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Arabidopsis *RecQl4A* suppresses homologous recombination and modulates DNA damage responses

Mohammad B. Bagherieh-Najjar[†], Onno M.H. de Vries, Jacques Hille and Paul P. Dijkwel[*]

Molecular Biology of Plants, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

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[*]For correspondence (fax +31 50 363 8126; e-mail p.p.dijkwel@biol.rug.nl).

[†]Present address: Department of Biology, Faculty of Science, Gorgan University of Agricultural Sciences and Natural Resources, Shahid Beheshti Ave, Gorgan, Iran.

Summary

The DNA damage response and DNA recombination are two interrelated mechanisms involved in maintaining the integrity of the genome, but in plants they are poorly understood. *RecQ* is a family of genes with conserved roles in the regulation of DNA recombination in eukaryotes; there are seven members in Arabidopsis. Here we report on the functional analysis of the Arabidopsis *RecQl4A* gene. Ectopic expression of Arabidopsis *RecQl4A* in yeast *RecQ*-deficient cells suppressed their hypersensitivity to the DNA-damaging drug methyl methanesulfonate (MMS) and enhanced their rate of homologous recombination (HR). Analysis of three *recQl4A* mutant alleles revealed no obvious developmental defects or telomere deregulation in plants grown under standard growth conditions. Compared with wild-type Arabidopsis, the *recQl4A* mutant seedlings were found to be hypersensitive to UV light and MMS, and more resistant to mitomycin C. The average frequency of intrachromosomal HR in *recQl4A* mutant plants was increased 7.5-fold over that observed in wild-type plants. The data reveal roles for Arabidopsis *RecQl4A* in maintenance of genome stability by modulation of the DNA damage response and suppression of HR.

Keywords: Arabidopsis, DNA repair, genome instability, DNA recombination, RecQ.

Introduction

DNA damage can be generated by exogenous genotoxic agents or by endogenous assaults arising during natural cell metabolism. If not removed, lesions in single-strand DNA such as strand breaks, gaps, damaged bases and DNA adducts may lead to DNA double-strand breaks, which are considered to be serious threats to genome stability. Double-strand breaks can be repaired by two major pathways: non-homologous end joining and homologous recombination (HR). Non-homologous end joining does not require an extensive homology between the ends of recombinant DNA molecules, thus any two available DNA ends can be resealed in an inherently error-prone manner. In contrast, HR takes advantage of large sequence homologies found in the genome to recombine the broken ends (reviewed in Jackson, 2002; Lowndes and Murguia, 2000; Pastink *et al.*, 2001).

Several Arabidopsis genes that function in recombinational repair of DNA damage have been described. The RMN (*Rad50*/*Mre11*/*Nbs1*) complex is implicated in non-homologous end joining, HR and the repair of arrested replication forks. *mre11* mutant plants show hypersensitivity to genotoxic stress and lengthened telomeres (Bundock and Hooykaas, 2002). Disruption of *rad50* leads to the shortening of telomeres in cell culture (Gallego and White, 2001), enhanced frequency of intrachromosomal HR (Gherbi *et al.*, 2001), increased sensitivity to methyl methanesulfonate (MMS) and plant sterility (Gallego *et al.*, 2001). Having similar effects to *rad50*, the *MIM* gene is a member of the *SMC*-like family that plays a role in the maintenance of chromosome structure. The Arabidopsis *mim* mutant shows enhanced sensitivity to genotoxic stress and exhibits reduced intrachromosomal HR (Mengiste *et al.*, 1999).

The Arabidopsis genome contains seven *RecQ*-like genes (Hartung *et al.*, 2000; accession no. AJ421618). In other organisms homologs of *RecQ* play vital roles in DNA recombination/repair and replication. *Escherichia coli* *RecQ*-deficient cells exhibit decreased levels of HR and elevated levels of non-homologous end joining (Hanada *et al.*, 1997; Nakayama *et al.*, 1985). *RecQ*-deficient (*sgs1*Δ)

cells in budding yeast (*Saccharomyces cerevisiae*) show genetic hyper-recombination phenotypes and reduced fidelity of chromosome segregation during mitosis and meiosis, and exhibit premature ageing (Sinclair and Guarente, 1997; Watt *et al.*, 1995). In addition, *sgs1*Δ cells show hypersensitivity to the DNA-damaging drug MMS and hydroxyurea, an inhibitor of DNA synthesis (Frei and Gasser, 2000; Yamagata *et al.*, 1998). The human genome contains at least five members of the *RecQ* family (*BLM*, *WRN*, *RECQL*, *RECQL4* and *RECQL5*). Mutations in *BLM*, *WRN* and *RECQL4* are responsible for Bloom, Werner and Rothmund–Thomson syndromes, respectively (Ellis *et al.*, 1995; Gray *et al.*, 1997; Kitao *et al.*, 1999). Although these syndromes are phenotypically different, they all have in common genome instability and predisposition to cancer. Cells derived from patients show hypersensitivity to DNA-damaging drugs, elevated rates of genetic recombination, increased chromosomal breaks and, in most cases, a reduced lifespan. Where studied, RecQ proteins exhibit ATP-dependent 3′ to 5′ DNA helicase activity, and are able to unwind DNA duplex molecules, Holliday junctions (HR intermediate structures), DNA–RNA heteroduplexes and G–G paired tetraplex structures formed at G-rich regions like telomeres (reviewed by Bachrati and Hickson, 2003; Nakayama, 2002). However, little is known about the functions of plant RecQ-type proteins. We have previously shown that the transcripts of seven Arabidopsis *RecQ* genes are detectable in all plant organs, but most of them exhibit specific expression profiles in various tissues or in plants exposed to various stress conditions (Bagherieh-Najjar *et al.*, 2003). We have also shown that Arabidopsis *RecQsim* and its homologs in rape and rice contain an unusual insert inside the helicase domain and that Arabidopsis *RecQsim* was able to suppress the MMS hypersensitivity of a haploid *sgs1*Δ mutant. Here, we report on the *in planta* detailed functional analysis of a plant *RecQ* gene by characterization of three independent Arabidopsis *recQl4A* mutants. We demonstrate an important role for *RecQl4A* in response to DNA damage and in regulation of DNA rearrangements.

