A Homolog of *ScRAD5* Is Involved in DNA Repair and Homologous Recombination in Arabidopsis[1][W]

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Rad5 is the key component in the Rad5-dependent error-free branch of postreplication repair in yeast (*Saccharomyces cerevisiae*). Rad5 is a member of the Snf2 ATPase/helicase family, possessing as a characteristic feature, a RING-finger domain embedded in the Snf2-helicase domain and a HIRAN domain. Yeast mutants are sensitive to DNA-damaging agents and reveal differences in homologous recombination. By sequence comparisons we were able to identify two homologs (*AtRAD5a* and *AtRAD5b*) in the *Arabidopsis thaliana* genome, sharing about 30% identity and 45% similarity to yeast Rad5. AtRad5a and AtRad5b have the same kind of domain organization with a higher degree of similarity to each other than to ScRad5. Surprisingly, both genes differ in function: whereas two independent mutants of *Atrad5a* are hypersensitive to the cross-linking agents mitomycin C and cis-platin and to a lesser extent to the methylating agent, methyl methane sulfonate, the *Atrad5b* mutants did not exhibit any sensitivity to all DNA-damaging agents tested. An *Atrad5a/Atrad5b* double mutant resembles the sensitivity phenotype of the *Atrad5a* single mutants. Moreover, in contrast to *Atrad5b*, the two *Atrad5a* mutants are deficient in homologous recombination after treatment with the double-strand break-inducing agent bleomycin. Our results suggest that the *RAD5*-dependent error-free branch of postreplication repair is conserved between yeast and plants, and that AtRad5a might be functionally homologous to ScRad5.

The genomic material of all organisms is constantly exposed to damaging agents of exogenous and endogenous origins. Alterations of bases (e.g. oxidation or methylation), single- or double-strand breaks (DSBs) or cross-links within or between DNA strands are the consequences. To preserve the genomic integrity such damages have to be repaired. A number of different repair pathways are available in all organisms.

Based on the epistasis analysis with radiation-sensitive mutants, DNA repair genes have been assigned for the yeast (*Saccharomyces cerevisiae*) to three groups: the *RAD3*, *RAD52*, and *RAD6* group (for review, see Friedberg et al., 2006). The *RAD3* group is responsible for nucleotide excision repair and operates mainly to remove DNA damages induced by UV irradiation (Prakash and Prakash, 2000). The *RAD52* group (homologous recombination [HR]) is involved in the repair of DSBs often caused by ionizing radiation (Kaytor and Livingston, 1994; Aylon and Kupiec, 2004). The *RAD6* group (postreplication repair [PRR]) is required to overcome damages during replication and arrested replication forks. Interestingly, in all three DNA repair groups a member of the DNA helicase-like *SNF2* gene family (Eisen et al., 1995; Flaus et al., 2006) is present, namely *RAD16* (in *RAD3* group; Bang et al., 1992), *RAD54* (in *RAD52* group; Emery et al., 1991), and *RAD5* (in *RAD6* group).

Components involved in PRR in yeast have been identified by genetic and biochemical studies. The pathway is controlled by a heterodimer of Rad6, an ubiquitin-conjugating (E2) enzyme (UBC; Jentsch et al., 1987) and Rad18, an ubiquitin-ligase (E3) enzyme with a characteristic RING-finger motif (Bailly et al., 1994, 1997). PRR consists of three branches: depending on its downstream components, it can be either error-prone (one branch) or error-free (two independent branches; Ulrich, 2005). In the error-prone branch, replication across damages is mediated by the low-fidelity translesion polymerase, Pol ζ (consisting mainly of gene products of *REV3* and *REV7*; Nelson et al., 1996). In one of the two error-free branches Pol η, another translesion polymerase, is able to replicate across T-T dimers with high accuracy (Johnson et al., 1999). The second error-free branch of PRR is dependent on the E3 RING-finger-containing protein Rad5, which is required for polyubiquitylation of proliferating cell nuclear antigen (PCNA). It has been suggested that distinct modifications on PCNA might direct the switch of different PRR modes (Hoege et al., 2002).

The yeast Rad5 protein possesses a RING-finger motif embedded in the conserved helicase motifs belonging to the Snf2 family of helicase-related proteins (Johnson et al., 1992; Flaus et al., 2006). Members of the Snf2 family (Eisen et al., 1995; Pazin and Kadonaga, 1997) are known to be involved in the destabilization of the nucleosome structure to permit the binding

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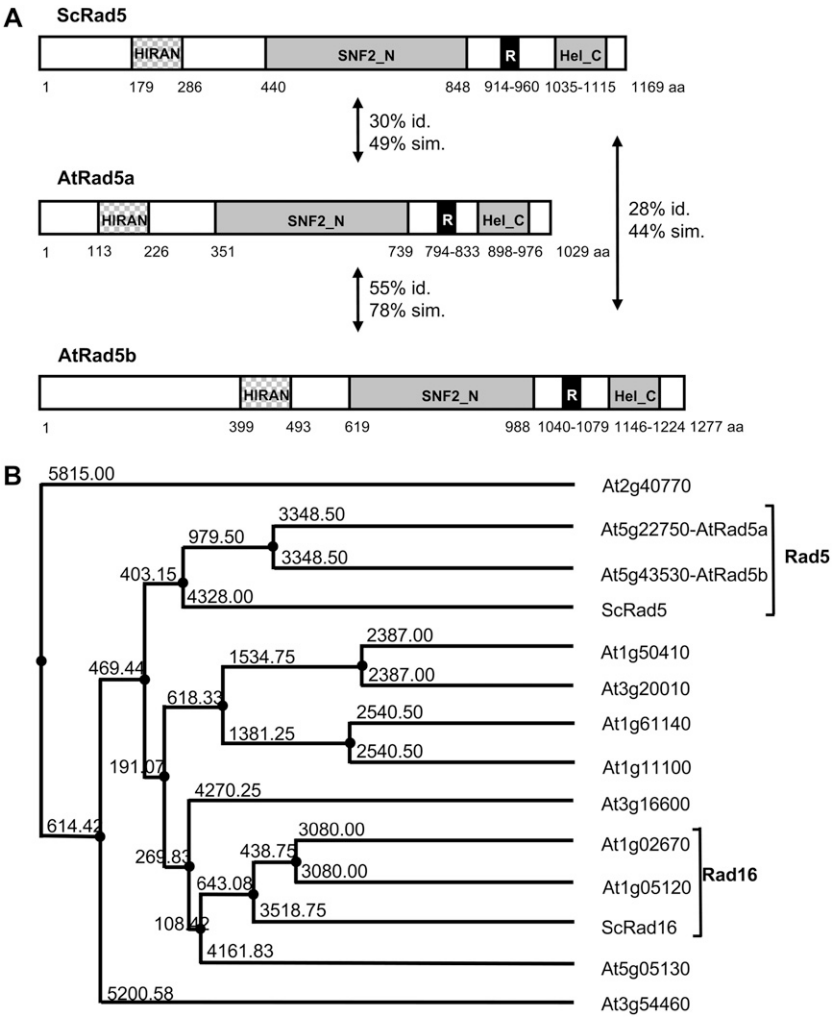
of specific proteins to chromatin, e.g. Rad54 and Tid1 (Shinohara et al., 1997; Petukhova et al., 1999) or to alter the contacts between proteins and DNA by hydrolyzing ATP, e.g. Mot1 (Auble et al., 1997; Sprouse et al., 2006). Whereas a DNA-dependent ATPase activity of Rad5 has been detected already a dozen years ago (Johnson et al., 1994), a helicase function specific for replication fork regression could only be demonstrated recently (Blastyák et al., 2007). Rad5 physically interacts with different proteins involved in PRR, such as Rad18, Ubc13, an E2 enzyme, and PCNA (Ulrich and Jentsch, 2000; Hoege et al., 2002). Upon treatment of cells with DNA-damaging agents, the Rad6-Rad18 heterodimer mediates the monoubiquitylation of PCNA at its Lys residue 164. The ubiquitin chain is further extended via Lys-63 linkage with the E3-ligase activity of Rad5 in cooperation with another heterodimer consisting of Mms2 (a variant form of an E2 ubiquitin conjugating enzyme that does not show E2 activity by itself; Broomfield et al., 1998; Xiao et al., 1999) and Ubc13 (an E2 enzyme; Brusky et al., 2000), resulting in polyubiquitylated PCNA (Hoege et al., 2002). The polyubiquitylation then triggers the Rad5-dependent branch of error-free PRR probably by means of a strand-switching mechanism (Ulrich, 2005). Thus, replication with the help of the replicative polymerase Pol δ can be continued. Mutants of *ScRAD5* are sensitive to DNA-damaging agents (Johnson et al., 1992; Ulrich and Jentsch, 2000; Friedl et al., 2001) and show increased genomic instability of direct repeats (Liefshitz et al., 1998). Altered recombination has also been detected in *rad5* mutants, however, detailed mechanistic understanding is yet lacking (Ahne et al., 1997; Liefshitz et al., 1998; Friedl et al., 2001).

