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Functional Conservation of the Yeast and Arabidopsis *RAD54*-Like Genes

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ABSTRACT

The *Saccharomyces cerevisiae RAD54* gene has critical roles in DNA double-strand break repair, homologous recombination, and gene targeting. Previous results show that the yeast gene enhances gene targeting when expressed in *Arabidopsis thaliana*. In this work we address the trans-species compatibility of Rad54 functions. We show that overexpression of yeast *RAD54* in Arabidopsis enhances DNA damage resistance severalfold. Thus, the yeast gene is active in the Arabidopsis homologous-recombination repair system. Moreover, we have identified an *A. thaliana* ortholog of yeast *RAD54*, named *AtRAD54*. This gene, with close sequence similarity to *RAD54*, complements methylmethane sulfonate (MMS) sensitivity but not UV sensitivity or gene targeting defects of *rad54*Δ mutant yeast cells. Overexpression of *AtRAD54* in Arabidopsis leads to enhanced resistance to DNA damage. This gene's assignment as a *RAD54* ortholog is further supported by the interaction of AtRad54 with AtRad51 and the interactions between alien proteins (*i.e.*, yeast Rad54 with AtRAD51 and yeast Rad51 with AtRad54) in a yeast two-hybrid experiment. These interactions hint at the molecular nature of this interkingdom complementation, although the stronger effect of the yeast Rad54 in plants than AtRad54 in yeast might be explained by an ability of the Rad54 protein to act alone, independently of its interaction with Rad51.

DNA repair and recombination are vital processes to maintain genome integrity, genetic variation, correct meiotic cellular division, and normal life of the organism. Many DNA repair proteins are well-conserved among eukaryotes, including plants (Britt and May 2003). Nevertheless, there are also differences between species regarding preferences in usage and efficiency of different DNA repair pathways. For example, in plants, the nonhomologous end-joining (NHEJ) pathway is more efficiently used and is thus preferred over homologous recombination (HR) for double-strand break (DSB) repair (Gorbunova and Levy 1999). The same also applies for integration of exogenous DNA into the genome, which in plants is executed mostly via a nonhomologous DNA repair pathway (Puchta *et al.* 1996; Mengiste and Paszkowski 1999). Homologous recombination involves tight regulation on many levels and many important and well-conserved components participate in the process. One such component that acts at a critical point during HR is the yeast gene *RAD54* and its homologs in mammals, chicken, Drosophila, and fission yeast (Kanaar *et al.* 1996; Muris *et al.* 1996; Bezzubova *et al.* 1997; Kooistra *et al.* 1999). This gene belongs to the *RAD52* epistasis group and in yeast affects mostly mitotic cell recombinational repair between sister chromatids, while having little effect on meiosis. Another important homolog of *RAD54* that acts primarily in meiosis in yeast is the gene *TID1/RDH54*, which acts in recombinational repair between homologous chromosomes (Arbel *et al.* 1999). The effect of Rad54 on recombination and repair is thought to occur via recruitment by Rad51, at sites of DNA breaks, assisting the strand invasion and homology search process (Alexeev *et al.* 2003; Mazin *et al.* 2003). The Rad54 protein has motifs similar to those found in the switch2/sucrose nonfermenting2 (Swi2/Snf2) superfamily (Eisen *et al.* 1995), members of which are chromatin modification-related proteins. DNA-dependent ATPase, ATP-dependent chromatin remodeling activities, and ability to translocate on dsDNA (Heyer *et al.* 2006) have been found for the Rad54 protein, but helicase activity has not been shown for this protein, nor for any other member of the Swi2/Snf2 family (Peterson and Tamkun 1995).

Mutations and disruptions of *RAD54* homologs have been studied in different species. In *Saccharomyces cerevisiae* (Kunz and Haynes 1981), chicken (Bezzubova *et al.* 1997), mouse cells (Essers *et al.* 1997), and the fission yeast *Schizosaccharomyces pombe* (Muris *et al.* 1996), these have a deleterious effect on DNA repair. The mutant cell lines are sensitive to methylmethane sulfonate (MMS) and ionizing radiation and integrate exogenous DNA very ineffectively (Arbel *et al.* 1999). In

Sequence data from this article have been deposited with the GenBank Data Libraries under accession no. DQ912973 for *AtRAD54*.

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Drosophila, a *RAD54* homolog was found to be involved in resistance to X-rays and in recombination repair (Kooistra *et al.* 1999). It was also found that the human homolog of *RAD54* can partially relieve the MMS-sensitive phenotype of *S. cerevisiae rad54*Δ cells (Kanaar *et al.* 1996). These findings suggest that a functional homolog of *RAD54* could also be found in other organisms, like the plant *Arabidopsis thaliana*.

Recently, it was shown that expression of the budding yeast gene *RAD54* in Arabidopsis plants is associated with enhanced frequencies of gene targeting (Shaked *et al.* 2005), suggesting that *RAD54* orthologs may be involved in DNA repair in the plant system and that some degree of conservation exists between the yeast and plant systems. Orthologs of *RAD54* may be used to further manipulate recombination and gene targeting levels in plants, thus facilitating research and genetic manipulations in agriculture.

Another recent study (Osakabe *et al.* 2006) had identified a putative Arabidopsis ortholog of *RAD54*, which interacts with *AtRAD51* in a yeast two-hybrid system. When mutated, the mutant lines for this gene were sensitive to different kinds of DNA damage, and showed reduced levels of inter-/intrachromosomal recombination.

In the present study, we provide additional evidence that the same Arabidopsis gene is a functional homolog of yeast *RAD54*.