Results

Arabidopsis RecQl4A *complements the MMS hypersensitivity and hyperhomologous recombination phenotypes of the yeast sgs1Δ mutant*

In contrast to Arabidopsis, which contains seven *RecQ* genes, the budding yeast *S. cerevisiae* contains only one *RecQ* gene (*SGS1*). Human *WRN* and *BLM* as well as Arabidopsis *RecQsim* have been shown to suppress at least one of the *sgs1*Δ phenotypes (Bagherieh-Najjar *et al.*, 2003; Yamagata *et al.*, 1998). We first examined whether all Arabidopsis *RecQ*-like genes were able to compensate for the absence of *SGS1*. The *RecQ* cDNA fragments containing the complete open reading frame (ORF) were cloned 3′ of the *GAL1* promoter, and a diploid *sgs1*Δ strain along with its respective wild type were transformed with either the resulting construct or the empty vector. Subsequently, the MMS hypersensitivity and the rate of heteroallelic interchromosomal HR were monitored in the transformants by the MMS spot assay and the restoration rate of the *his1* gene, respectively, as described by Ui *et al.* (2001). Consistent with our previous results (Bagherieh-Najjar *et al.*, 2003), *RecQsim* partially suppressed the MMS hypersensitivity of *sgs1*Δ cells (data not shown). However, the increased rate of heteroallelic interchromosomal HR was not suppressed by the *RecQsim* gene. Interestingly, of the seven Arabidopsis *RecQ* genes only *RecQl4A* was able to suppress both the MMS hypersensitivity and hyper-recombination phenotypes of *sgs1*Δ cells to near wild-type levels (Figure 1). These results showed that *RecQl4A* can function in response to DNA damage and in suppression of interchromosomal HR in *sgs1*Δ cells, and suggested that *RecQl4A* may exhibit a similar function in plants.

Identification of Arabidopsis recQl4A *mutants*

Three independent transferred DNA (T-DNA) insertional mutant alleles (*recQl4A-1*, *recQl4A-12* and *recQl4A-13*) were isolated from two different accessions (Figure 2a). The *recQl4A-1* mutant was identified in a screen of the Arabidopsis T-DNA insertion lines in the Wassilewskija (WS) background available at the University of Wisconsin Arabidopsis

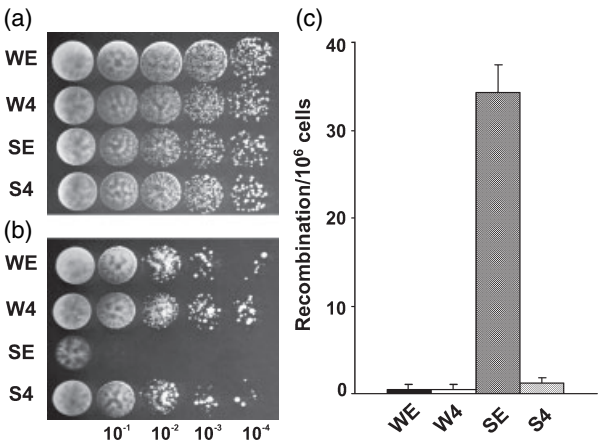

Figure 1. Complementation of the MMS hypersensitivity and hyper-recombination phenotypes of *sgs1*Δ cells by the Arabidopsis *RecQl4A* gene.
A diploid *sgs1* mutant and its corresponding wild type were transformed either with the empty vector (SE and WE, respectively) or a plasmid containing the Arabidopsis *RecQl4A* cDNA (S4 and W4, respectively).
(a, b) Exponentially growing cultures were serially diluted and spotted on minimal medium plates, supplemented with 0 (a) or 0.01% MMS (b) and photographed after 3 days of growth at 30°C.
(c) The rate of heteroallelic homologous recombination in the transformants. The error bars represent the standard errors of the means. Three independent transformants were examined and similar results were obtained in each case.