Several of the DNA repair pathways (e.g. nucleotide excision repair and HR) are conserved in higher plants (Britt, 1999; Kimura and Sakaguchi, 2006). After the recent identification of the translesion polymerases (Pol η, Pol ζ, and Pol κ) in Arabidopsis (*Arabidopsis thaliana*), the existence of at least two branches of PRR seems to be evident for plants, too (Sakamoto et al., 2003; Garcia-Ortiz et al., 2004; Takahashi et al., 2005; Santiago et al., 2006). However, until recently no indication had been provided for the existence of a Rad5-dependent branch of PRR in plants. Wen et al. (2006) identified and cloned two *UBC13* homologs from Arabidopsis, *AtUBC13A* and *AtUBC13B*, and performed an initial functional characterization with the help of heterologous systems. The authors showed physical interaction between the Arabidopsis Ubc13 and the yeast or human Mms2 protein. The *AtUBC13* genes were also able to complement the yeast *ubc13* null mutant for spontaneous mutagenesis and sensitivity to DNA-damaging agents. Thus, indirect hints for the existence of a Rad5 branch of PRR in plants could be supplied. Here we report on the identification and functional characterization of two closely related *RAD5* homologs in Arabidopsis. The fact that one of the genes is required for DNA repair and DSB-induced HR can be taken as a strong hint that a Rad5 branch of PRR is functional in plants, too.

RESULTS

Identification of Homologs of *ScRAD5* in Arabidopsis and Other Model Plants

A characteristic feature of ScRad5 protein is the unusual localization of a RING-finger domain within the Snf2-helicase domain combined with an N-terminal HIRAN domain. The HIRAN domain seems to mediate DNA binding (Iyer et al., 2006). To identify putative *RAD5* homologs in the Arabidopsis genome, a database search was carried out using TBLASTN and the protein sequence of ScRad5 as the template (GenBank M96644). The search resulted in a number of hits, of which the two most significant were At5g22750 and At5g43530, both possessing the conserved domain feature (Fig. 1A). Upstream of the Snf2 family N-terminal domain, a HIRAN domain could be detected by Pfam database searching. At5g22750 shares 30% identity and 49% similarity and At5g43530 shares 28% identity and 44% similarity with ScRad5 over the entire protein. A higher identity was obtained when amino acid sequence of functional domains was compared. At5g22750 and At5g43530 both share with ScRad5 to about the same extent of identity in the Snf2 family N-terminal domain (30%), the RING-finger (40%), and the helicase C-terminal domain (50%). We refer to the genes of At5g22750 and At5g43530 as *AtRAD5a* and *AtRAD5b*, respectively. These two genes, together with 10 others, had been assigned before to form a putative Arabidopsis *RAD5/RAD16*-like gene family (Shaked et al., 2006). As depicted in Figure 1B a closer relationship between At5g22750, At5g43530, and ScRad5 can be seen with a phylogenetic tree comparing the sequences of the 12 most closely related Arabidopsis genes and ScRad5. The high conservation (identity 55%, similarity 78%) between AtRad5a and AtRad5b and the similar intron-exon structures (data not shown) indicate that the two Arabidopsis genes arose from a duplication event late during plant evolution (Hartung et al., 2002). To obtain the full-length complementary DNA (cDNA) for *RAD5a* and *RAD5b*, RACE-PCR was applied (Matz et al., 1999). According to our analysis the open reading frame (ORF) of *RAD5a* has a length of 3,090 bp, contains 20 exons, and encodes a protein of 1,029 amino acids (Figs. 1A and 2A). This is in line with the sequence of a cDNA clone (AK228695) from the GenBank. The ORF of *RAD5b* has a length of 3,834 bp, contains 18 exons, and encodes a protein of 1,277 amino acids (Figs. 1A and 2D). Sequence homology among ScRad5, AtRad5a, and AtRad5b is mainly limited to the conserved helicase domain, the RING-finger, and the central part of the HIRAN domain (Fig. 1C). Snf2 family N-terminal domain and the helicase C-terminal domain of AtRad5a and AtRad5b were also compared with that from five representative members of other Snf2 subfamilies in the Arabidopsis genome: At3g19120 (AtRad54), At2g18760 (a homolog of Ercc6/Rad26), At1g08600 (Atrx), At2g46020 (AtBrm), and At5g44800 (Mi-2). Here a lower sequence identity of about 23% and 38% in the Snf2 family



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HIRAN-central
ScRAD5      FDIQYDREIGRVSEDIAQILYPL 253
AtRAD5a     FSTKDSGEIGRIPNEWARCLLPL 194
AtRAD5b     FSTKRCGEIGRLPMEWSNWAVSL 461
            *. :   ****:. : :.   .*

Helicase I                                  Helicase Ia
ScRAD5      GGILSDEMGLGKTV 540              ScRAD5      LIVVPMSLL 597
AtRAD5a     GGILADAMGLGKTV 427              AtRAD5a     LIVCPMTLL 501
AtRAD5b     GGILADAMGLGKTV 695              AtRAD5b     LIICPMALL 752
            ****:* *******                              **: **:**

