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Involvement of the Arabidopsis *SWI2/SNF2* Chromatin Remodeling Gene Family in DNA Damage Response and Recombination

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ABSTRACT

The genome of plants, like that of other eukaryotes, is organized into chromatin, a compact structure that reduces the accessibility of DNA to machineries such as transcription, replication, and DNA recombination and repair. Plant genes, which contain the characteristic ATPase/helicase motifs of the chromatin remodeling Swi2/Snf2 family of proteins, have been thoroughly studied, but their role in homologous recombination or DNA repair has received limited attention. We have searched for homologs of the yeast *RAD54* gene, whose role in recombination and repair and in chromatin remodeling is well established. Forty Arabidopsis *SWI2/SNF2* genes were identified and the function of a selected group of 14 was analyzed. Mutant analysis and/or RNAi-mediated silencing showed that 11 of the 14 genes tested played a role in response to DNA damage. Two of the 14 genes were involved in homologous recombination between inverted repeats. The putative ortholog of *RAD54* and close homologs of *ERCC6/RAD26* were involved in DNA damage response, suggesting functional conservation across kingdoms. In addition, genes known for their role in development, such as *PICKLE/GYMNOS* and *PIE1*, or in silencing, such as *DDM1*, turned out to also be involved in DNA damage response. A comparison of *ddm1* and *met1* mutants suggests that DNA damage response is affected essentially by chromatin structure and that cytosine methylation is less critical. These results emphasize the broad involvement of the *SWI2/SNF2* family, and thus of chromatin remodeling, in genome maintenance and the link between epigenetic and genetic processes.

THE DNA recombination and repair machinery is usually well conserved during evolution and plants seem to have the same complement of repair enzymes as other species (Britt and May 2003). Nevertheless, there are significant differences between species. For example, homologous recombination (HR) is less efficiently used for double strand break repair in plants than in yeast, while nonhomologous end joining is a prominent pathway (Gorbunova and Levy 1999). Similarly, the integration of exogenous DNA into chromosomes proceeds essentially via a nonhomologous DNA recombination pathway (Puchta and Hohn 1996; Mengiste and Paszkowski 1999). Mutations that are lethal in other species are viable in plants, *e.g.*, *RAD50* (Gallego *et al.* 2001), *MRE11* (Gallego *et al.* 2001; Bundock and Hooykaas 2002), or *AtERCC1* (Hefner *et al.* 2003; Dubest *et al.* 2004). Telomere maintenance also differs from other species even though the same machinery is involved (Gallego and White 2001; Bundock *et al.* 2002; Riha and Shippen 2003).

Thanks to the Arabidopsis genome project it is now possible to address aspects of HR and DNA repair that have received limited attention so far in plants, such as the connection between chromatin structure and genome maintenance. The genome of plants, like that of other eukaryotes, is organized into chromatin, a compact structure that limits the accessibility of DNA to various machineries such as transcription, replication, and DNA recombination and repair. Disrupting the nucleosome–DNA interactions or remodeling of chromatin via ATP-dependent proteins might thus stimulate HR and DNA repair. In support of this possibility, it was shown that alteration in the expression of *MIM*, a gene encoding a chromatin structural component related to the *SMC* family (structure maintenance of chromosomes), could affect the rate of intrachromosomal recombination in Arabidopsis (Hanin *et al.* 2000). *BRU1* is an additional example of an Arabidopsis gene linking heterochromatin stability to gene silencing as well as to DNA repair (Takeda *et al.* 2004).

The link between chromatin remodeling and recombination is emphasized in the *RAD54* gene of the yeast *Saccharomyces cerevisiae* (see review by Tan *et al.* 2003). The Rad54 protein has motifs similar to those found in the switch2/sucrose non-fermenting2 (Swi2/Snf2) superfamily (Eisen *et al.* 1995), members of which are chromatin-related proteins. The common feature of these proteins, which unites all family members, is the presence of an ~400-amino-acid stretch of highly conserved ATPase/helicase motifs (Eisen *et al.* 1995). Another