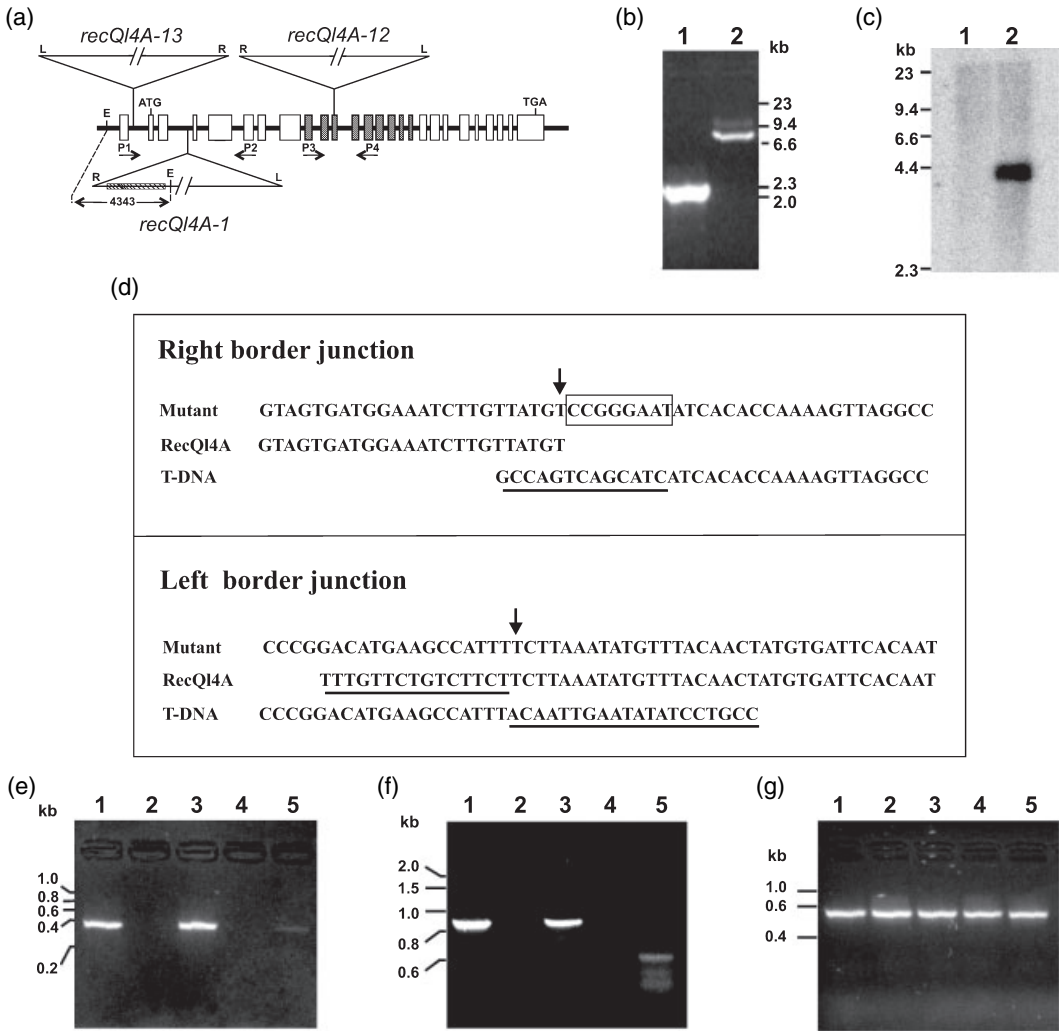

Figure 2. Molecular characterization of the T-DNA insertions in the *RecQl4A* gene.
(a) Schematic diagram of the *RecQl4A* locus. The position of the T-DNA in three independent lines is indicated. Boxes, exons; gray boxes, exons that encode the helicase domain; L, left border; R, right border; P, primers used in PCR and RT-PCR; E, *Eco*RI sites. The shaded rectangle represents the probe used for Southern analysis.
(b) PCR amplification of the whole T-DNA, using primers P1 and P2. Lane 1, wild type WS; lane 2, *recQl4A-1* mutant.
(c) DNA gel blot of the wild-type WS (lane 1) and *recQl4A-1* mutant (lane 2), using a 1.7 kb GUS fragment of the T-DNA as the probe.
(d) Organization of the junctions of the inserted T-DNA in *recQl4A-1*. Nucleotide sequences of the mutant plant are aligned with that of the *RecQl4A* gene and the T-DNA. Arrows indicate the integration sites. The filler nucleotides are shown in the box. Deleted nucleotides are underlined.
(e), (f) Gene expression analysis performed by RT-PCR. Lane 1, wild-type WS; lane 2, *recQl4A-1*; lane 3, wild-type Col-0; lane 4, *recQl4A-12*; lane 5, *recQl4A-13*. Primers P3 and P4 amplify a 0.4 kb cDNA fragment (e). Primers P1 and P2 a 0.9 kb cDNA fragment (f).
(g) The RT-PCR control as demonstrated by amplification of a cDNA fragment of Arabidopsis *Tubβ6*.

Knockout Facility (Krysan *et al.*, 1999). PCR reactions (Figure 2b), DNA blot analysis (Figure 2c) and the nucleotide sequences of the T-DNA junctions (Figure 2d) together with the segregation pattern of the kanamycin-selectable marker (not shown) revealed that a single copy of the T-DNA is inserted in the third intron of the *RecQl4A* gene. The other two alleles were obtained from the Syngenta Arabidopsis Insertion Library (SAIL) sequenced T-DNA lines and have the Columbia (Col-0) background (Sessions *et al.*, 2002). As shown in Figure 2(a), the T-DNA in the *recQl4A-12* line is inserted in the eleventh exon, in the middle of the predicted helicase domain. The insertion in the *recQl4A-13* line mapped to the first intron that is located in the 5′ untranslated region (5′ UTR).

The expression of the *RecQl4A* gene in the wild-type and mutant alleles was studied by RT-PCR and Arabidopsis *Tubβ6* was used as a control (Figure 2g). Using primers P3 and P4 that span the integration site of the T-DNA in the *recQl4A-12* line, the *RecQl4A* cDNA was amplified from the wild type. In contrast, no amplification was detected in the *recQl4A-1* or *recQl4A-12* lines but some signal was detected in the *recQl4A-13* mutant (Figure 2e). After 40 cycles of PCR

using primers P1 and P2 that span the integration sites of the T-DNA in the *recQl4A-1* and *recQl4A-13* lines, the *RecQl4A* cDNA was not amplified in any of the mutant lines (Figure 2f). These results suggested that *recQl4A-1* and *recQl4A-12* are knockout mutants, while the T-DNA insertion in the *recQl4A-13* line might result in reduced gene expression instead of a complete disruption.

When grown under standard growth conditions all three homozygous *recQl4A* mutant alleles were fertile and no obvious phenotypic differences between them and their heterozygous or wild-type siblings were observed. This revealed that *RecQl4A* is not essential for growth and development under standard growth conditions.