We have cloned the gene and have shown that it partially relieves DNA repair defects of yeast *rad54*Δ cells. Our identification of this gene as *AtRAD54* was further supported by its interaction with *AtRAD51* in a yeast two-hybrid system, similar to a former work (Osakabe *et al.* 2006). This plant Rad54 homolog also interacts with the yeast Rad51 in a two-hybrid experiment, showing conservation of molecular mechanisms across kingdoms. We also show that the yeast *RAD54* gene, when overexpressed in Arabidopsis, enhances resistance of the plant to radiation of different kinds and interacts with AtRad51 in a yeast two-hybrid system. Moreover, overexpressing *AtRAD54* in plants results in the same phenotype as overexpression of the yeast *RAD54* in Arabidopsis, namely, increased resistance to ionizing radiation compared to the wild type. These results show conservation as well as partial compatibility between the yeast and plant systems in terms of HR repair.

MATERIALS AND METHODS

Cloning of plant genes and their expression in yeast: The plant gene At3g19210, in its protein-coding form (without introns), was cloned by PCR from cDNA of two-leaf-stage plants. Primers were designed according to the predicted sequence of the gene. (primer 1, 5′-CGGGATCCATGGAG GAAGAAGATGAAGAGATCT; primer 2, 5′-CGGAATTCTCA TACAAAATCATCATCGTGATTT). The primers contained *Bam*HI restriction sites. The PCR product was subcloned into the *Bam*HI restriction site in the pRS426-gal1p (Gal1 promoter, a galactose inducible promoter) yeast expression vector (Mumberg *et al.* 1995). Arabidopsis gene At5g63950 cDNA was ordered from SALK seed bank (clone no. R21465, see http://signal.salk.edu/index.html) and cloned directly into the pMBLArt binary vector. For expression in yeast, the cDNA was cut from the plasmid using *Eco*RI and cloned into a pDrive cloning vector using a PCR cloning kit (QIAGEN). The fragment was cut using *Eco*RI, and recloned into the pRS426-gal1p plasmid using the same site. A control gene (*RAD54* of *S. cerevisiae*) was amplified with large upstream and downstream regions, cut using *Spe*I and *Sal*I and cloned into pRS426 and pRS426-gal1p plasmids, into the same restriction sites. Both vectors contained the *URA3* marker (all inserts were verified by sequencing). The vectors were transformed into yeast cells using the LiAc method (Schiestl and Gietz 1989).

Yeast strains: Strain MKP15, used for the complementation experiments, was of YPH background, with the *rad54*Δ mutation inserted into strain YPH857 by the one-step replacement method (Rothstein 1991); MKP15 has the genotype *MAT***a**, *can1^r, leu2, ade2, trp1, ura3, his3, rad54*Δ*::HIS3*.

For the intragenic recombination assay we used the following strains:

OH1X2: *MAT***a**/*MAT*α, *ho:LYS2/ho:LYS2, lys2/lys2, ura3/ura3, ADE2/ade2, TRP1/trp1, leu2::hisG/leu2::hisG, his4B::LEU2/his4X::LEU2-Bam*HI-*ura3* (mutated on 5-FOA).

AA9X10: *MAT***a**/*MAT*α, *ho:LYS2/ho:LYS2, lys2/lys2, rad54::ura3* (mutated on 5-FOA)/ *rad54::ura3* (mutated on 5-FOA), *ura3/ura3, leu2::hisG/leu2::hisG, his4B:LEU2/his4X::LEU2-Bam*HI-*ura3* (mutated on 5-FOA).

For the two-hybrid experiment we used Clontech (Mountain View, CA) strain AH109.

Genotoxicity assay for yeast cells: MMS sensitivity was tested by growing yeast cells of strains MKP15 and YPH857 to logarithmic phase in liquid SC −Ura medium (either with glucose or with galactose), counting, and plating in serial dilutions on SC +glucose +MMS (0.06%), SC +galactose +MMS (0.06%), or on complete (SC) medium with glucose (Sherman 1991). After 4 days incubation at 30°, appearance of colonies was assayed and recorded.

UV sensitivity was tested by growing yeast cells of the above two strains to logarithmic phase in liquid SC −Ura medium (either with glucose or with galactose), counting them, and plating on SC, or SC +galactose plates. After 2 hr at 30°, the plates were irradiated with UV (0.85 J/m^2/sec, standard General Electric 15-watt lamp) for the indicated times, wrapped immediately in aluminum foil (to prevent photorepair), and incubated at 30° for 4 days, after which colonies were counted.

Integration assay for yeast: A *Pvu*I-*Aat*II fragment from the plasmid pRS404, containing the *TRP1* marker and flanking sequences was either cut from the plasmid or amplified by PCR (the results of the two methods were indistinguishable) and transformed into recipient cells also containing *URA3* 2μ plasmids, either pRS426 or pRS426-Gal1p (a galactose inducible promoter) with the yeast or plant *RAD54* genes. To prevent plasmid loss, yeast cells were grown on medium lacking uracil (SC −Ura). The DNA fragment (500 ng) was transfected into yeast cells (2×10^7/ml) of strains MKP15 and YPH857 by the LiAc method (Schiestl and Gietz 1989). Before plating the cells, the cloned genes on the host plasmids were induced by incubating the cells in SC −Ura +galactose liquid medium for 60 min. The cells were subsequently plated on selective plates (either SC −Trp or SC −Trp +galactose). After 4 days of incubation at 30°, the number of emerging

colonies was recorded. Fifty plates were analyzed for every strain. To evaluate transformation efficiency of each strain, the same yeast strains were transformed with 50 ng of uncut DNA of the plasmid pRS424, also harboring the *TRP1* marker and plated on SC −Trp medium. The relative integration efficiency of each strain was calculated by dividing the number of colonies obtained in the transformation with the fragment by the number of colonies in the transformation with the uncut plasmid (pRS424), adjusted to the amounts of DNA used.