Helicase II                                 Helicase III
ScRAD5      RIIIDEGHNIRNRTTVTS 694          ScRAD5      KWVLTGTPIINRLDDLYSLVKF 726
AtRAD5a     RIVLDEAHTIKNSKSQIS 586          AtRAD5a     RWCLTGTPIQNNLEDLYSLLRF 618
AtRAD5b     RIVLDEAHTIKSWKTQAA 835          AtRAD5b     RWCLTGTPLQNKLEDLYSLLCF 867
            **::**.*.*:. .:  :              :* *****: *.*:*****: *

RING-finger
ScRAD5      CSICTTEPMDLDKALFTECGHSFCEKCLFEYIEFQNSKNLGLKCPNC 960
AtRAD5a     CPICLEA---LEDAVLTPCAHRLCRFCLLASWRNSTSG----LCPVC 833
AtRAD5b     CPICLES---ADDPVLTPCAHRMCRECLLTSWRSPSCG----LCPIC 1079
            *.**       :..::* *.* :*.:**:   .  ..      ** *

Helicase IV                                 Helicase V
ScRAD5      VIFSQFSTYLDILEKELT 1033         ScRAD5      ILLLSLKAGGVGLNLTCASHAYMMD 1096
AtRAD5a     ILFSQWTAFLDLLQIPLS 900          AtRAD5a     VLLMSLKAGGVGINLTAASNAFVMD 957
AtRAD5b     IVFSQWTSFLDLLEIPLR 1148         AtRAD5b     ILLMSLKAGGVGLNLTAASSVFLMD 1205
            ::***::::**:*:  *               :**:********:***.** .::**

Helicase VI
ScRAD5      QAIDRLHRIGQTNSVKVMRFI 1126
AtRAD5a     QAVMRIHRIGQTKEVKIRRFI 987
AtRAD5b     QAIMRIHRIGQKRTVFVRRFI 1235
            **: *:*****.. * : ***
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Figure 1. Sequence comparison of AtRad5a, AtRad5b, and ScRad5. A, Schematic representation of the domain arrangement. The HIRAN domain on the N-terminal part, the centrally localized Snf2 family N-terminal domain (SNF2_N), followed by the embedded C3HC4-type RING-finger (R), and the helicase C-terminal domain (Hel_C) are conserved in all three proteins. B, Phylogenetic tree of Arabidopsis *RAD5/RAD16* genes. Protein sequences of 12 Arabidopsis Rad5/Rad16 candidates and the yeast Rad5 and Rad16 (ScRad5 and ScRad16) were aligned with the program ClustalW2 using standard parameters, and the tree was derived from calculated average distances using BLOSUM62. The two genes sharing the highest homology with the yeast Rad5 and Rad16 are indicated, respectively. The distances to the nodes are given. C, Sequence alignments of the characteristic domains. Central part of the HIRAN domain including the invariant Gly residue, the helicase motifs I, Ia, II, and III (which constitute the SNF2_N domain), the RING-finger, and the helicase motifs IV, V, and VI (which constitute the helicase C-terminal domain) are aligned. Identical amino acids are indicated by an asterisk (*); conserved and semi-conserved substitutions are indicated by a colon (:) and period (.), respectively.

N-terminal domain and the helicase C-terminal domain, respectively, was obtained. Both the HIRAN domain and the embedded RING-finger characteristic of AtRad5 are absent in the members of the other Snf2 subfamilies. These results indicate that the *AtRAD5* genes differ from the members of other Snf2 subfamilies in Arabidopsis.

We were wondering at what point in time during evolution the duplication of *RAD5* might have occurred. We searched for the presence of *RAD5* homologs in two other model plants, rice (*Oryza sativa*) and the moss *Physcomitrella patens*, for both of which the complete genome sequence is available (Ouyang et al., 2007; http://www.mossgenome.org). Searching the genome database we identified two entries (GenBank accession nos. EAZ23279 and CAE04094) in rice showing high homology to both AtRad5a and AtRad5b. The protein sequence of EAZ23279 had a slightly higher identity (identity 56%/similarity 67%) than CAE04094 (identity 53%/similarity 69%) to AtRad5a. Vice versa, the sequence of CAE04094 shared higher homology (identity 56%/similarity 72%) with AtRad5b (identity between AtRad5b and EAZ23279 44%; similarity 59%). We therefore tentatively referred to EAZ23279 as OsRad5a and to CAE04094 as OsRad5b. Interestingly, a lower identity of 43% and a similarity of 57% were observed between the two rice homologs, compared to the higher homology between homologs from both plants (AtRad5a to OsRad5a and AtRad5b to OsRad5b). Notably, in *Physcomitrella* we could identify only one closely related Rad5 homolog, the *Physcomitrella* protein identification number 207739. (We suggest referring to this protein as PpRad5. For protein identification, see the *Physcomitrella* Web site [http://genome.jgi-psf.org/cgi-bin/searchGM?db=Phypa1_1].) PpRad5 showed a similar homology to both AtRad5a and AtRad5b. It is tempting to speculate that the duplication of *RAD5* occurred during plant evolution and before the split of mono- and dicotyledonous plants. However, we cannot fully exclude the alternative explanation that one *RAD5* homologs was lost from the *Physcomitrella* genome.

Expression of *AtRAD5a* and *AtRAD5b*

Transcription levels of *AtRAD5a* and *AtRAD5b* were determined by real-time PCR. Both genes are expressed in whole seedlings and in adult tissues (flowers, leaves, and stems of 5- to 6-week-old plants), however, the expression level of *AtRAD5b* is lower with about 10% of that of *AtRAD5a*. No obvious tissue-specific expression of either gene was detected. A slightly higher transcript level of both genes is present in flowers and a slightly lower one is present in leaves among tissues analyzed. Mutagen treatments with methyl methane sulfonate (MMS), UV, γ-ray, or bleomycin (see ''Materials and Methods'') virtually did not alter the expression of *AtRAD5b*, however, γ-ray and bleomycin caused a moderate increase (about 2-fold) of *AtRAD5a* transcripts (data not shown).

Atrad5a But Not *Atrad5b* Mutants Are Sensitive to Mutagens

Yeast *rad5* mutants are sensitive to a range of mutagens, e.g. UV, MMS, and γ-irradiation (Johnson et al., 1992; Ulrich and Jentsch, 2000; Friedl et al., 2001). To define the individual functions of *RAD5a* and *RAD5b* in Arabidopsis, T-DNA insertion mutants were identified.

