainly involved in the resolution of aberrant DNA structures arising during replication but not in the repair of DSBs with two free ends.

Another important aspect in the functional classification of RecQ homologues is whether their mutations, in combination with mutations in other genes, cause new proliferation defects or suppress existing ones. A prominent example is the combination of mutations of certain RecQ homologues with the endonuclease MUS81 mutants leading to synthetic lethality. This was shown for *S. cerevisiae* for the combination *sgs1/mus81* (24); for *S. pombe*, for the combination *rqh1/slx3* (23); and for *Drosophila blm/mus81* (9). Recently, we demonstrated that *Atrecq4A* shows synthetic lethality when combined with *Atmus81* (25). These results indicate that the respective RecQ homologues are required to process aberrant replication intermediates that cannot be processed properly if *MUS81* is mutated (39).

Mutations in the *TOP3* or *TOP3 α* gene result in proliferation defects and lethality in several eukaryotes (12–14). This phenotype is partially suppressed in *S. cerevisiae* and *S. pombe* if combined with a genetic mutation in the respective RecQ helicases SGS1 and RQH1, respectively (14, 21, 22). The *top3 α -1* mutant we used for crossings with *recq4A-4* and *recq4B-2* showed a very severe phenotype with lethality at the cotyledon stage. The mutant line *recq4A-4* could partially suppress this phenotype; the double mutant is sterile and mitotically impaired but viable. Thus, to our knowledge, for the first time in a multicellular eukaryote the suppression of a *top3 α* phenotype by the additional mutation of a RecQ helicase could be demonstrated.

In summary, we supply three lines of evidence that AtRECQ4A has a similar function in plants as SGS1 has in yeast and BLM has in mammals: (i) if mutated in *RECQ4A*, plants show an enhanced frequency of homologous crossovers; (ii) double mutants of *RECQ4A* and *MUS81* are lethal in *Arabidopsis* (25); and (iii) mutation of *RECQ4A* in a *top3 α* background changes the lethal phenotype to viability.

AtRECQ4B: A Surprisingly Different Sister. In contrast to *RECQ4A* neither *RECQ4B* mutant showed any sensitivity to DNA-damaging agents. Moreover, also in all other aspects tested, *RECQ4B* differed from *RECQ4A*. The mutants of RECQ4B did not show lethality in a *mus81* background, nor did they suppress the lethal *top3 α -1* phenotype. However, the most surprising finding was that RECQ4B has an antagonistic function in homologous recombination in comparison with RECQ4A: HR

was not enhanced but reduced in the mutant background with both recombination substrates. Thus, AtRECQ4B is the first reported case of a eukaryotic RecQ homologue that is positively involved in homologous recombination. It is tempting to speculate about the different molecular roles of RECQ4A and RECQ4B: Together with TOP3 α , RECQ4A seems to be involved in Holliday junction (HJ) resolution, which prevents CO recombination. In contrast, RECQ4B might be actively involved in the initiation or stabilization of recombination intermediates. One could imagine that RECQ4B with its helicase activity is involved in the formation of D-loop structures. This suggestion is in line with recent evidence that RecQ homologues might be actively involved in synthesis-dependent strand annealing (38, 40). Alternatively, RECQ4B might be able to stabilize formation of HJs by binding to these structures directly.

RECQ4A and 4B: Separation of Function During Plant Evolution? The *RECQ4A/RECQ4B* gene pair can be found exclusively in dicotyledonous plants, whereas rice and the moss *P. patens* possess one RECQ4A orthologous gene only (1). Therefore, two putative scenarios can be envisaged about how the different functions of both gene products evolved: (i) after the duplication of the functional SGS1 homologue (ancestral RECQ4), one copy retained its function of CO suppression (RECQ4A), whereas the second (RECQ4B) became involved in promoting recombination, most probably by taking over the function of another unknown helicase; (ii) the ancestral protein RECQ4 possessed both functions (CO suppression and promotion) and the duplicated proteins specialized in different directions. We favor the second explanation because of different indications reported in the literature.

Early work on RecQ in *E. coli* and *S. cerevisiae* demonstrated that in both organisms RecQ (or SGS1) acts as a suppressor of illegitimate recombination and that even BLM can take over this function in a yeast *sgs1* mutant (4, 41, 42). In a recent study, the group of Susan Rosenberg (43) was able to demonstrate that the RecQ helicase of *E. coli* is involved in the net accumulation of replication intermediates that might be resolved by HR. A combination of *uvrD* with *ruvA*, *B*, or *C* mutants in *E. coli* was shown to be inviable because of the presence of RecQ. The RuvAB complex is involved in branch migration and RuvC complex is involved in the resolution of HJs (44). UvrD is a DNA helicase required for mismatch repair and nucleotide excision repair, which resolves bimolecular recombination intermediates by removing RecA from single-stranded DNA (45, 46). Interestingly, the mutation of the unique bacterial RecQ helicase rescued the viability of the *ruvA/uvrD* cells (43). These results led the authors to suggest that, in general, RecQ homologues have two different functions: they might not only be involved in CO suppression, for example, by resolving of dHJs (the classical paradigm), but also in the promotion of recombinogenic DNA structures, such as dHJs (the new “second” paradigm). Whereas their study supplied first indications that the new paradigm might be valid for bacteria, we now clearly demonstrate that this paradigm can be applied to eukaryotes, too. We predict that in the long run other examples besides AtRECQ4B will be discovered in the eukaryotic kingdom.