Intragenic recombination assay: Diploid yeast cells containing the heteroallelic *his4B/his4X* mutations were transformed either with *AtRAD54* or with the yeast *RAD54* harboring plasmids (mentioned above, pRS426-gal1p-*AtRAD54* and pRS426-*ScRAD54*). Recombination was monitored by the appearance of His^+ colonies on SC −His +Glucose or SC −His +galactose plates.

Expression of *ScRAD54* in Arabidopsis and genotoxicity assays in seedlings: *Cloning of ScRAD54:* To express the full length of the *S. cerevisiae* gene *RAD54* in Arabidopsis, we amplified it with primers containing the *Eco*RI and *Xba*I sites, cloned it into corresponding sites on the pArt7 vector (Gleave 1992), isolated the *Not*I insert from this construct, and cloned it into the same site of the pMBLArt binary vector, containing gluphosinate (BASTA) plant resistance, giving rise to plasmid pHS-35S*RAD54*.

Plant material and Agrobacterium-mediated transformation: Agrobacterium-mediated transformation was done in wild-type Arabidopsis plants (ecotype Columbia). Plant transformation was done by floral dipping and transformants (T_0) were selected by BASTA selection. T_0 plants were grown to maturity and the resulting T_1 seeds were used for further analysis.

γ- and UV irradiation procedure: To test γ-irradiation sensitivity of seedlings, seeds were surface sterilized, soaked overnight in distilled water at 4°, and then irradiated by a dose of 30 or 40 krad, provided by a ^{60}Co source from a Gammabeam 150 machine (Nordion, Kanata, Ontario, Canada) at the Radiation unit of the Weizmann Institute of Science. Plants were grown on 1/2 MS medium + 2% sucrose for 10 days. Growth conditions were 16 hr of light/day, at 25°. Seedlings were considered resistant to γ-irradiation if they developed at least two true leaves following irradiation, whereas sensitive seedlings had no true leaves or only one. All the seeds used in this experiment (transgenic plants or WT), when not irradiated, had germination rates >99%. This assay is similar to that described elsewhere (Hefner *et al.* 2003).

Yeast two-hybrid assay: Use was made of the Two-Hybrid kit (Clontech). Bait plasmid pAS1 was used to clone *AtRAD54* (in *Xho*I sites, keeping correct orientation of the genes with respect to the vector) and yeast *RAD54* (in the *Nco*I and *Bam*HI restriction sites). Prey plasmid pACT was used to clone both *AtRAD51* (Siaud *et al.* 2004) and yeast *RAD51* (with restriction sites *Xho*I and *Bam*HI). Plasmids pACT containing yeast *RAD51* and pAS1 containing *AtRAD54* were cotransformed into wild-type AH109 yeast cells using the LiAc method (Schiestl and Gietz 1989). Similarly, pAS1 containing yeast *RAD54* and pACT containing *AtRAD51* were cotransformed into the same yeast cells. We also cotransformed into yeast *AtRAD51* in pACT together with *AtRAD54* in pAS1 and yeast *RAD51* on pACT together with yeast *RAD54* on pAS1, as positive controls. Interaction between each pair of protein-coding plasmids was monitored by two steps of plate selection, first on SC −Leu −Trp medium, followed by SC −Leu −Trp −His +3AT [the latter is a potent histidine uptake antagonist, 3-amino-Triazole (Sigma, 5 mm final concentration)].

Overexpression of *AtRAD54* in Arabidopsis: To express the *AtRAD54* cDNA in plants, we isolated it from the pRS426 plasmid harboring the *AtRAD54* cDNA (previously cloned for yeast-expression purposes), using *Bam*HI restriction nuclease and cloned it into the same sites of the pArt7 vector. The new insert was isolated by *Not*I and cloned into the same site of the pMBLArt binary vector, giving rise to plasmid pHS-35S*AtRAD54*.

RT–PCR: RNA was extracted from mid-log-phase yeast cells using the RNeasy kit (QIAGEN). RT was performed by using the Superscript II enzyme (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Results were analyzed using standard 1% agarose TBE gel.

Western blot analysis: Extraction of proteins from yeast cells was done according to Knop *et al.* (1999), as was Western blotting. As first antibody we used the anti-AtRad54 antibody described in Osakabe *et al.* 2006 (kindly donated by S. Toki).

RESULTS

Identification of Arabidopsis *RAD54* homologs: A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) on the Arabidopsis genome using the sequence of yeast Rad54 protein (blastp) yielded ∼40 significant hits. These hits included all the previously analyzed *RAD54*-related-SWI2/SNF2 Arabidopsis genes (Verbsky and Richards 2001).

The Arabidopsis gene At3g19210 had the highest resemblance of its predicted protein sequence to the yeast Rad54 protein (*E* score value, E^{-120}), exceeding by far the other hits. We cloned the cDNA of this gene by PCR (as described below and in materials and methods) along with that of another member of the family (At5g63950, with significance score E^{-59} for resemblance of the predicted protein to that of yeast Rad54).

Gene and protein structure of At3g19210: Using the published genomic sequence of *A. thaliana* (TAIR) (http://www.arabidopsis.org/), we designed primers for cloning the cDNA of the gene At3g19210, by RT–PCR on a two-leaf-stage cDNA library. The cDNA thus obtained was sequenced and the sequence indicated that the gene comprises an open reading frame (ORF) of 2730 bp. The ORF of At3g19210 encodes a 910-amino-acid (aa) protein; *i.e.*, it is 58 aa longer than the length predicted by TAIR. At3g19210 is composed of 21 exons and 20 introns (Figure 1A), the boundaries of which are slightly different from the predicted gene structure (NM_112808). The ORF's full sequence is provided in supplemental Figure 1. This ORF's sequence is identical to that previously published (Osakabe *et al.* 2006).