T-DNA insertion mutants were screened on the SIGnAL Web site (Salk Institute Genomic Analysis Laboratory; Alonso et al., 2003). For each gene, two independent mutants were identified, propagated, and homozygous mutant lines were obtained. Figure 2 shows the insertion sites for mutant lines SALK_124891 (designated as *rad5a-1*) and SALK_047150 (designated as *rad5a-2*) in detail as determined via PCR. These two mutants carry left borders on both ends of the T-DNA insertion, indicating an integration of at least two T-DNAs in an inverted orientation. The T-DNA insertion in *rad5a-1* is located in the sixth exon, between the HIRAN domain and the helicase motif I. The insertion leads to a small deletion of 28 nucleotides and to an ectopic insertion of seven nucleotides in the exon. The T-DNA insertion in *rad5a-2* is located in the 15th intron, immediately downstream of the RING-finger. Here, a small deletion of four nucleotides in the intron is accompanied by a 31-nucleotide insertion. To monitor the expression level of *Atrad5a* in the mutants, right transcription (RT)-PCR experiments were performed with homozygous mutants using primers binding upstream, across, and downstream of the insertion sites (Fig. 2, A and C). For both mutant lines, expression upstream of the insertion could be detected at a similar level as in the wild type. Expression downstream of the insertion is decreased in *rad5a-1*, whereas wild-type level was detected in *rad5a-2*. By using primers across the insertions no full-length transcripts could be detected in both mutants. Figure 2D depicts the insertion sites for mutant lines SALK_126949 (designated as *rad5b-1*) and SAIL_1253_A04 (designated as *rad5b-2*). The *rad5b-1* mutant carries a left and a right border of T-DNA on each end, whereas *rad5b-2* carries left borders of T-DNAs on both ends, indicative of a tandem integration in inverted orientation of the latter. The T-DNA insertion in *rad5b-1* is located in the junction of second exon and intron, immediately downstream of the HIRAN domain. The insertion leads to a big deletion of 2.7 kb of the *RAD5b* gene and to a small insertion of 13 nucleotides. The T-DNA insertion in *rad5b-2* is located in the ninth exon, immediately downstream of the helicase motif Ia resulting in a small deletion of 13 nucleotides. The *RAD5b* expression upstream of the insertion sites in both *Atrad5b* mutants is similar to that in the wild type. Both mutants show a higher level of expression behind the insertion in comparison to the wild-type level (Fig. 2, D and F). However, expression across the insertion sites could not be detected in both *Atrad5b* mutants, indicative for the absence of full-length mRNA.

The *rad5a-1*, *rad5a-2* and *rad5b-1*, *rad5b-2* homozygous mutants were viable and did not show any deviation

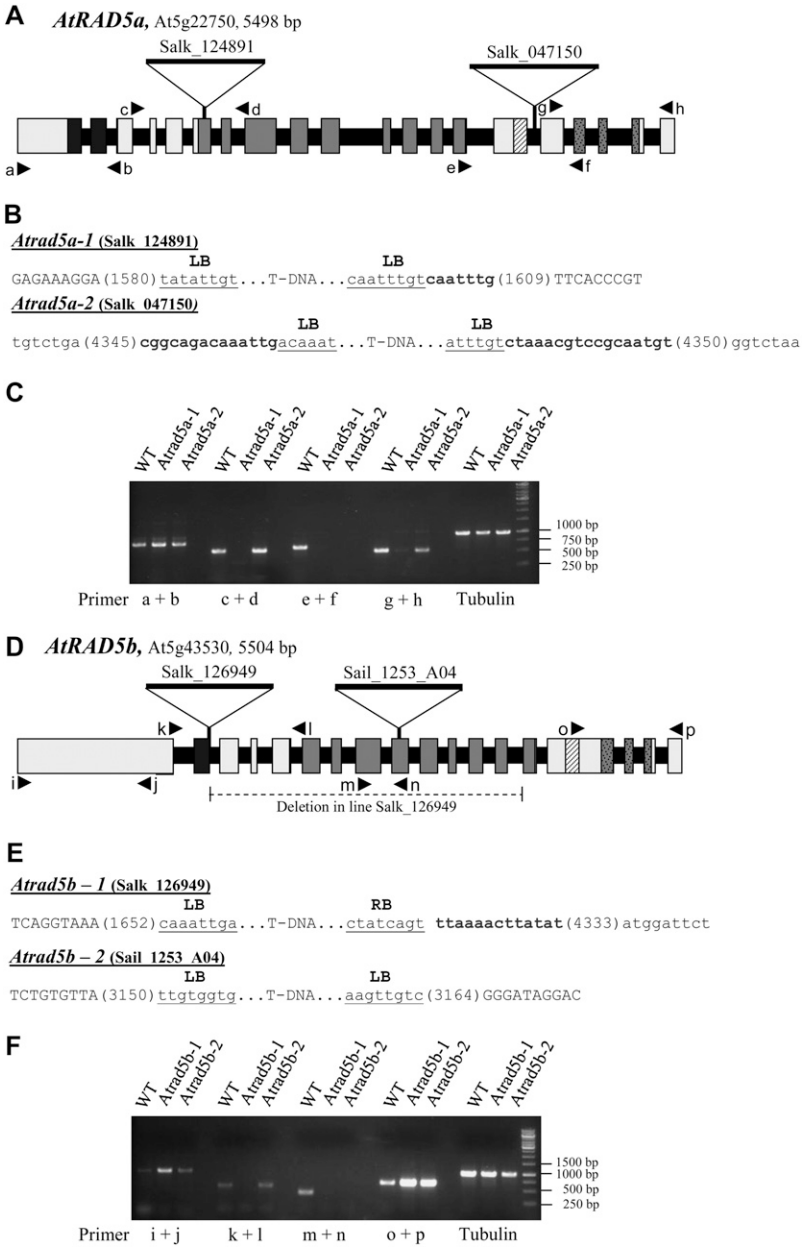

Figure 2. Gene structure and T-DNA insertion mutants of *AtRAD5a* and *AtRAD5b*. A, The *AtRAD5a* gene consists of 20 exons (shown as boxes). The exons coding for the functional domains are indicated as follows: the HIRAN domain (dark gray), the Snf2 family N-terminal domain (gray), the C3HC4-type RING-finger (hatched), and the helicase C-terminal domain (dots). Mutants *Atrad5a-1* and *Atrad5a-2* with the T-DNA insertion in the sixth exon and in the 15th intron, respectively, are depicted. B, Sequence determination of the insertion sites. Precise localization of the T-DNA insertion within *AtRAD5a* is shown. Exon sequences are displayed as uppercase letters, intron sequences as lowercase letters, and left or right border (LB or RB) sequences of the T-DNA are underlined. Small insertions are indicated in bold-type letters. C, Semiquantitative expression analysis. Expression of different regions of *AtRAD5a* was determined via RT-PCR. For *Atrad5a-1* the following primer pairs were applied: primers a+b for expression upstream of T-DNA insertion, primers c+d for expression across T-DNA insertion, and primers e+f and primers g+h for expression downstream of the insertion. For *Atrad5a-2* the following primer pairs were applied: primers a+b and c+d for expression upstream of T-DNA insertion, primers e+f for expression across T-DNA insertion, and primers g+h for expression downstream of the insertion. D, The *AtRAD5b* gene consists of 18 exons (shown as boxes). The exons coding for the functional domains are indicated as in A. Mutants *Atrad5b-1* and *Atrad5b-2* with T-DNA insertion in the junction of the second exon and intron and in the ninth exon, respectively, are indicated. E, Sequence determination of the insertion sites. Precise localization of the T-DNA insertion within *AtRAD5b* is depicted as in B. The T-DNA insertion in *Atrad5b-1* leads to a large deletion of 2.7 kb of the *RAD5b* gene and to a small insertion of 13 nucleotides. F, Semiquantitative expression analysis of different regions of *AtRAD5b* was determined via RT-PCR. Primer combinations for expression upstream of T-DNA insertion (primers i+j and additionally for *Atrad5b-2* k+l), for expression across T-DNA insertion (primers k+l for *Atrad5b-1* and m+n for *Atrad5b-2*), and for expression downstream of the insertion (o+p and additionally for *Atrad5b-1* m+n). The expression of β-tubulin serves as a control. WT, Columbia wild type.