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yeast homolog of *RAD54, RDH54 (TID1)*, acts in meiosis and in repair between homologous chromosomes (Klein 1997; Shinohara *et al.* 1997). Other yeast *SWI2/SNF2* genes, *RAD26*, *RAD16*, and *RAD5*, are involved in various aspects of DNA repair, such as nucleotide excision repair and transcription-coupled repair (Eisen *et al.* 1995). Disruption of *RAD54* in *S. cerevisiae* (Arbel *et al.* 1999), of its homologs in chicken (Bezzubova *et al.* 1997) and mice cells (Essers *et al.* 1997), and in fission yeast, *S. pombe* (Muris *et al.* 1997), results in mutant lines that are sensitive to ionizing radiation and to methylmethane sulfonate and defective in homologous integration of exogenous DNA. Similarly, a Drosophila *RAD54* homolog is involved in X-ray resistance and in recombination repair (Kooistra *et al.* 1997). In humans, the UV-sensitivity disorder, Cockayne syndrome, is caused by a defect in *CSB*, a *RAD26* homolog with ATP-dependent chromatin remodeling activity (Troelstra *et al.* 1992; Citterio *et al.* 2000). These studies suggest functional conservation of the *RAD54*-like genes.

In plants, the *SWI2/SNF2*-like family has been studied (Verbsky and Richards 2001; Li *et al.* 2002); however, a detailed functional analysis of most members is still missing. Those members studied so far are involved in a diverse range of biological activities, an updated compilation of which is described in the Plant Chromatin Database (http://www.chromdb.org). For example, some *SWI2/SNF2* members play a role in gene silencing: (1) mutations in the gene *DDM1* (*decreased DNA methylation 1*) cause a gradual demethylation of the genome and the release from gene silencing in Arabidopsis (Jeddeloh *et al.* 1999); (2) another *SWI2/SNF2* Arabidopsis gene, *MOM1* (*Morpheus molecule 1*), is also required for gene silencing (Amedeo *et al.* 2000); (3) recently, the gene *DRD1* was shown to be required for RNA-directed DNA methylation (Kanno *et al.* 2004, 2005). Other *SWI2/SNF2* members play a role in development: (1) the gene *PICKLE* (also known as *GYMNOS*) affects cell transition from the embryonic to the vegetative state (Ogas *et al.* 1999) and controls differentiation of the carpels in Arabidopsis (Eshed *et al.* 1999); (2) the *SPLAYED* gene is a regulator of reproductive development (Wagner and Meyerowitz 2002); (3) the gene *PIE1* is a regulator of genes controlling flowering in Arabidopsis (Noh and Amasino 2003); (4) The *AtBRM* gene controls shoot development and flowering (Farrona *et al.* 2004); and (5) the CHR11 gene controls female gametophyte development (Huanca-Mamani *et al.* 2005). Recently, the involvement of the Arabidopsis *SWI2/SNF2* gene family in DNA recombination and repair was shown for the first time for the Arabidopsis ortholog of *INO80* (Fritsch *et al.* 2004).

In this study, we have analyzed 14 of the 40 Arabidopsis *SWI2/SNF2* gene family members with regard to their role in DNA damage response and recombination. The analysis of mutants and RNAi lines showed sensitivity to γ- or UV radiation for most genes; two lines had reduced rates of somatic recombination between inverted repeats. We discuss the conservation of *SWI2/SNF2* functions across kingdoms, the link between genetic and epigenetic maintenance of the genome, and the role of chromatin remodeling and cytosine methylation in DNA damage response.

MATERIALS AND METHODS

Sequence alignments: Similarity searches for *S. cerevisiae* Rad54 and other Swi2/Snf2 proteins were done using BLAST package version 2.0 (BLASTN, BLASTP, BLASTX, BLASTTN) on the NCBI server (http://www.ncbi.nlm,nih.gov/BLAST/) or on the Arabidopsis information resource (http://www.arabidopsis.org/home.html) and the Arabidopsis database server (http://genome-www.stanford.edu/Arabidopsis/). Sequences used in this work were downloaded from NCBI databases. ClustalW and ClustalX programs generated multiple sequence alignments with some minor manual adjustments for Macintosh iBook computer.