Nine domains that are conserved among the Arabidopsis Swi2/Snf2 proteins were identified in At3g19210 by combining results obtained by three different bioinformatics methods (Shaked *et al.* 2006). Eight of these domains were similar in sequence and in order to the yeast Rad54 protein (Figure 1B). The yeast Rad54 protein and the protein encoded by At3g19210 show 38% identity and 54% similarity. The domains shared by these two proteins are similar among the Arabidopsis Rad54-like proteins. These include domains a, b, c, f, g, and h that are homologous to the Snf2-helicase-like domain. Domain e is homologous to a DNA-dependent ATPase, with a very strong DEAH signature (Eisen *et al.*

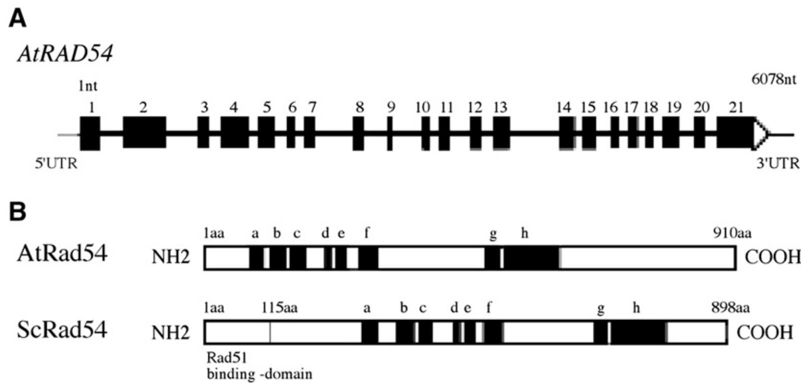

FIGURE 1.—Gene and primary protein structure of *AtRAD54*. The *AtRAD54* gene (A) is composed of 21 exons (shown as solid boxes) and 20 introns. The total gene length (5′-UTRs, exons, and introns) is 6078 bp. The length of coding sequence is 2733 bp. The encoded protein AtRad54 (B) is shown above the yeast Rad54 protein. Conserved domains (a–h) are shown as solid boxes and are drawn to scale.

1995). The function of domain d is still unknown. Another functionally important domain in the yeast gene is the region necessary for the interaction between Rad54 and Rad51 that is located within the NH2-terminal 115 residues (Jiang *et al.* 1996). This region is not conserved in any of the plant Rad54-like proteins (Figure 1B).

Complementation by plant genes of DNA repair deficiency in *rad54Δ* mutant yeast cells: The human ortholog of yeast *RAD54* has been shown to partly complement the DNA repair defect of yeast *rad54Δ* cells (Kanaar *et al.* 1996). To test whether members of the *A. thaliana RAD54*-like gene family are able to similarly function as a Rad54 substitute, we introduced cDNA constructs of two such genes into *rad54Δ* cells and tested for complementation of their DNA repair defect. This defect prevents *rad54Δ* cells from growing on media containing MMS, a mutagen that damages DNA (Chlebowicz and Jachymczyk 1979). The *rad54Δ* mutants are also more sensitive to UV radiation than wild-type cells, because of the involvement of Rad54 in the repair of some UV-damaged DNA molecules (Cole *et al.* 1987; Keszenman-Pereyra 1990).

Two Arabidopsis genes were cloned as cDNAs and transferred to a yeast expression vector with the inducible promoter of the gene *GAL1*: At3g19210, which is the *A. thaliana* gene closest to yeast *RAD54*, and At5g63950, another gene from the same SWI2/SNF2 family in Arabidopsis, but more distantly related to yeast *RAD54*. The *GAL1* promoter is active only in the presence of galactose in the medium. As shown in Figure 2, the plant gene At3g19210 remedies the inability of *rad54Δ* cells to grow on MMS plates; yeast cells containing the plant gene grew (divided) on galactose-containing MMS medium and not on glucose MMS medium, unlike the original *rad54Δ* cells, which did not grow on either medium. At3g19210 was thus named *AtRAD54*, being a true ortholog of yeast *RAD54*. RT–PCR (data not shown) and Western analyses (Figure 2g) both confirmed that the *AtRAD54* cDNA and protein were specifically expressed in medium containing galactose but not in a medium containing glucose. The other plant gene, At5g63950, did not complement the MMS sensitivity of *rad54Δ* cells in similar experiments (Figure 2), suggesting that the ability to alleviate the repair deficiency of *rad54Δ* is not a general property of *SWI2/SNF2* Arabidopsis genes but rather a specific property of *AtRAD54*.

We also tested these two plant genes for complementation of the repair deficiency of *rad54Δ* cells following UV radiation. Both genes, At3g19210 and At5g63950, did not complement the UV repair defect of the yeast *rad54Δ* cells (Figure 3).

It was previously shown that *rad54Δ* cells of budding yeast are defective in targeted integration of DNA fragments introduced by transfection (Arbel *et al.* 1999), probably due to the defect in the homologous recombination pathway. We have tested whether *AtRAD54* complements the *rad54Δ* defect in this respect. The assay was based on integration of a fragment from plasmid pRS404 into the genome of yeast strains containing another plasmid, pRS426 (see materials and methods). The results in Figure 4 show that the integration efficiency of the fragment in strain *rad54Δ* + pRS426 was reduced to 0.1% compared to 3.9% in strain yPH87 + pRS424 (wild type). The plasmid harboring *ScRAD54* partially rescued the *rad54Δ* deficiency by increasing the integration efficiency up to 1.4%. Thus, the *rad54Δ* mutation is indeed responsible for the low integration frequency in the yeast mutant, as originally proposed by Arbel *et al.* (1999). However, neither *AtRAD54* nor At5g63950 complemented the DNA integration defect of *rad54Δ* yeast cells. We also tested whether the presence of the *AtRAD54* gene elevated the generation of His^+ colonies in a heterozygous heteroallelic *his4B/his4X* strain. This strain harbors a different mutation on each allele of *HIS4*. A recombination event between the two mutation sites results in a functional allele and generation of a His^+ colony. Therefore an elevation in the level of His^+ colony generation might indicate a functional role of the gene in HR (see Xu *et al.* 1997 and materials and methods). The plant *AtRAD54* gene did not cause elevation of the level of His^+ colonies when transformed into either a *rad54Δ* strain or a *RAD54* strain (data not shown). It thus seems that the plant ortholog *AtRAD54* complements some DNA re-