from the wild-type phenotype under standard growth conditions. To elucidate possible roles of Rad5a and Rad5b in DNA repair, all mutant lines were challenged with a series of mutagens. The seeds were spread onto solid germination medium (GM) supplemented with the respective chemical. Growth of the seedlings was analyzed 21 d after germination. Severe impairment in growth could be detected for *rad5a-1* and *rad5a-2* mutants challenged with the cross-linking agent mitomycin C (MMC; Fig. 3, A and D). Even at a low concentration (1 μg/mL) of MMC growth of both lines (measured as dry weight) was already reduced to about 40% compared to the wild type (Columbia; data not shown). At 3 μg/mL, MMC the dry weight of these mutants dropped down to about 20% of the wild-type level. Although the mutant lines have been able to form their first real leaves, the leaves did not develop and remained small. We treated *rad5a-1* and *rad5a-2* with another cross-linking agent, cis-platin, and again hypersensitivity could be demonstrated (Fig. 3, B and D). Moderate sensitivity to a methylation agent, MMS, was found for both *rad5a* mutants as well (Fig. 3, C and D). After treatments with bleomycin or γ-irradiation, *rad5a-1* and *rad5a-2* did not display a significant sensitivity (data not shown). In contrast to the *rad5a* mutants, no sensitivity could be detected for any mutagens tested with both *rad5b* mutants (Fig. 3). We generated a double mutant, *rad5a-2/rad5b-2*, to test whether putative genetic interactions between both genes might exist. The double mutant showed no deviation from

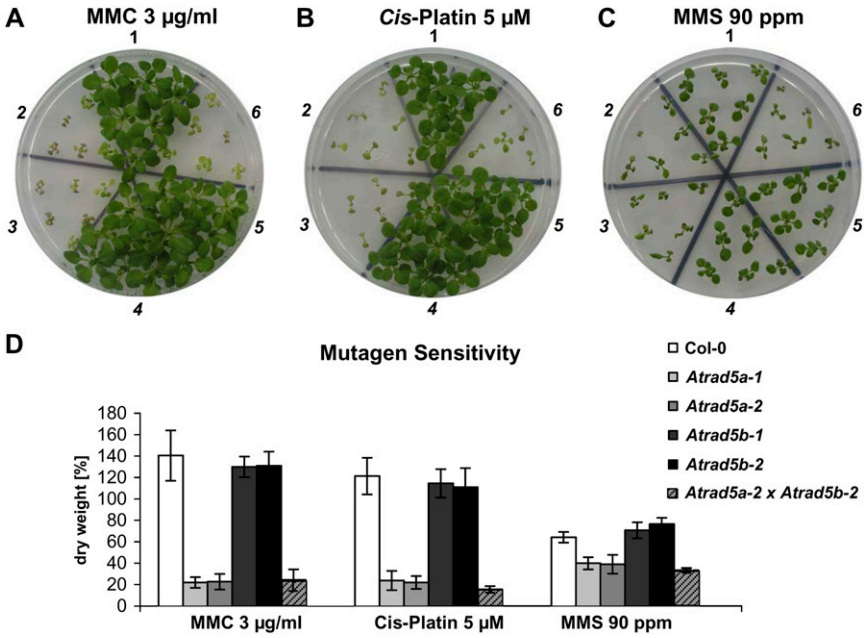

Figure 3. *Atrad5a* but not *Atrad5b* mutants are sensitive to cross-linking agents and MMS. *Atrad5a* and *Atrad5b* mutants as well as the double mutant *Atrad5a-2/Atrad5b-2* were tested for their sensitivity to the cross-linking agents MMC (A), cis-platin (B), and methylating agent MMS (C). Line 1, Columbia wild type; line 2, *rad5a-1*; line 3, *rad5a-2*; line 4, *rad5b-1*; line 5, *rad5b-2*; line 6, *rad5a-2/rad5b-2*. Seeds were plated onto GM containing 3 μg/mL MMC, 5 μM cis-platin, or 90 ppm (117 μg/mL) MMS, after 20-d seedlings were analyzed. D, The diagram shows the sensitivities of the respective mutants upon treatment with MMC, cis-platin, and MMS at the indicated concentrations. Dry weight of treated seedlings was compared with that of untreated ones. The results were obtained from at least three independent experiments and error bars indicating SD are given.

the wild-type phenotype and no apparent meiotic defects as judged by the efficiency of the seed set under standard growth conditions and exhibited, after treatment with DNA-damaging agents, a phenotype resembling those of the *rad5a* mutants (Fig. 3). Thus, AtRad5a has an important role in the repair of DNA cross-links and methylated bases. In contrast, functions of AtRad5b seem to be dispensable in this respect.

The *rad5a* Mutants Exhibit a Hyporecombination Phenotype after Induction of DSBs by Bleomycin

In addition to a defect in DNA repair, yeast *rad5* mutants also showed a change in their recombination behavior (Ahne et al., 1997; Liefshitz et al., 1998; Friedl et al., 2001). To determine whether the frequency of HR is also affected in the Arabidopsis *rad5a* and *rad5b* mutants, we determined HR frequencies in mutants by means of a well-established in planta recombination assay (Swoboda et al., 1994). For this assay, the mutants were crossed with the transgenic line 651 that harbors a recombination substrate in its genome. The substrate consists of two fragments of the GUS gene (*uidA*) interrupted by a hygromycin resistance gene. The two GUS fragments share an overlap of 566 bp in an inverted orientation. A functional GUS gene can be restored by intra- as well as interchromosomal recombination (Schuermann et al., 2005). Cell clusters expressing GUS activity can be visualized as blue sectors after histochemical staining. Plants homozygous for *rad5a*, or *rad5b* and the transgene 651 were screened and used for the assay. Seven-day-old seedlings were transferred from solid GM to liquid medium with or without mutagens. Recombination events were counted in 14-d-old seedlings. Under standard conditions (GM without mutagen), HR frequencies comparable to those of the wild type were obtained from both *rad5a* mutants (Fig. 4, A and B; Table I). However, after induction of DSBs by bleomycin (5 μg/mL) both lines revealed a strong decrease with an approximate 5-fold reduction of HR (Fig. 4, C and D; Table I). In contrast, with or without bleomycin, the number of HR events in *651/rad5b-1* and *651/rad5b-2* seedlings did not differ from the control (Table I). Thus, AtRad5a but not AtRad5b is involved in the regulation of HR after induction of DSBs. As the *Atrad5a* mutants were sensitive to cross-linking agents we also tested whether HR was disturbed after MMC treatment. Due to the high sensitivity of both *Atrad5a* mutants to MMC (see mutagen sensitivity above), we applied a lower concentration (1 μg/mL) of MMC that does not cause drastic growth defects. Indeed, HR was induced by this treatment. However, in contrast to bleomycin, no significant difference in the induction between the wild type and mutants was found (see Supplemental Table S1). This can be taken as an indication that the function of AtRad5a in cross-link repair is not directly linked to HR.