The response of RecQl4A*-deficient mutants to genotoxic stress*

A deficiency in genes involved in DNA repair or recombination in Arabidopsis may lead to increased sensitivity of the mutant plants to genotoxic stress (reviewed in Reiss, 2003). The effects of UV light, MMS and mitomycin C (MMC) on the *recQl4A* mutant plants were examined. UV irradiation generates cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidinone dimers, both of which can block DNA replication (Courcelle *et al.*, 2003; Seigneur *et al.*, 1998). Two different pathways, called photorepair and light-independent repair, are mainly involved in the repair of UV-induced DNA damage. Therefore, 10-day-old wild-type and *recQl4A* mutant seedlings germinated and grown on agar plates were exposed to various doses of UVC light and were allowed to recover either in the light or in the dark. The growth of wild-type WS and Col-0 plants was completely abolished at 600 and 900 mJ cm^{-2} UVC, respectively (data not shown). Thus, the WS plants were more sensitive to the applied UV light than the Col-0 plants. As shown in Figure 3(a,b), and as expected, in both accessions the recovery from UVC-induced DNA damage in the presence of light was more efficient than in the dark. The fresh weight of *recQl4A-1* plants exposed to 400 mJ cm^{-2} UVC light was less than that of wild-type WS plants after either light or dark recovery (Figure 3a). Comparable results were obtained when the other two mutant alleles in the Col-0 background were exposed to 600 mJ cm^{-2} UVC light (Figure 3b). Interestingly, the *recQl4A-13* mutant showed an intermediate hypersensitivity to the applied treatment. This is in agreement with the notion that in this line the *RecQl4A* gene might not be completely disrupted (see above). In addition, the mutant plants were also found to be hypersensitive to UVB irradiation (data not shown). The data show that the disruption of the *RecQl4A* gene is associated with the observed hypersensitivity to UV light, and suggest that *RecQl4A* may be involved in repairing UV-induced damage to DNA in Arabidopsis.

Methyl methanesulfonate is a monofunctional DNA alkylating agent. If MMS-induced DNA lesions are not repaired by the base excision repair (BER) pathway they may cause fork arrest during DNA replication (Tercero and Diffley, 2001). Six-day-old wild-type and mutant seedlings germinated and grown in the absence of MMS were transferred to a liquid medium supplemented with various concentrations of MMS and were grown for an additional 2 weeks. As illustrated in Figure 3(c,d), the wild-type Col-0 plants were more resistant to this genotoxic agent than the WS plants. Nevertheless, as the concentration of MMS increased, the growth of all three *recQl4A* mutant lines was more retarded than that of the corresponding wild type (Figure 3c,d). The extent of the observed hypersensitivity to MMS was

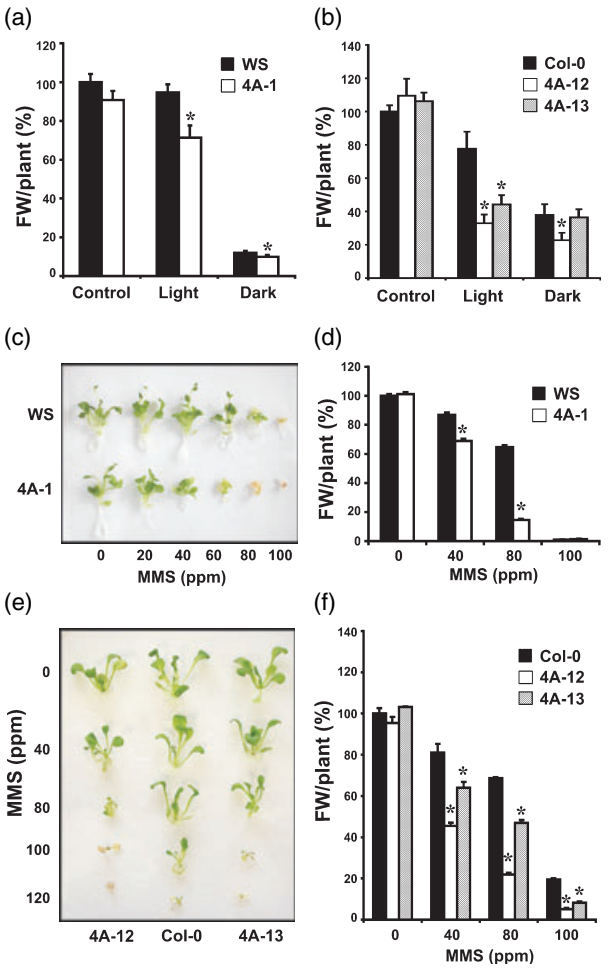

Figure 3. Hypersensitivity of *recQl4A* mutants to UV light and MMS.
(a, b) Effect of UVC light on 10-day-old wild-type (WS, Col-0) and *recQl4A* mutants (4A-1, 4A-12, 4A-13) that were exposed to either 400 mJ cm^{-2} UVC light (a) or 600 mJ cm^{-2} UVC light (b) and were allowed to recover either in the dark or in the light for 48 h. The fresh weight (FW) was measured 2 weeks post-recovery.
(c–f) Response of the wild-type (WS, Col-0) and *recQl4A* mutants (4A-1, 4A-12, 4A-13) to increasing concentrations of MMS; FW, fresh weight. The error bars represent the standard errors of the means. Asterisks indicate a significant difference from the wild type in the same treatment (two-tailed Student's *t*-test, $P < 0.01$, $n \geq 35$).

quantified by measuring the fresh weight (Figure 3e,f) and dry weight (not shown) of the seedlings. The results confirmed that the *recQl4A* mutant seedlings were more sensitive to MMS than wild type. The hypersensitivity of the *recQl4A-13* mutant to MMS was less than that of the *recQl4A-12*, similar to the UV experiment. Taken together, this led us to conclude that the observed MMS hypersensitivity is coupled to the disruption of the *RecQl4A* gene.