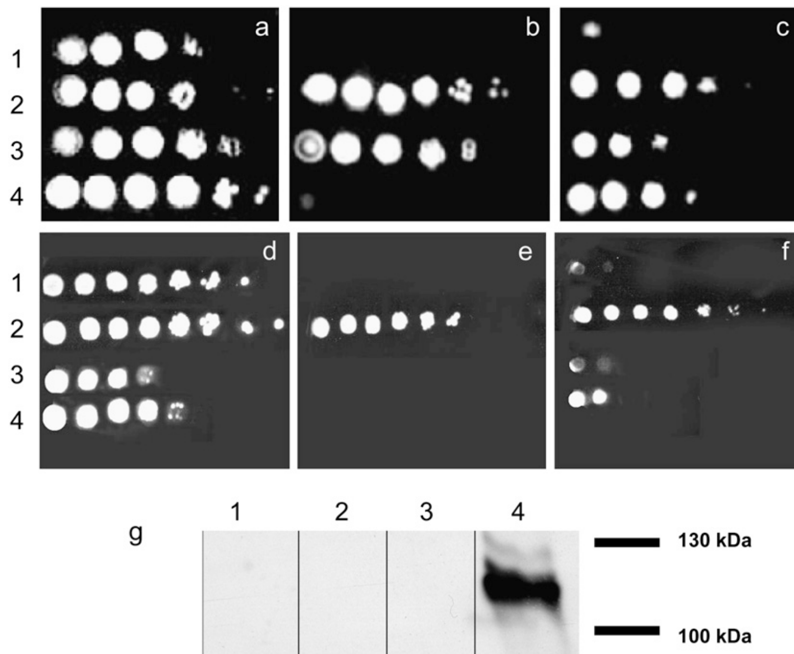

Figure 2.—Plant gene At3g19210 complements DNA-repair deficiency in *rad54*Δ yeast cells (strain MKP15). Drops of cell cultures grown to mid-logarithmic phase, were placed on plates containing SC +glucose (a and d), SC +glucose +MMS (0.06%) (b and e), or SC +galactose +MMS (0.06%) (c and f) media. Cell-culture drops were serially diluted (×10, ×100, etc.), starting from 10^7 cells/ml. (a–c) Top row (1), *rad54*Δ strain (MKP15); row 2, WT strain (YPH857); row 3, *rad54*Δ strain + *ScRAD54*; row 4, *rad54*Δ strain + At3g19120. (d–f) Top row (1), *rad54*Δ strain; row 2, *rad54*Δ strain + *ScRAD54*; rows 3 and 4 (two bottom rows), *rad54*Δ strain + At5g63950. (g) Specific expression of *AtRAD54* in yeast cells: Western blot analysis with an anti-AtRad54 antibody. 1, *rad54*Δ cells (strain MKP15) without plasmid grown in glucose-containing medium; 2, *rad54*Δ cells without plasmid grown in galactose-containing medium; 3, *rad54*Δ cells with *AtRAD54* plasmid(pRS426-*Gal1* promoter-*AtRAD54*) grown in glucose-containing medium; 4, *rad54*Δ cells with *AtRAD54* plasmid (pRS426-*Gal1* promoter-*AtRAD54*) grown in galactose-containing medium.

pair deficiencies in *rad54*Δ yeast cells but does not fully replace the gene in all aspects of recombination/repair.

Activity of yeast Rad54 in Arabidopsis: To test functioning of yeast Rad54 in Arabidopsis, we examined whether expression of *RAD54* improved resistance of Arabidopsis plants to ionizing radiation. We subcloned the yeast *RAD54* into a binary vector, under the regulation of the 35S promoter and introduced it into wild-type Arabidopsis plants. Seeds of independently derived transgenic plants expressing the pHS-35S*RAD54* construct (all seedlings derived from these seeds expressed the yeast *RAD54* mRNA, as shown in supplemental Figure 2 by an RT–PCR assay) were γ-irradiated and allowed to germinate (Figure 5A). Seeds of the transgenic plants were more resistant to γ-irradiation than their wild-type progenitors, as shown by the increased fraction of seedlings developing to the true-leaves stage following exposure to 40 krad (Figure 5B): 7.7% for the wild type *vs.* 14% (plant no. 6) to 96% (plant no. 5) in plants expressing the yeast *RAD54* gene. Irradiation of 60 krad resulted in total death of progeny of the wild-type plants whereas seedlings of transgenic plant nos. 2, 5, and 8 (which showed the highest resistance to 40 krad) were relatively resistant to 60 krad, with 6.8, 4.4, and 9% of seedlings developing two true leaves or more, respectively (Figure 5C). The differences in radiation

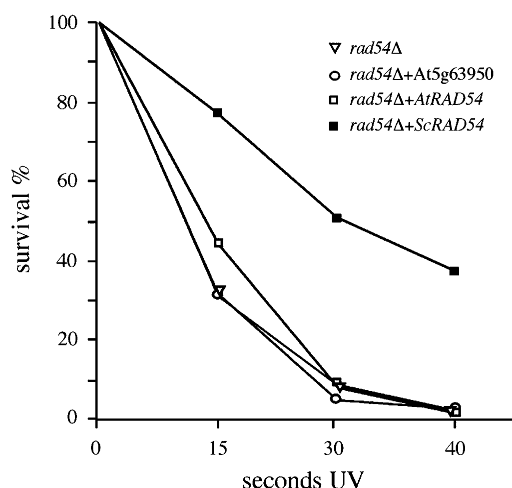

Figure 3.—Plant DNA repair genes do not complement UV repair defect of *rad54*Δ cells (MKP15). Shown are the numbers of colonies that survived after UV irradiation for different lengths of time. Irradiation was performed as described in materials and methods.