DISCUSSION

By sequence comparisons we identified in Arabidopsis two *ScRAD5* homologs, *AtRAD5a* and *AtRAD5b*. Both genes are more closely related to each other than to *ScRAD5* and seem to have arisen by a duplication event. The fact that rice harbors two *RAD5* homologs in its genome but moss *Physcomitrella* only one indicates that the duplication event apparently occurred during plant evolution after the separation of moss and higher plants but before the divergence of mono- and dicotyledonous species. However, we cannot totally exclude

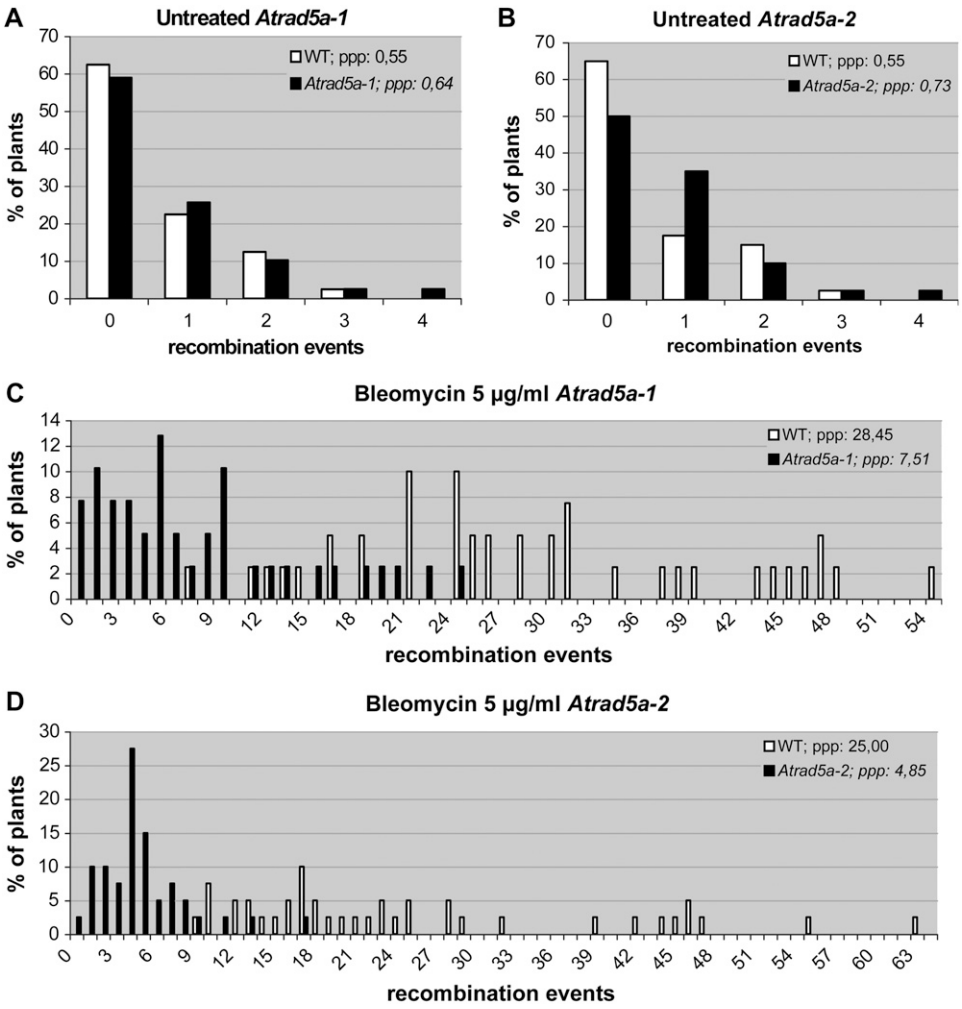

Figure 4. HR in the *Atrad5a* mutants. The representative diagrams show the percentage of seedlings with a given number of blue sectors reflecting HR events from individual experiments. A, Untreated *651/Atrad5a-1* line. B, Untreated *651/Atrad5a-2* line. C, Bleomycin (5 μg/mL) treated *651/Atrad5a-1* line. D, Bleomycin (5 μg/mL) treated *651/Atrad5a-2* line. *651/Atrad5a* seedlings are displayed as black bars; the control siblings homozygous for *651/AtRAD5a* are shown as white bars. ppp, Points per plant.

the possibility that a homolog has been lost in *Physcomitrella*.

The DNA Repair Function of Rad5 Is Conserved in Arabidopsis

In yeast, *rad5* mutants are sensitive to UV radiation and other DNA-damaging agents (Johnson et al., 1992; Ulrich and Jentsch, 2000; Friedl et al., 2001). We were able to demonstrate that Arabidopsis *rad5a* mutants are extremely sensitive to the cross-linking agents MMC and cis-platin even at very low concentrations (Fig. 3). We have to stress that although both MMC and cis-platin damage DNA by inducing cross-links, the treatment with the former results preferentially in interstrand cross-links and with the latter in intrastrand cross-links (Eastman, 1985; Rink et al., 1996). Thus, distinct repair mechanisms might be involved in the repair of both kinds of damage (Moggs et al., 1997; Dronkert and Kanaar, 2001; Lee et al., 2006). It has been reported before that cross-links might be repaired by PRR. A defect of the *REV3* gene encoding the catalytic subunit of DNA polymerase Pol ζ involved in the error-prone branch of PRR results in sensitivity to cross-links in Arabidopsis (Sakamoto et al., 2003). Furthermore, AtRev1 and AtRev7, two other PRR components, seem to contribute to the repair of cis-platin-induced damage in Arabidopsis, although only to a minor extent (Takahashi et al., 2005). Our results can be taken as a hint that PRR can contribute to repair cross-links in plants. In addition, a moderate sensitivity to a methylating agent, MMS, was observed for the *Atrad5a* mutants, a sensitivity that had previously been reported for the *Scrad5* mutants.

AtRad5 Is Involved in DSB-Induced HR in Somatic Cells

Alterations in recombination were described for yeast *rad5* mutants (Ahne et al., 1997; Liefshitz et al., 1998). However, depending on the recombination substrates used, different effects on recombination frequencies were detected, and a specific role of ScRad5 in respect to HR could not be defined. Liefshitz et al. (1998) reported that recombination between direct repeats and gene conversion have been increased in *Scrad5* mutants. In contrast, loss of ScRad5 decreased HR frequencies when the repair of plasmid with a

Table I. *Somatic HR frequencies in mutants and segregated control plants: Atrad5a-1, Atrad5a-2, Atrad5b-1, and Atrad5b-2*

Plants, Number of plants tested; events, total number of blue sectors; ppp, the mean value of blue sectors per plant for each experiment (ppp1, control; ppp2, mutant; *calculated from the means of the three experiments). Bleomycin concentration, 5 μg/mL. The variation observed between the HR frequencies of control plants after induction with bleomycin might be due to the fact that experiments RAD5a-1, RAD5a-2 and RAD5b-1, RAD5b-2 were performed independently with different batches of bleomycin. Therefore, the comparison of the ratios (right column) is most relevant for the evaluation of the role of the mutant phenotypes on HR.