Blocks analysis: Multiple alignment of the 26 Arabidopsis sequences, of the 40 sequences that showed the most significant similarity to *S. cerevisiae RAD54*, was performed by integrating three multiple alignment methods. First, we used the BlockMaker (Henikoff *et al.* 1995), an automated system that finds blocks in a group of protein sequences and is an extension of the Gibbs algorithm (Lawrence *et al.* 1993) and of the Motif algorithm (Smith *et al.* 1990), which identifies spaced triples. The second program that was used to analyze conserved blocks was the automated MEME program (Grundy *et al.* 1996), which uses an expectation maximization algorithm. Finally, we used the interactive MACAW program (Schuler *et al.* 1991) to visualize and precisely define the conserved blocks. The Gibbs algorithm (Lawrence *et al.* 1993) found nine blocks in 21 sequences. The Motif algorithm (Smith *et al.* 1990) defined only four blocks that were conserved in 24 sequences. Using the MEME method, which uses an expectation maximization algorithm, we found seven conserved regions in the 26 sequences. Results from the three methods were integrated using the MACAW program.

Phylogenetic tree: A phylogenetic tree was built on the basis of the 40 Arabidopsis sequences that showed similarity to *RAD54*, using different approaches. The sequences were fully aligned and the multiple sequence alignments were carried out using the ClustalW (1.4) program with the standard parameters and the BLOSUM series matrix. Then the conserved regions were used to build trees. We used the PHYLIP algorithm, based on the ClustalW alignment, to build and bootstrap neighbor-joining trees. The internal control was provided by the bootstrap resampling technique (Felsenstein 1985): the number on each branch is the number of bootstrap trees that support this grouping (out of 100). Another tree was automatically constructed from blocks alignment (http://www.blocks.fhcrc.org) and was used for comparison with the other trees. All trees (on the basis of full length alignment, conserved regions alignment, or blocks alignment) gave rise to the same phylogenetic relationships among the 40 *RAD54*-like members.

Plasmids: To produce RNAi lines for the *RAD54*-like genes, constructs were made that contained sense and antisense arms, namely short fragments of ~200–300 bp, isolated from the 3′ end of 13 selected Arabidopsis *SWI2/SNF2* genes, choosing sequences that are not conserved in the other gene family members. These fragments were isolated using 13 primer pairs containing tails of *Xho*I and *Kpn*I restriction sites

for the sense arm and 13 primer pairs containing tails of *Bam*HI and *Cla*I restriction sites for the antisense arm. The two arms were cloned into pKannibal (Wesley *et al.* 2001), a vector designed to produce hairpin RNAs, and the resulting insert was further isolated as a *Not*I restriction fragment and cloned into the pMBLArt binary vector, containing glufosinate (BASTA) plant resistance (Eshed *et al.* 2001). These plasmids were transformed into Arabidopsis plants to generate the RNAi silencing effects.

Plant material and Agrobacterium-mediated transformation: Agrobacterium-mediated transformation was done either in wild-type Arabidopsis plants (ecotype Columbia) or in Arabidopsis plants, line N1IC4-651 (Puchta *et al.* 1995). Plant transformation was done by floral dipping (Clough and Bent 1998) and transformants (T0) were selected by BASTA selection. T0 plants were grown to maturity and the resulting T1 seeds were used for further analysis.

Histochemical staining procedure for intrachromosomal recombination assay: Histochemical staining for β-glucuronidase (GUS) activity was usually done with six to eight true-leaves plants (3 weeks after germination) after seed surface sterilization (Jefferson 1987). Plants were grown on 1/2 MS (Murashige and Skoog 1962) medium plus 2% sucrose. Growth conditions were 16 hr of light at 25°. Plants were harvested and incubated for 16 hr at 37° in sterile staining buffer containing 0.5 mg/ml of 5-bromo-4-chloro-3-indolyl glucuronide (X-Glu) substrate (DUCHEFA, Haarlem, The Netherlands) in final concentration of 100 mm phosphate buffer (pH 7.0), 15 mm EDTA, 0.1% Triton X-100 and 5 mm of potassium ferricyanide and potassium ferrocyanide trihydrate (SIGMA). Bleaching was done at room temperature in 70% ethanol.

γ- and UV irradiation procedure: To test γ-irradiation response, seeds were surface sterilized, imbibed overnight in distilled water at 4°, and irradiated at 30 krad supplied 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 plus 2% sucrose for 10 days. Growth conditions were 16 hr of light at 25°. Plants with resistance to γ-irradiation developed at least two true leaves after 10 days, while plants that were sensitive to γ-irradiation had one or no true leaves at all. The percentage of plants with two or more true leaves 10 days after irradiation was used as a quantitative estimate of resistance. Experiments were done only with batches of seeds that showed germination rates of >99%. This assay is similar to that described by Hefner *et al.* (2003).