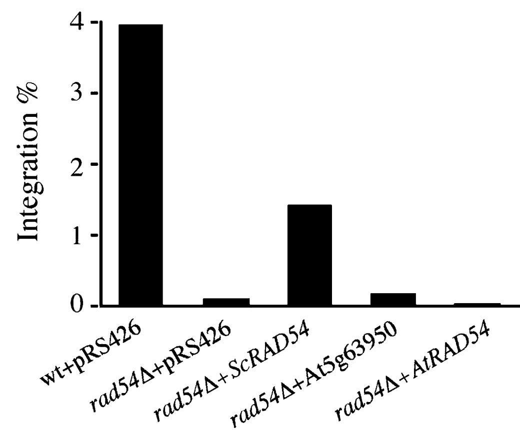

Figure 4.—Integration efficiency of DNA fragments in different mutant yeast strains. Plotted are integration efficiencies of a transfected DNA fragment (calculated as explained in the materials and methods) in strains harboring different plasmids containing plant and yeast genes.

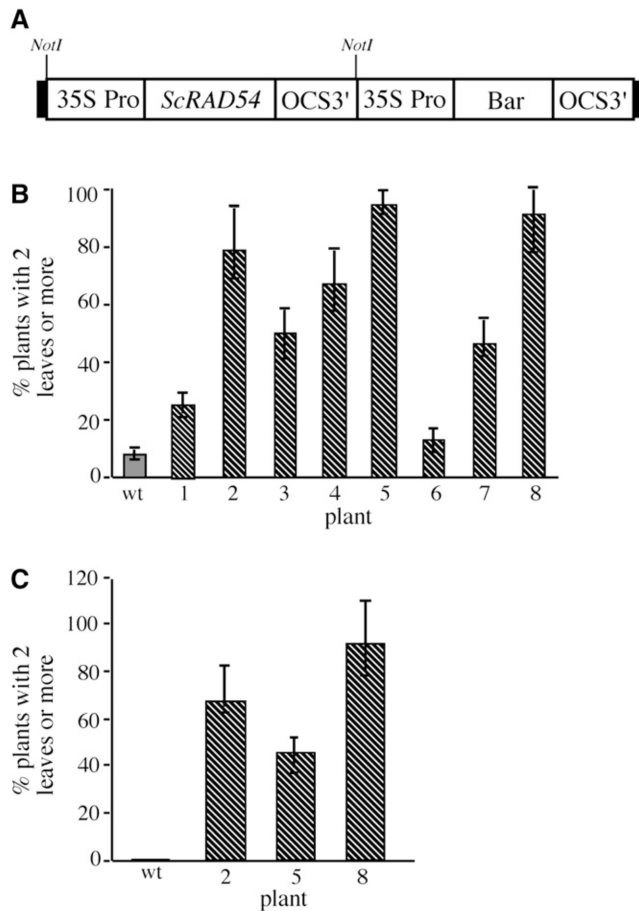

FIGURE 5.—γ-Irradiation resistance of Arabidopsis lines expressing the yeast *RAD54* gene. (A) Structure of the T-DNA vector that was used for transformation of Arabidopsis. 35S, cauliflower-mosaic virus promoter under which yeast *RAD54* ORF was expressed. OCS3′, terminator sequence from the *Octopine synthase* gene. Bar, the *Phosphinothricin* gene which confers resistance to the BASTA herbicide. RB and LB, right and left borders of the pMBLArt T-DNA binary vector. (B) The response of T_1 progeny of transformants of the vector in A and of wild-type (WT) Columbia control to 40 krad γ-irradiation. Plants were assayed as described in MATERIALS AND METHODS. Bars represent the standard errors of the means from 3 to 4 experiments. A total of 100 to 150 seedlings were monitored for each line in each experiment. (C) The response of T_1 progeny of transformants of the vector in A and of wild-type (WT) Columbia control to 60 krad γ-irradiation. Plants were assayed as described in MATERIALS AND METHODS.

resistance between independent transgenic plants (or their seedling progeny) might be due to position effects (resulting from the different integration sites of the yeast gene) or to the number of copies integrated.

In previous work (SHAKED *et al.* 2005), it was shown that expression of yeast *RAD54* in Arabidopsis cells causes increases of the rates of targeted (homologous) integration by two orders of magnitude. Therefore, we conclude that in the plant context yeast Rad54 might increase the efficiency of the plant homologous recombination system and that the yeast protein is very active in this alien context, possibly replacing or acting in addition to the native *AtRAD54*.

Yeast two-hybrid assays: As it is known that the yeast Rad54 protein interacts with Rad51 in a yeast two-hybrid system (JIANG *et al.* 1996), we examined whether the plant protein AtRad54 interacts similarly with the plant AtRad51 and whether this interaction is conserved between proteins of the two species. Use was made of the Two-Hybrid kit (Clontech). We found (as shown in Figure 6) that yeast Rad54 interacted with AtRad51, and that AtRad54 interacted with yeast Rad51. The plant proteins AtRad54 and AtRad51 also showed significant interaction, as did their yeast homologs Rad54 and Rad51 (as previously shown by JIANG *et al.* 1996 and by OSAKABE *et al.* 2006). These results (Figure 6) support the designation of *AtRAD54* as the true ortholog of yeast *RAD54* and show that the molecular function of the *RAD54* homologs is conserved across different kingdoms.