Control			Mutant Line			Ratio
Plants	Events	ppp1	Plants	Events	ppp2	ppp2/ppp1
RAD5a-1			*rad5a-1*			
No genotoxic stress			No genotoxic stress			
40	37	0.93	40	68	1.70	1.83
40	22	0.55	39	25	0.64	1.16
40	19	0.48	40	18	0.45	0.94
Mean		0.65 ± 0.24			0.93 ± 0.67	1.31*
Bleomycin			Bleomycin			
40	1383	34.58	40	229	5.73	0.17
40	1138	28.45	39	293	7.51	0.26
40	845	21.13	40	144	3.60	0.17
Mean		28.05 ± 6.73			5.61 ± 1.96	0.20*
RAD5a-2			*rad5a-2*			
No genotoxic stress			No genotoxic stress			
40	22	0.55	40	29	0.73	1.32
40	21	0.53	40	20	0.50	0.94
40	14	0.35	40	26	0.65	1.86
Mean		0.47 ± 0.11			0.63 ± 0.12	1.37*
Bleomycin			Bleomycin			
40	1000	25.00	40	189	4.73	0.19
40	846	21.15	40	192	4.80	0.23
40	617	15.50	40	212	5.33	0.34
Mean		20.55 ± 4.78			4.95 ± 0.33	0.25*
RAD5b-1			*rad5b-1*			
No genotoxic stress			No genotoxic stress			
40	28	0.70	40	35	0.88	1.26
40	65	1.63	40	48	1.20	0.73
39	28	0.72	40	24	0.60	0.83
Mean		1.02 ± 0.53			0.89 ± 0.30	0.94*
Bleomycin			Bleomycin			
40	2072	51.80	40	1596	39.90	0.77
40	2521	63.03	40	2596	64.90	1.03
40	2135	53.38	40	2266	56.65	1.06
Mean		56.07 ± 6.08			53.82 ± 12.74	0.95*
RAD5b-2			*rad5b-2*			
No genotoxic stress			No genotoxic stress			
40	41	1.03	40	33	0.83	0.81
40	52	1.30	40	36	0.90	0.69
40	38	0.95	39	23	0.59	0.62
Mean		1.09 ± 0.18			0.77 ± 0.16	0.71*
Bleomycin			Bleomycin			
40	2515	62.88	40	2605	65.13	1.04
40	3232	80.80	40	3248	81.20	1.00
40	2208	55.20	40	2091	52.28	0.95
Mean		66.29 ± 13.14			66.20 ± 14.49	1.00*

169-bp gap in the *URA3* gene by using the homologous sequence in the chromosome was analyzed (Ahne et al., 1997). In our study, we found a strong reduction in chromosomal HR frequencies of an inverted repeat for the *Atrad5a* mutants after induction of DSBs via bleomycin treatment. Therefore, indeed, Rad5 might only be required for the efficient performance of HR in case a DSB is induced. It was suggested by Ahne et al. (1997) that ScRad5 might play an important regulatory role in avoiding nonhomologous end joining in yeast. The data obtained in this study are in line with this hypothesis. It is interesting to compare the phenotype of other Arabidopsis DNA repair mutants with that of the *Atrad5a* mutants. Interestingly, mutants of genes

involved in HR or its regulation (e.g. *bard1*, *rad51C*, and *rad54*) are also sensitive to cross-linking agents (Abe et al., 2005; Osakabe et al., 2006; Reidt et al., 2006; Shaked et al., 2006). Moreover, they are deficient in intrachromosomal HR, but in contrast to *rad5a* not only after bleomycin treatment but also without DSB induction. In contrast, for *Atmus81* (Hartung et al., 2006) a defect in HR was only found after DSB induction. The *Atmus81* mutants were also sensitive to cross-linking agents, however, the *Atmus81* mutants showed a higher sensitivity than *Atrad5a* mutants to MMS. Although, to our knowledge, the kind of DNA repair phenotype of *Atrad5a* had not been previously described for other Arabidopsis mutants, it will be of great interest to test (with the help of double mutants) whether *AtRAD5a* is indeed not epistatic to the other known factors involved in the repair of DSBs, cross-links, or methylation-induced DNA damages. The fact that we did not detect a defect in HR when the *Atrad5a* insertion lines were treated with MMC can be taken as strong indication that AtRad5a is involved at least in two different DNA repair pathways, one dependent on and the other independent of HR factors.

Due to the presence of the Snf2 helicase/ATPase domain and the RING-finger E3 ubiquitin ligase domain in ScRad5, it has been speculated that these domains might have distinct (as well as overlapping or coordinating) functions. By means of disrupting the Walker-A box via substitution of two conserved amino acids that caused the loss of ATPase activity of ScRad5, Chen et al. (2005) demonstrated the involvement of the Snf2 helicase/ATPase domain in DSB repair. A mutation in the RING-finger domain that abolishes the interaction of ScRad5 with its E2 enzyme, however, did not impair DSB repair in yeast. To address the individual contribution of the AtRad5a domains to repair cross-links and to perform HR, the complementation of *Atrad5a* mutants with a correspondingly modified *AtRAD5a* gene might provide further details for dissection of the AtRad5 domain functions.

Other *RAD5*/*RAD16*-Like Genes in Arabidopsis

It is puzzling that higher plants maintain two *RAD5* homologs. Whereas *AtRAD5a* is necessary for DNA repair and the regulation of HR, *AtRAD5b* seems to be dispensable at least under our experimental conditions. According to our transcriptional analysis, both *AtRAD5a* and *AtRAD5b* are expressed in different tissues nearly in a ubiquitous manner, though expression of the latter one is weaker. This finding together with the fact that a *RAD5b* homolog is also present in monocotyledones and that all critical residues are conserved in the ATPase as well as in the RING-domain strongly disfavors the idea that the gene is nonfunctional. Because we were not able to elucidate the role of *AtRAD5b* with our experimental approaches, further experiments are required to define a function for this gene. Strictly speaking, our study could not supply any data that *AtRAD5b* is indeed also a functional *ScRAD5* homolog. Nevertheless, we cannot exclude that AtRad5b is involved in postreplication repair. Indeed, also in the case of AtRad5a our evidence on the involvement in PPR is indirect, based on a similar behavior of the *rad5* mutant of yeast in DNA repair and recombination.