Further, the response of the mutant seedlings to MMC was monitored. MMC induces DNA cross-linking and subsequent formation of DNA double-stranded breaks (De Silva *et al.*, 2000; McHugh *et al.*, 2000). Six-day-old wild-type and mutant seedlings germinated and grown in the absence of MMC were transferred to a liquid medium supplemented with various concentrations of MMC. As shown in Figure 4, the wild-type WS and Col-0 plants failed to grow with concentrations of 40 and 120 μM MMC, respectively. Surprisingly, the mutant plants showed an enhanced resistance to the increasing concentrations of MMC, as compared with their respective wild type (Figure 4). Measurements of fresh and dry weights confirmed that the sensitivity of the *recQl4A* mutants to MMC was less than that of the wild types (Figure 4c and data not shown). The *recQl4A-13* mutant had an intermediate phenotype. Thus, inactivation of *RecQl4A* leads to an increased resistance to MMC.

Taken together, the data indicate that *RecQl4A* is involved in the response of the plant to genotoxic stress and that none of the other Arabidopsis *RecQ* homologs is able to fully compensate for the absence of *RecQl4A*.

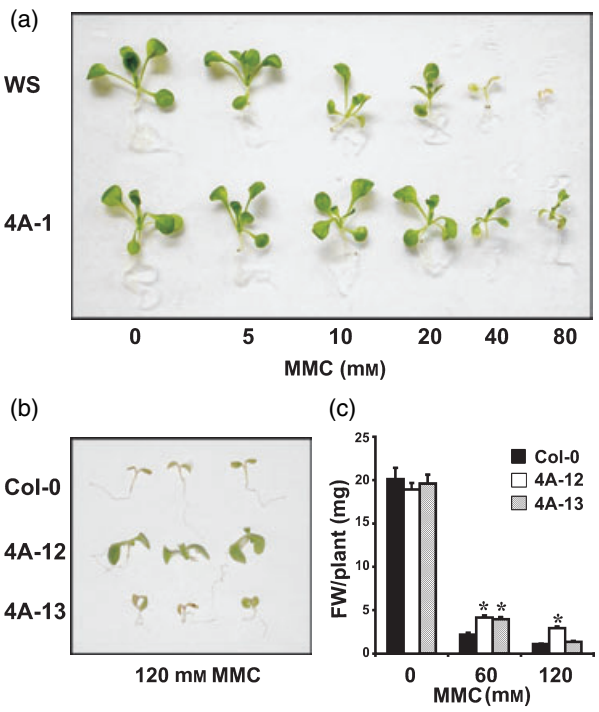

Figure 4. Hyper-resistance of *recQl4A* mutants to MMC.
(a) Wild-type (WS) and *recQl4A* mutant (4A-1) seedlings were treated with the indicated concentrations of MMC.
(b) Wild-type (Col-0) and mutant (4A-12, 4A-13) seedlings were treated with 120 mM MMC.
(c) The fresh weight (FW) of plants treated with the indicated concentrations of MMC. The error bars indicate the standard errors of the means. Asterisks indicate a significant difference from the wild type in the same treatment (two-tailed Student's *t*-test, $P < 0.01$, $n \geq 26$).

Telomere status in Arabidopsis recQl4A *mutants*

Many Arabidopsis genes involved in the responses of the plant to DNA damage and DNA recombination also play crucial roles in the maintenance of telomeres (Bundock and Hooykaas, 2002; Gallego *et al.*, 2003; Riha *et al.*, 2002). Telomeres are capping structures at the ends of eukaryotic chromosomes and are composed of Tx(A)Gy tandem repeats bound to a complex array of proteins (reviewed in Blackburn, 2001). In the absence of telomeres, the ends of the chromosomes are subjected to the DNA repair/recombination machinery and cell-cycle checkpoint pathways are activated. A possible involvement of *RecQl4A* in telomere homeostasis in Arabidopsis was examined by determining the length of telomeric repeat tracks in the second generation of the mutant plants. Terminal restriction fragments (TRFs) were generated by cleavage of genomic DNA with *Mbo*I and analyzed by Southern hybridization with a telomeric probe; the results are presented in Figure 5. As expected (Riha *et al.*, 2002), the telomeric smear in the Col-0 ecotype ranged from about 2–4 kb and in the WS ecotype was about twice the size of that in Col-0. No obvious changes in telomeric repeat tracks were detected in the mutant lines, when compared with wild-type plants (Figure 5). The result revealed that a deficiency in *RecQl4A* does not markedly alter telomere homeostasis in the second generation of mutants grown under standard growth conditions. Maintenance of telomeres in yeast and animal cells is mainly mediated by two pathways: the telomerase reverse transcriptase (TERT)-dependent pathway and the alternative telomere maintenance (ALT) pathway (Henson *et al.*, 2002; Nugent and Lundblad, 1998). RecQ family members, such as Sgs1, BLM and WRN, have been shown to participate in the ALT pathway of telomere maintenance (Bai and Murnane, 2003; Cohen and Sinclair, 2001; Johnson *et al.*, 2001). It is possible that RecQl4A acts in a pathway similar to the ALT pathway which might only be active in the absence of a functional telomerase. Taking into account that telomerase activity has been detected in most Arabidopsis organs (Riha *et al.*, 1998; Fitzgerald *et al.*, 1999), any possible role of RecQl4A in telomere maintenance in Arabidopsis might be minor. Alternatively, the mechanisms of telomere maintenance in plants might be different from those in yeast and humans.