Role of *AtRAD54* in response to γ-irradiation: Following the identification of At3g19210 as a *RAD54* ortholog, we wanted to further test the function of this gene *in planta*, by suppressing or increasing its activity. RNAi was previously reported to downregulate the plant native gene (SHAKED *et al.* 2006). Here, overexpression of the plant gene was performed by fusing it to a strong promoter, with the aim of producing high resistance/tolerance to DNA damage. Sensitivity to γ-irradiation was scored as the fraction of seedlings with two or more true leaves (resistant seedlings). Seedlings with cotyledons only or with only one true leaf were considered as sensitive to DNA damage. In the wild type, 7% of the 30-krad irradiated seeds developed as resistant seedlings compared to <2% for the RNAi line in which *AtRAD54* was silenced (SHAKED *et al.* 2006). The RNAi line was also more sensitive than the wild-type line to UV radiation (data not shown).

For overexpression of *AtRAD54*, *AtRAD54* cDNA was cloned into a binary vector, under the regulation of the 35S promoter, and introduced into wild-type Arabidopsis plants. Seeds of independently derived transgenic plants expressing the pHS-35S*AtRAD54* cDNA construct (Figure 7) were γ-irradiated prior to sowing. Seeds of the transgenic plants were more resistant to γ-irradiation than their wild-type progenitors when exposed to 40 krad (Figure 7). Irradiation of wild-type plants with 40 krad resulted in 0.65% of seedlings with two or more true leaves, whereas plants overexpressing *AtRAD54* cDNA showed much higher fractions of resistant seedlings, ranging from 11.1% (plant no. 3) to 30.4% (plant no. 2) (Figure 7). The differences in radiation resistance between independent transgenic plants (or their seedling progeny) might be due to position effects or to the number of copies of the introduced gene. Thus, overexpression of *AtRAD54* enhances resistance of Arabidopsis seedlings to γ-radiation. It is worth noting that such an overexpression did not enhance homolo-

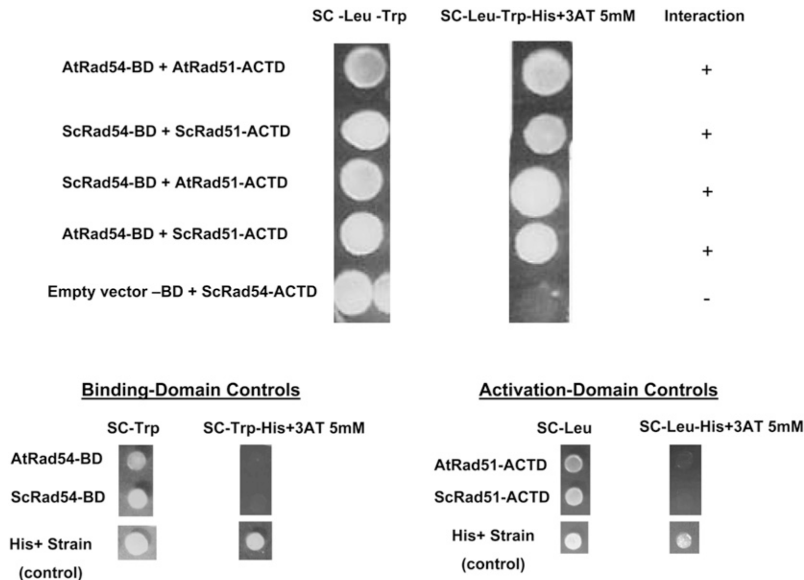

FIGURE 6.—(Top) Yeast two-hybrid system results. Strain AH109 was transformed with two plasmids: one containing the Gal4BD conjugated construct and the other with the Gal4ACTD conjugated construct. The cells were grown on SC −Leu −Trp medium for selection of the plasmids and then on SC −Leu −Trp −His +3AT to test for interaction between the different insert proteins coded by the two plasmids. (Bottom) Single-domain controls: strain AH109 was transformed with each of the single-domain plasmids alone and grown either on SC −Trp or SC −Leu plates for selection. Lack of activation of the *HIS3* reporter gene is seen as absence of growth on either SC −Trp −His +3AT or on SC −Leu −His +3AT plates. A His^+ strain was added to all plates as a positive control for growth.

gous recombination rates in another work (OSAKABE *et al.* 2006).

DISCUSSION

In this work we provide evidence that At3g19210 (*AtRAD54*) is the functional ortholog of *RAD54*. In addition, we show that the yeast *RAD54* gene, in plants, retains some of its DNA repair activities and that the plant gene *AtRAD54* is active in yeast, complementing some of the DNA repair defects of the *rad54*Δ yeast mutant. We discuss below the evidence for orthology and the possible mechanistic basis for trans-species compatibility.

The gene At3g19210 (*AtRAD54*) was identified here as an Arabidopsis *RAD54* ortholog because (i) *AtRAD54* is more similar to yeast *RAD54* than to any plant gene; (ii) the cDNA of *AtRAD54* remedies the MMS-sensitivity phenotype of *rad54*Δ yeast cells; (iii) we show here that overexpression of either gene in Arabidopsis enhances plant resistance to γ-radiation, while RNAi downregulation of *AtRAD54* was previously shown to confer sensitivity (SHAKED *et al.* 2006); and (iv) our yeast two-hybrid experiments show that *AtRAD54* interacts with *AtRAD51*, like the corresponding yeast homologs (JIANG *et al.* 1996). Taken together, these data strongly support the identification of *AtRAD54* as a functional *RAD54* ortholog and further emphasize its role in DNA damage repair.

During completion of this work we have learned about independently reached results concerning the same Arabidopsis *AtRAD54* cDNA clone (OSAKABE *et al.* 2006). In their work, OSAKABE *et al.* (2006) showed that this cDNA interacted with *AtRad51* in a yeast two-hybrid system, as also shown by our data; they also showed sensitivity of mutant *AtRAD54* plants to genotoxic agents. Our work both confirms and extends the work of OSAKABE *et al.* (2006), by showing functional complementation of the yeast *rad54*Δ mutation by the plant gene *AtRAD54*, and by showing that the *AtRAD54* overexpressing plants are more radiation resistant than control plants.