To test UV-C response, seeds were surface sterilized and grown on 1/2 MS medium plus 2% sucrose for 2 weeks. UV-C was supplied by a UV Stratalinker (Stratagene model 1800; Startagene, La Jolla, CA). UV-C–irradiated plants were treated with a range of 100–400 kJ/m^2 of UV-C (254 nm), placed under dark conditions for 48 hr, and then returned to normal lighting conditions. UV sensitivity was assayed 72 hr after transfer back to the growth chamber under normal lighting conditions. Plants that showed typical curly and necrotic leaves were defined as UV-C sensitive as described by Preuss and Britt (2003).

RESULTS

Identification and characterization of Arabidopsis *RAD54* homologs: *Identification and phylogeny of the RAD54 homologs:* To identify genes with a Rad54-like function in Arabidopsis, we performed a search for Rad54-homologous sequences as described in materials and methods. This search resulted in 40 hits with significant similarity to the yeast Rad54 protein. These hits include all the previously analyzed *SWI2/SNF2* Arabidopsis genes (Verbsky and Richards 2001), with few additional members found in this search. The phylogenetic relationship among *SWI2/SNF2* genes was performed using different methods (see materials and methods). The phylogenetic tree we obtained (Figure 1) is almost identical to that previously published (Verbsky and Richards 2001) and is in agreement with the tree and annotations of the Plant Chromatin Database (http://www.chromdb.org). The Arabidopsis locus At3g19210/CHR25 had the highest significance *E*-score value (E^{-120}) in comparison to *S. cerevisiae* Rad54, whereas Mom (At1g08060/CHR15) had the lowest value (2×10^{-5}). At3g19210/CHR25 was closer to the yeast *RAD54* gene than to any other Arabidopsis gene; it is therefore

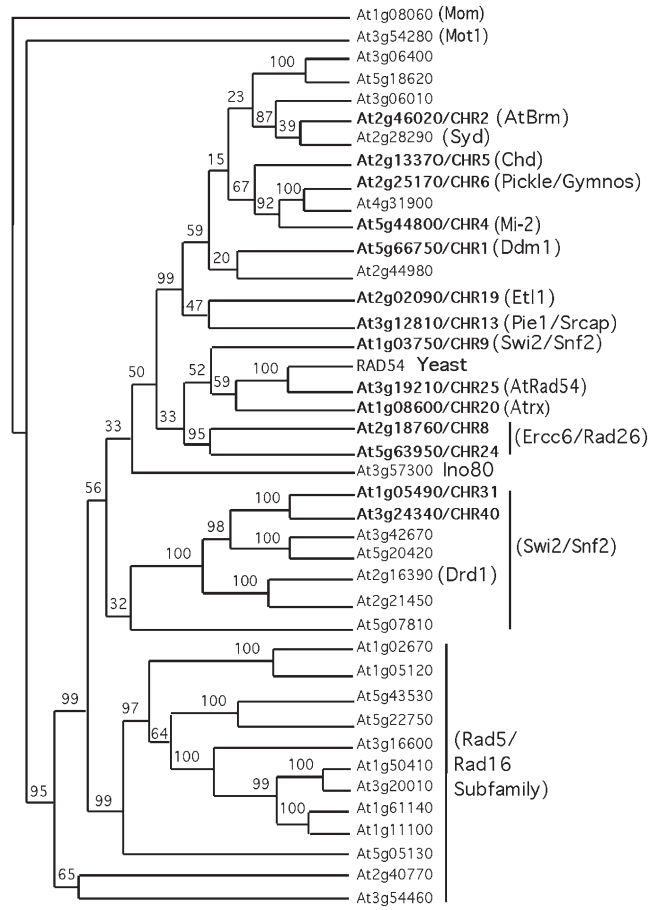

Figure 1.—The phylogenetic relationships among 40 Arabidopsis Swi2/Snf2-like proteins and the yeast Rad54 are described as a neighbor-joining tree produced and bootstrapped by PHYLIP. The tree contains Swi2/Snf2 chromatin remodeling proteins. Proteins with a known function are indicated on the right in parentheses. The Arabidopsis locus numbers shown in boldface type were selected for functional analysis. The CHR (chromatin remodeling) number, as given by the Plant Chromatin Database, is given for the 14 selected genes. Branch length was set arbitrarily. The number at each branching point represents the bootstrap values for specific nodes. Values <80 are not significant.