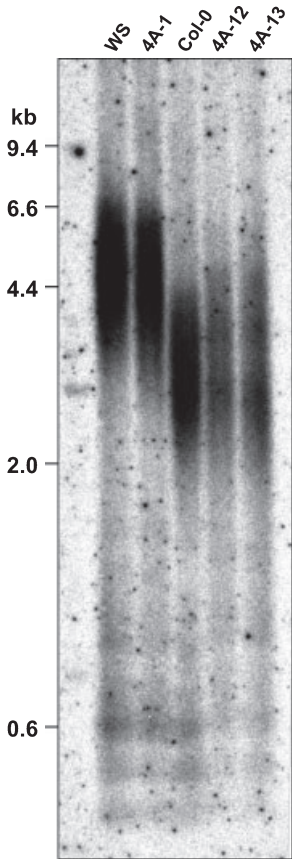

Figure 5. Telomere length analysis of *RecQl4A*-deficient plants.
DNA was isolated from the wild-type (WS, Col-0) and *recQl4A* mutants (4A-1, 4A-12, 4A-13) and digested with *Mbo*I. The telomeric repeat sequence $(TTTAGGG)_6$ was used as a probe.

Somatic intrachromosomal homologous recombination is increased in recQl4A *mutant plants*

To further characterize the mechanism by which the response of plants to genotoxic stress is regulated by *RecQl4A*, an *in planta* recombination assay was used to monitor the frequency of intrachromosomal HR. The assay system employed a transgenic recombination trap in the Arabidopsis C24 background, line N1DC1 no.11 (L11), consisting of direct repeats of overlapping non-functional parts of a chimeric β-glucuronidase (GUS) gene separated by a hygromycin-selectable marker (Swoboda *et al.*, 1994). The 1213 bp overlap between the two fragments of the GUS gene, together with the hygromycin-selectable marker, can be removed by HR, leading to restoration of the GUS gene. Such HR events can be detected as blue spots or sectors when plants are histochemically stained. The *recQl4A-1* mutant (kanamycin resistant) was crossed to L11 and the resulting F_3 seed populations were collected separately. Portions of the seeds were germinated on agar plates supplemented with either hygromycin or kanamycin. The genotype of the seeds was determined on the basis of the segregation pattern of the selectable markers. To minimize the influence of other segregating traits, six independent F_3 families either wild type for *RecQl4A* (*RecQl4A*$^{+/+}$ *L11*$^{+/+}$; *RecQl4A*$^{+/+}$ *L11*$^{+/-}$) or mutant (*recQl4A*$^{-/-}$ *L11*$^{+/+}$; *recQl4A*$^{-/-}$ *L11*$^{+/-}$) were analyzed. One F_3 family that was wild type and two that were mutant for the *RecQl4A* gene were segregating for the chimeric GUS gene. The plants were grown in the presence of hygromycin, such that all green plants had at least one copy of the GUS reporter construct. In total, 290 hygromycin-resistant plants were histochemically stained and the number of blue spots per plant was counted under a dissecting microscope. A plant with the *recQl4A*$^{-/-}$ *L11*$^{+/+}$ genotype is shown in Figure 6(a). The T-DNA in the *recQl4A-1* allele also contains a GUS gene; however, no GUS expression was detected in *recQl4A*$^{-/-}$ *L11*$^{-/-}$ seedlings (data not shown). Thus, we concluded that all the blue spots detected represent HR events. These events will mainly be the result of intrachromosomal recombination, but we cannot exclude the possibility that some blue spots represent interchromosomal HR between sister chromatids. The number of blue spots per plant was determined in each family and the average frequencies of HR were calculated and are shown in Figure 6(b). The data demonstrate that in

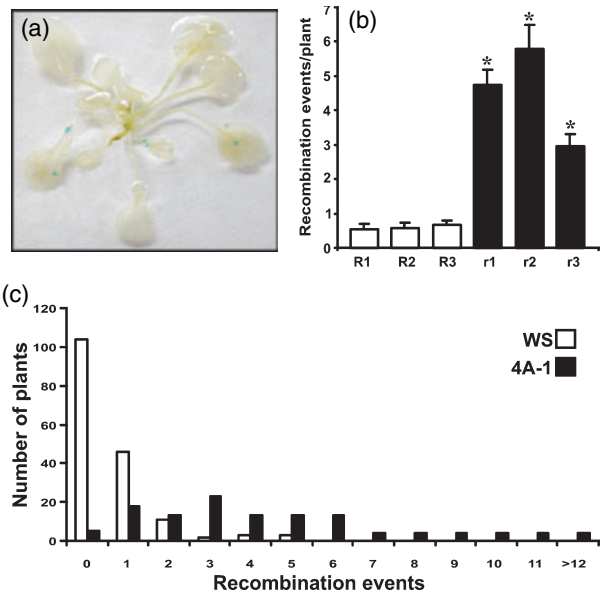

Figure 6. Increased rate of somatic intrachromosomal HR in *recQl4A* mutants.
(a) An example of a *recQl4A*$^{-/-}$ *L11*$^{+/+}$ seedling after GUS staining. The blue spots represent intrachromosomal HR events.
(b) The frequency of HR was compared between independent progenies of plants that were either wild type (R) or homozygous for the *recQl4A-1* mutation (r) and contained at least one copy of the GU-US reporter construct. R1, *RecQl4A*$^{+/+}$ *L11*$^{+/+}$; R2, *RecQl4A*$^{+/+}$ *L11*$^{+/+}$; R3, *RecQl4A*$^{+/+}$ *L11*$^{+/-}$; r1, *recQl4A*$^{-/-}$ L11$^{+/+}$; r2, *recQl4A*$^{-/-}$ *L11*$^{+/-}$; r3, *recQl4A*$^{-/-}$ *L11*$^{+/-}$. The error bars represent the standard errors of the means. Asterisks indicate a significant difference from R1, R2 and R3 (two-tailed Student's *t*-test, $P < 0.01$, $n \geq 37$).
(c) Distribution of the plants with indicated frequencies of recombination in wild-type (WS) and *recQl4A-1* (4A-1) mutant populations.

both $L11^{+/+}$ and $L11^{+/-}$ families the rate of HR in the mutant was higher than that in the wild type. Furthermore, a clear shift towards higher numbers of recombination events in individual mutant plants was observed (Figure 6c). Statistical analysis, performed on pooled populations, revealed that the average frequency of HR per plant in the *recQI4A-1* mutant (4.5 ± 0.3) was 7.5 ± 1 times higher than that in the wild type (0.6 ± 0.1).