Because of the duplicated *RECQ4* gene, *A. thaliana* seems to be an ideal model system to further elucidate the molecular basis of both paradigmatic functions of RecQ helicases. It will be very interesting to define which parts of the highly homologous RECQ4A and B proteins are responsible for their antagonistic functions by complementation of the mutants with use of chimeric genes. Future analysis should also reveal whether RECQ4A and 4B have different biochemical properties *in vitro*, and if they interact with different factors *in vivo* to explain their antagonistic behavior.

Methods

See SI Tables 1–3 for details of the primers used and data of the sensitivity and HR assays.

Analysis of T-DNA Insertion Lines. Seeds were obtained from the GABI (line GABI_203C07: At1g10930, *recq4A-4*; and GABI_399C04: At1g60930, *recq4B-1*) and SALK collections (line SALK_011357, *recq4B-2*, SALK_139357; At5g63920, *top3 α -1*) (27, 28). Seeds derived from heterozygous plants were cultivated in soil, and PCR assays with primers flanking the T-DNA insertions were used to screen 3- to 4-week-old plants (Fig. 1*A* and *B*; primers 2 and LB for *recq4A-4*; 5 and LB or LB1 for *recq4B-1* and 2, respectively). Plants homozygous for the T-DNA insertion (or heterozygous in the case of *top3 α -1*) were propagated further. The exact integration sites were determined by PCR with use of primer combinations specific for the left or right border of the respective T-DNA and genomic sequences within the respective gene (Fig. 1; primers 2, 2R, 5, 5R, 8, LB, and LB1). PCR products were purified and sequenced (GATC Biotech).

RNA Extraction and RT-PCR of the T-DNA Insertion Lines. RNA from young *Arabidopsis* plantlets was isolated by using the RNeasy Plant Mini Kit from Qiagen according to the instructions of the manufacturer. Reverse transcription and RT-PCR were performed according to the SMART protocol from Clontech by using 2 μ g of total RNA. The cDNA produced was used for different PCRs with primers 1 to 9R to evaluate the mRNA level of the respective interrupted gene in front of, spanning, and behind the T-DNA insertion sites (SI Fig. 5 and SI Table 1).

Mutagen Assays. Homozygous mutant plants from all three lines, the double mutant and Col-0 wild type were sterilized by using 4% NaOCl solution and plated on a germination medium (GM). After 7 days the small seedlings were transferred into six-well plates containing 5 ml of pure liquid GM per well, or liquid GM with different amounts of the respective mutagen methylmethanesulfonate or *cis*-platin. Ten seedlings were transferred to each well. After 13 days in the respective mutagen solution, the seedlings were taken out and pressed on paper towels to remove any excess liquid. Finally, the weight of the seedlings was determined by using a fine scale balance. All experiments were performed at least four times, and for each experiment the mean value of each line was compared with the wild-type mean value given for each mutagen concentration (Fig. 2*A–C*; SI Table 2).

Homologous Recombination Assay. Homozygous plants for the T-DNA insertion in the respective gene were used for crossings with the two HR-reporter lines 651 and IC9C (34, 35). After crossing and propagation of the heterozygous F₁ generation, the F₂ generation was screened by PCR for double-homozygous plants and also for plants, in which the respective homozygous wild-type gene (*RECQ4A* or *4B*) and the homozygous reporter construct locus were combined. The latter plants were used as internal controls for the recombination assays. For HR assays, the seedlings were treated in the same manner as for the mutagen assays, but instead of transferring them to six-well plates after 7 days, 30–35 seedlings each were transferred into halved Petri dishes containing 10 ml of either pure liquid GM, or liquid GM with 5 μ g of bleomycin, respectively. After 5 additional days in liquid culture the seedlings were transferred to a staining solution (47). After 2 more days, the seedlings were incubated in 70% ethanol for 12 h, and subsequently, the number of blue sectors on each plant was determined by using a binocular microscope. The HR assays were repeated independently at least five times, and the mean values were calculated (Fig. 3; SI Table 3).

DAPI Staining of Mitotic Anaphase Figures. Inflorescences were collected and incubated in a mixture of methanol and acetic acid in a ratio of 3 to 1. In this fixative the plant material can be stored at -20°C until preparation, which is done, at the earliest, the next day and, at the latest, after a few weeks.

For usage, the plant material was washed in 0.01 M citrate buffer (4 ml of 0.1 M citric acid and 6 ml of 0.1 M sodium citrate, 90 ml of distilled water, pH 4.8) for 5 min and then incubated for 30 min in citrate buffer containing 2% cellulase and 0.2% pectinase (Sigma Aldrich) at 37°C . This digestion was processed in a moist chamber, followed by washing the inflorescences twice for 5 min with citrate buffer.

Buds with a maximum size of 0.5 mm were prepared in 15 μl of acetic acid (45%). After adding a coverslip, the pollen grains

were gently squeezed out of the dissected anthers by gentle, manual pressure. To enable fast removal of the coverslip with the help of a razor blade, the slides were quick-frozen in liquid nitrogen for 30 s. The air-dried preparation was then stained with Vectashield mounting medium containing 1.5 $\mu\text{g/ml}$ DAPI (48). By using a fluorescence microscope (Zeiss Axio Imager M1) with an appropriate DAPI filter, mitotic anaphase stages were detected and counted, both accurate stages and stages showing misarranged DNA fragments or chromosomes, respectively.

We thank Manfred Focke and Shaun Lampi for their thorough reading of the manuscript and Sabine Zeiler for technical assistance. This work was supported by Deutsche Forschungsgemeinschaft Grants HA5055/1-1 and PU137/8-1.

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