Actually, the case of *AtRAD5a* and *AtRAD5b* genes is somehow reminiscent of the situation of Arabidopsis *RECQ4a* and *RECQ4b* genes (Hartung et al., 2000). Both genes are also derived from a recent duplication. Whereas *RECQ4a* is required for repairing DNA damage induced by MMS and MMC, no sensitivity could be detected for the *recq4b* mutants (Bagherieh-Najjar et al., 2005; Hartung and Puchta, 2006; Hartung et al., 2007). In addition, in the fission yeast (*Schizosaccharomyces pombe*) two *RAD54* homologs, *RHP54* and *TID1*, which also belong to the *SNF2* family, exist. Whereas *RHP54* is involved in DNA repair, no evident impairment upon mutagen treatments has been detected for the *tid1* mutant during haploid vegetative growth (Muris et al., 1997; Catlett and Forsburg, 2003). Instead, meiotic defects have been observed for the *tid1* mutants. For Arabidopsis *rad5a* and *rad5b* mutants and the double mutant, *rad5a-2/rad5b-2*, no apparent meiotic defects were observed as judged by the morphology of flowers and germination efficiency of the seeds.

Besides *AtRAD5a* and *AtRAD5b*, 10 other genes showed homology to *ScRAD5* to varying degrees in our homolog searching in the Arabidopsis genome (Fig. 1B). One additional gene (At5g05130) sharing a lower homology with *ScRAD5* than with *AtRAD5a* and *AtRAD5b* exists, for which the Rad5 typical domain arrangement (HIRAN/SNF2/RING-finger/helicase) is also conserved in the deduced protein sequence. However, no information about the function of At5g05130 is available, to our knowledge, until now.

Recently, the human protein Shprh (Motegi et al., 2006; Unk et al., 2006) that was able to perform polyubiquitylation of PCNA in vitro, has been suggested to be a functional homolog of Rad5 in human. Sequence homology between Shprh and Rad5 (from yeast) is only moderate and mainly confined to the helicase domain and the RING-finger. The HIRAN domain characteristic to Rad5, is absent in Shprh. Interestingly, the Arabidopsis ORF At2g40770 has the highest homology to human Shprh. However, the elucidation of the biological role of At2g40770 has not been reported yet.

Furthermore, in the Arabidopsis genome eight other genes are present, which, besides being similar to *RAD5*, have a high homology to *RAD16*. This is not surprising because *ScRAD5* and *ScRAD16* are homologous, too. The homology is restricted to the RING-finger and the Snf2 helicase domain (Bang et al., 1992; Eisen et al., 1995). A HIRAN domain is absent in ScRad16 as well as in the respective Arabidopsis homologs. We, therefore, assume that at least some of the protein products of these genes might be involved in excision repair in plants as is ScRad16 in yeast. Thus, there are several genes related to *RAD5* present in the Arabidopsis genome awaiting their functional characterization.

MATERIALS AND METHODS

Database Screening and Sequence Analysis

Sequence searches were performed using the WU-BLAST 2.0 program at The Arabidopsis Information Resource Web site. Multiple sequence alignment was done using ClustalW2, and the protein domain architectures were obtained from the Pfam database on the internet. To create a phylogenetic tree, protein sequences of 12 Arabidopsis (*Arabidopsis thaliana*) Rad5/Rad16 candidates and the yeast (*Saccharomyces cerevisiae*) Rad5 and Rad16 (ScRad5 and ScRad16) were aligned with the program ClustalW2 using standard parameters and the tree was derived from calculated average distances using BLOSUM62. Sequence analysis was performed with DNASTAR (Lasergene).

RNA Isolation and Full-Length cDNA Determination

DNA-free total RNA was isolated using the RNA isolation kit from QIAGEN. Removal of genomic DNA was achieved by in-column DNase-I digestion. First-strand cDNA was synthesized with M-MuLV reverse transcriptase using oligo$(dT)_{18}$ as primer (Fermentas). To obtain the *RAD5a* and *RAD5b* full-length cDNA from Arabidopsis a SMART protocol from Clontech was applied (Matz et al., 1999).

Transcriptional Analysis of *AtRAD5a* and *AtRAD5b* by Real-Time PCR

Expression levels of *AtRAD5a* and *AtRAD5b* were determined with real-time PCR using 60S RP L27A (At1g70600) as a reference gene in 2-week-old seedlings and in flowers, leaves, and stems of 5- to 6-week-old plants. Transcriptional response of *AtRAD5a* and *AtRAD5b* to genotoxins: MMS (100 ppm [130 μg/mL], 6 h; Sigma), UV (0.3 J, harvest 3 h postirradiation), γ-ray (75 Gy, harvest 3 h postirradiation), or bleomycin (3 μg/mL, 6 h; Duchefa) in 2-week-old seedling was conducted as previously described (Chen et al., 2003). Sequences of primers for real-time PCR are given in Supplemental Table S2.

Characterization of the Arabidopsis Insertion Mutants

The *rad5a-1* (SALK_124891), *rad5a-2* (SALK_047150), and *rad5b-1* (SALK_126949) T-DNA insertion lines (all in Columbia background) were obtained from the SALK collection (Alonso et al., 2003) and the *rad5b-2* (SAIL_1253_A04) insertion line was obtained from the GARLIC collection of Syngenta Biotechnology. Homozygous mutants were identified with suitable primer combinations via PCR. Primers used for expression analysis of *RAD5a* and *RAD5b* in Columbia wild type and mutants are listed in Supplemental Table S3.

To generate the *rad5a-2 rad5b-2* double mutant, respective homozygous plants were crossed and the homozygous double mutant was identified in the F_2 progeny by PCR. For the HR assay different *651/rad5* lines were obtained via crossing homozygous *rad5a or rad5b* mutant lines with plants homozygous for the transgene *651*, which carries a scorable recombination substrate (Puchta et al., 1995). Siblings homozygous for both the *651* transgene and the *RAD5a* (or *RAD5b*) gene were used as control lines.

Growth Conditions and Mutagen Tests

Seeds of Arabidopsis were sterilized in 6% sodium hypochlorite for 7 min, and rinsed several times with sterile water. Plants were grown in chambers at 22°C under white light (16-h light/8-h dark). For mutagen tests sterilized seeds were spread onto solid GM-agar (Duchefa) containing different mutagens (MMS and cis-platin, Sigma; MMC and bleomycin, Duchefa). After 21 d, the effect of the respective mutagen on plant growth was evaluated. Phenotypes (e.g. plant growth rate and appearance of leaves, flowers, and seeds) of adult plants (6–8 weeks old) of the mutants were compared with those of the wild type (Columbia).

Quantification of Recombination Events in the Presence or Absence of Mutagens

One-week-old seedlings were transferred to liquid GM medium the day after the mutagen was added. Five days later the seedlings were subjected to GUS staining. Histochemical staining was performed as described by Swoboda et al. (1994). Blue sectors were counted using a binocular after the plants had been decolorized with 70% ethanol.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AK228695 (*RAD5a*) and NM_123719 (*RAD5b*).

Supplemental Data

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

Supplemental Table S1. Somatic HR frequencies in *rad5a* mutant lines and segregated control plants with and without MMC.

Supplemental Table S2. Real-time PCR primers for the transcriptional analysis of *AtRAD5a* and *AtRAD5b* genes.

Supplemental Table S3. Primers used for expression analysis of *Atrad5a* and *Atrad5b* mutants.

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