Discussion

Arabidopsis *recQI4A* mutant plants were employed to study the interplay between response to DNA damage, DNA recombination and telomere homeostasis in plants.

Here we showed that the *recQI4A* mutant plants were hypersensitive to UV light and MMS and exhibit an enhanced frequency of HR. Interestingly, it has been reported that transient and stable expression of the *E. coli RecQ* gene in rice can increase the frequency of extrachromosomal HR events (Li *et al.*, 2004). Thus, over-expression of the *E. coli RecQ* gene in rice may have a different effect from the absence of endogenous *RecQI4A* in Arabidopsis. Furthermore, the *E. coli* gene might interfere with the proper function of the intrinsic rice *RecQ* genes or the mechanisms controlling extrachromosomal and intrachromosomal HR might be different. Nevertheless, the data presented here suggest that RecQI4A is involved in cellular responses to DNA damage. Cellular responses to DNA damage include direct removal of DNA lesions, so-called DNA repair, and the activation of cell-cycle arrest. RecQI4A might act directly in the removal of certain types of DNA lesions. In the absence of a functional RecQI4A, alternative DNA repair processes could then generate recombinogenic DNA structures leading to increased HR. In support of this notion, human WRN has been suggested to act in unwinding DNA repair intermediates of the BER pathway (Harrigan *et al.*, 2002). Alternatively, or in addition, RecQI4A might directly function in halting cell-cycle arrest. In somatic cells, unrepaired DNA lesions can stop DNA replication by inducing replication fork arrest (reviewed in Lovett, 2003; Michel *et al.*, 2001; Osborn *et al.*, 2002; Rothstein *et al.*, 2000). The mechanism by which arrested replication forks are reset has recently been established in *E. coli*. When a replication fork meets a DNA lesion it moves backwards such that the newly synthesized strands anneal together and the parental strands re-anneal, thereby forming a recombinogenic DNA structure called a Holliday junction (Courcelle *et al.*, 2003). Depending on the type of DNA lesion, there are two possible ways in which Holliday junctions can be processed. If the lesion only affects one of the DNA strands reversion of Holliday junctions can lead to re-establishment of the replication fork. This so-called reverse branch migration re-establishes the fork in a non-recombinogenic pathway. Alternatively, the Holliday junctions can be cleaved at the branch point. This is associated with the activation of DNA recombination and is essential if the lesion affects both strands of the template DNA (reviewed in Cromie *et al.*, 2001; McGlynn and Lloyd, 2002). Sgs1, WRN and BLM have all been shown to locate at the sites of arrested replication forks, bind Holliday junctions and aid reverse branch migration which leads to suppression of DNA recombination (Frei and Gasser, 2000; Sengupta *et al.*, 2003; Wu and Hickson, 2003). Accordingly, the increased DNA recombination observed in RecQI4A-deficient plants might be explained by assuming a role for RecQI4A in suppressing DNA recombination at the sites of arrested replication forks. In this scenario, the absence of a functional RecQI4A does not allow for proper removal of UV- and MMS-induced DNA lesions in a non-recombinogenic way. This may increase HR and DNA rearrangements and consequently hypersensitivity to UV light and MMS. *In vitro* analysis of the effect of purified RecQI4A protein on the repair of various types of DNA damage and its ability to unwind aberrant DNA structures may help to clarify the role that Arabidopsis RecQI4A plays in plant metabolism.

It was intriguing that *recQI4A* mutant seedlings were more tolerant to MMC than the wild type. MMC is a multifunctional alkylating agent which primarily induces cross-links between DNA strands that subsequently generate Holliday junctions during DNA replication (De Silva *et al.*, 2000; McHugh *et al.*, 2000). It is possible that the upregulation of HR, caused by inactivation of RecQI4A, led to the observed MMC hyper-resistance in the mutant seedlings. In support of this notion, HR has been shown to play a crucial role in removal of interstrand cross-links in *E. coli*, yeast and human cells (Pierce *et al.*, 1999; Simon *et al.*, 2000; Sladek *et al.*, 1989). Alternatively, the interplay between the inhibition of HR by RecQI4A and the induction of HR by MMC in wild-type plants might lead to accumulation of irreparable recombination intermediates, which may not be produced in *recQI4A* mutants. Therefore, HR might have two opposing effects on the maintenance of genome stability depending on the type of DNA damage and perhaps the stage in the cell cycle. While HR contributes to the repair of arrested replication forks in an error-free manner and is necessary for proper removal of certain types of 'obstacles' to DNA, such as MMC-induced cross-links, enhancement of HR might lead to increased rearrangement of DNA and genome instability during cell division.

Analysis of the genes involved in the rearrangement of DNA in plants is of special interest due to the persisting dilemma of targeted gene replacement (reviewed in Hanin and Paszkowski, 2003; Puchta, 2002). In higher plants, transgenes or T-DNAs are integrated predominantly by non-homologous end joining. Such a natural preference for non-homologous end joining and the low frequency of HR are the main obstacles to the development of efficient gene-targeting technology in plants (Hanin and Paszkowski, 2003). RecQI4A-deficient plants are fertile, do not show

telomere deregulation and exhibit enhanced levels of HR. Imamura *et al.* (2002) reported that in chicken B-cells, the absence of the *RecQ* homologs *WRN* and *BLM* resulted in an increase of HR-dependent sister chromatid exchange of 1.8- and 10-fold, respectively. Given that in these cells the efficiency of gene targeting also showed a respective 2.5- and 10-fold induction, manipulation of *RecQl4A* might help in developing new tools for gene targeting in plants.