Our work (here, and in SHAKED *et al.* 2005) suggests that some degree of functional complementation occurs between plant and yeast genes in both organisms: the plant gene *AtRAD54* in yeast cells and the yeast gene *RAD54* in plants. The partial cross-compatibility of the DNA repair systems in yeast and Arabidopsis is demon-

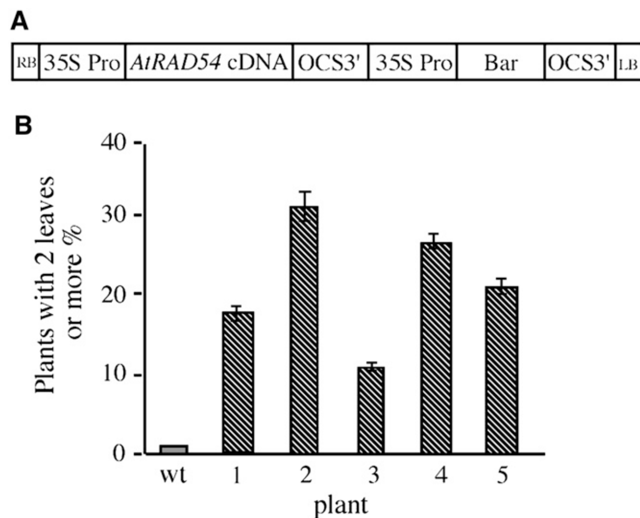

FIGURE 7.—γ-Irradiation resistance of Arabidopsis lines overexpressing the *AtRAD54* cDNA. (A) Structure of the vector used to express *AtRAD54* in plants. The same markers as in Figure 5 were used. (B) The response of T_1 progeny of transformants of the vector in A and of wild-type (WT) Columbia control to 40 krad γ-irradiation. Plants were assayed as described in MATERIALS AND METHODS. Bars represent the standard errors of the means from three to four experiments. A total of 100 to 150 seedlings were monitored for each line in each experiment.

strated by the overexpression of the yeast *RAD54* gene in Arabidopsis. The transgenic plants harboring overexpression plasmids of the yeast gene *RAD54* are much more resistant to γ-radiation than wild-type plants (Figure 5) and undergo gene targeting more efficiently than wild-type plants by up to two orders of magnitude (Shaked *et al.* 2005). From our results it appears that the yeast gene *RAD54* is very potent in Arabidopsis plants and that the plant *AtRAD54* is active in yeast in enabling growth of *rad54*Δ mutants on MMS medium; however, the latter cross-kingdom activity is only partial, as the plant gene did not remedy the UV-sensitivity phenotype nor the homologous DNA integration defect that characterize the yeast *rad54*Δ mutant. Similarly, the human *hRAD54* also only partly remedied *rad54*Δ DNA repair deficiency in yeast cells (Kanaar *et al.* 1996).

The data reported here provide some insight into the possible mechanistic basis for partial cross-species complementation. The positive interactions in the two-hybrid assays between alien Rad54 and Rad51 partner proteins, namely the yeast Rad54 with AtRad51, and AtRad54 with yeast Rad51, are compatible with the phenotypic complementation observed in the transgenic yeast (with plant *AtRAD54*) and transgenic plants (with the yeast gene). Indeed, several studies have shown that the interaction between Rad54 and Rad51 is essential for their function (Heyer *et al.* 2006). The two-hybrid results also explain perhaps the elevation in gene targeting frequencies found when *RAD54* was expressed in Arabidopsis plants (Shaked *et al.* 2005). Yet, there seems to be a higher potency of the yeast Rad54 protein in Arabidopsis, when compared to the activity of AtRad54 in yeast cells (which complements MMS resistance, but not UV resistance and gene targeting frequencies). There are two main explanations for this greater potency of the yeast Rad54 protein over AtRad54, which should be tested in future experiments. As the region in Rad54 necessary for its interaction with Rad51, which is located within the115 NH_2-terminal residues (Jiang *et al.* 1996), shares no sequence similarity with the AtRad54 protein (Figure 1B), yeast Rad54 might be interacting with additional components of the plant system, which normally AtRad54 does not. This possible difference in protein activity between the yeast and plant systems might also explain some of the differences in efficiency of HR between the two organisms (much higher in yeast). Alternatively, the similar effects on DNA damage response in yeast (MMS resistance) of *AtRAD54* and the yeast *RAD54* plasmids might be mediated by a repair function executed by the yeast Rad54 or AtRad54 alone (independent of interaction with Rad51), as also suggested (Osakabe *et al.* 2006) to explain the inability of overexpression of *AtRAD54* to enhance homologous recombination frequency in plants. It was also shown that the yeast Rad54 protein can mediate nucleosome movement (sliding along the DNA) *in vitro* (Alexeev *et al.* 2003). This chromatin remodeling activity was enhanced by the Rad51–ssDNA complex; nevertheless Rad54 was also active on its own in this respect, *in vitro* (Alexeev *et al.* 2003), suggesting the possibility of a role of Rad54, which is independent of its interaction with Rad51. A recent report (Bugreev *et al.* 2006) suggests an additional and novel role for Rad54. These authors propose that Rad54 functions alone to promote Holliday junction branch migration, a function which also might explain the strong effect of *RAD54* by itself in an alien system.

In conclusion, this study has led to the identification of an *A. thaliana RAD54* ortholog and has established its involvement in DNA repair in the plant and its compatibility with the yeast HR machinery. We have also shown compatibility between the yeast *RAD54* and the plant DNA repair system. We propose that the differences between species in the extent of alien-gene complementation could be caused by alteration in the partner proteins found in a heterologous environment and/or by the ability of Rad54 and AtRad54 to act independently at various stages of the recombination and repair processes.

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