Experimental procedures

Yeast complementation

The diploid wild-type MR101 (*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp-298/trp-298 his1-1/his1-7*) and *sgs1*Δ (ds1, MR101 *sgs1*Δ::*AUR1/sgs1*Δ::*AUR1*) strains used in this study were generous gifts from T. Enomoto (Ui *et al.*, 2001). A cDNA fragment of each Arabidopsis *RecQ*-like gene containing the complete ORF was amplified by RT-PCR, using gene-specific primers containing the *Kpn*I restriction site. Cloning of these fragments into the expression vector pYES2/CT (Invitrogen, Carlsbad, CA, USA) and transformation of the yeast strains were performed as previously described (Bagherieh-Najjar *et al.*, 2003). All subsequent experiments with the transformants were conducted on media lacking uracil (Ura). The MMS spot assay and measurement of the frequency of interchromosomal recombination between heteroalleles, *his1-1/his1-7*, was essentially performed as described by Ui *et al.* (2001).

Plant growth conditions and genotoxic treatments

Seeds were surface sterilized in 20% bleach/ethanol (EtOH) for 15 min, washed twice with 96% EtOH, dried and germinated on Murashige and Skoog (MS) agar plates (Valvekens *et al.*, 1992). Plates were kept at 4°C in the dark for 4 days, then transferred to a growth chamber at 21°C and 65% relative humidity with 16 h light (60 μmol m^{-2} sec^{-1})/8 h dark cycles. Alternatively, seeds were sown directly on soil and germinated and grown as above.

UVC. Ten-day-old seedlings germinated and grown on MS agar plates were exposed to various doses of UVC light (254 nm) using a UV-Stratalinker (Model 1800; Stratagene, La Jolla, CA, USA).

MMS and MMC. Six-day-old seedlings germinated and grown on MS plates were individually transferred to 96-well plates containing liquid MS medium supplemented with various concentrations of either MMS (Sigma, St. Louis, MO, USA) or MMC (Sigma) and incubated in a growth chamber. Photography, physiological measurements and the recombination assay were performed 3 weeks later.

Isolation and characterization of the recQl4A *mutant lines*

The *recQl4A-1* allele was identified from a PCR screen on pooled plant DNA available at the University of Wisconsin Arabidopsis Knockout Facility, as described by Krysan *et al.* (1999). The mutant alleles *recQl4A-12* and *recQl4A-13* were identified in the Syngenta Arabidopsis Insertion Library (SAIL) sequenced T-DNA lines (Sessions *et al.*, 2002). The T-DNA integration sites were confirmed by analysis of the PCR products, amplified using primers of DNA flanking the suggested T-DNA integrations sites and primers of the T-DNA borders.

Total RNA was isolated by using the RNeasy Mini Kit (Qiagen, Hilden, Germany), following the supplier's instructions. The reverse transcription reaction was performed by using either oligodinucleotide (oligo-dT) or gene-specific primers, as described previously (Bagherieh-Najjar *et al.*, 2003). PCR reactions were performed with Taq DNA polymerase (Roche Diagnostic, Mannheim, Germany) and primers P1 (5′-TTGAGCTCAAGTCGGTGGTGGCCATTTTA-3′), P2 (5′-TACTTGTTGAATGGTAGTGTTCC-3′), P3 (5′-GACATATCAGCTCCC-CGCTCT-3′) and P4 (5′-CTTCTTTCACACTGGCTGTTGC-3′).

Southern blot and terminal restriction fragment analyses

DNA from seedlings was extracted as described by Dellaporta *et al.* (1983). Two micrograms of DNA was digested either with 20 units of *Eco*RI (for Southern blot analysis) or with 20 units of *Mbo*I (for TRF analysis) in a final volume of 50 μl for 16 h. Digested DNA samples were directly electrophoresed in a 0.8% agarose/TEA gel (Tris 40 mM; sodium acetate 20 mM; EDTA 1 mM) overnight. The gels were blotted onto a positively charged nylon membrane (Hybond N+; Amersham Pharmacia, Uppsala, Sweden), as described by Sambrook and Russell (2001). The membrane was hybridized in phosphate–SDS buffer at 60°C and washed in phosphate–SDS washing solutions at 58°C (Sambrook and Russell, 2001). A 1.7 kb PCR fragment amplified from the GUS gene of the T-DNA present in the *recQl4A-1* mutant using primers (5′-CGTCCTGTAGAAACCCCAACC-3′) and (5′-AGGTCG-CAAAATCGGCGAAA-3′) was labeled with [α-^{32}P]ATP and used as the probe in Southern analysis. The telomeric repeat probe [5′-$(TTTAGGG)_6$] was 5′ end labeled using T4 polynucleotide kinase (Amersham Pharmacia) and [γ-^{32}P]ATP, following the supplier's instructions.

Intrachromosomal homologous recombination assay

The GUS recombination tester line N1DC1 no.11 (L11) used in this study, was a kind gift from H. Puchta and has been described elsewhere (Swoboda *et al.*, 1994). Three-week-old seedlings were histochemically stained for GUS essentially as described by Sundaresan *et al.* (1995). The number of blue spots in each seedling was counted under a dissecting microscope.

Statistical analysis

Averages and standard errors were calculated and the significance of the variations was confirmed with the Student's *t*-test, set for two-tailed paired analysis at either the 95% or 99% confidence level.

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