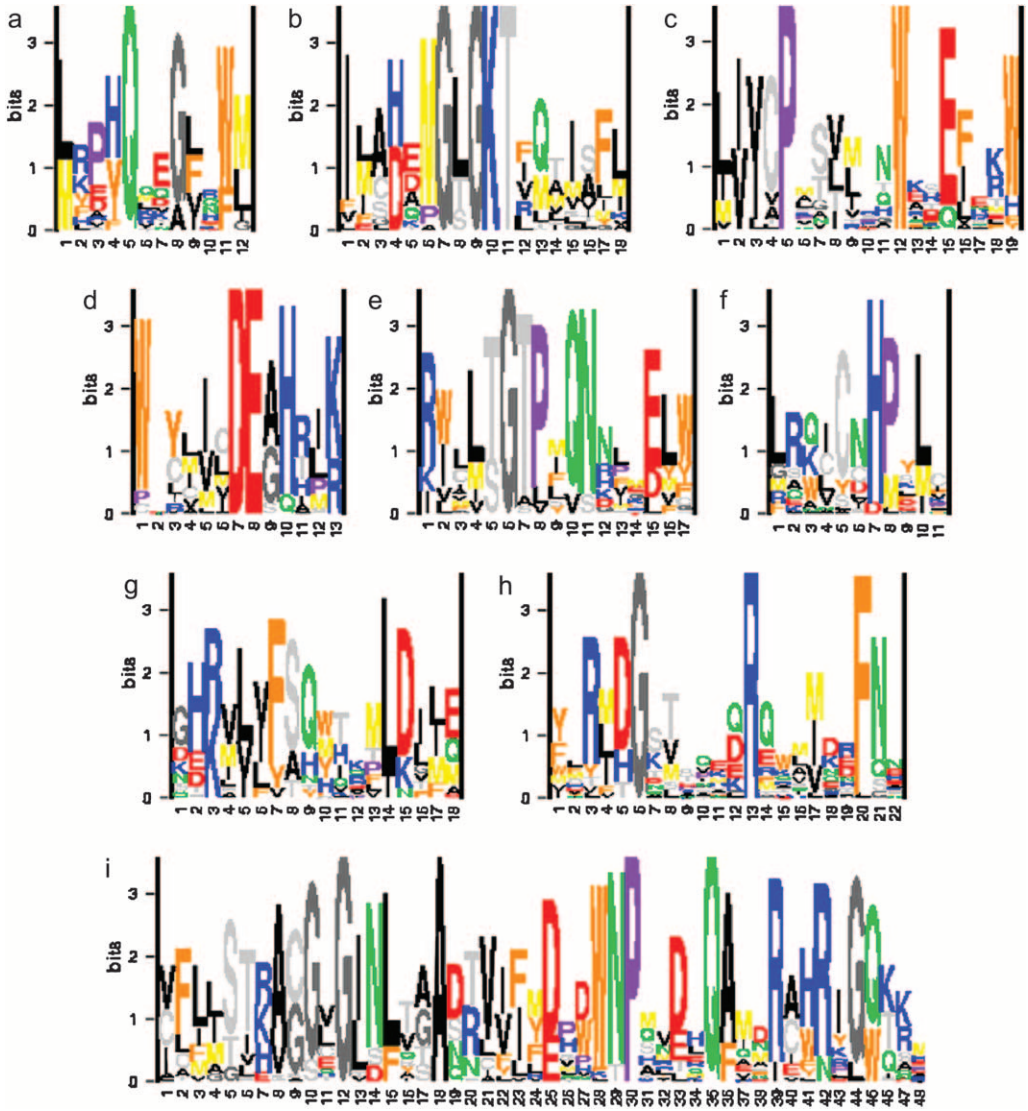

FIGURE 2.—Conserved blocks in *Arabidopsis thaliana* Swi2/Snf2 protein family members are shown. Nine conserved blocks (a–i) were built from 23 proteins, except for block g, which was found in 22 proteins only. The blocks are represented as logos. The height of each position, as calculated in bits of information, is proportional to its conservation, and residues at each position are shown at a height proportional to their conservation within that position. The colors of the amino acids (aa) represent the following: red, acidic; blue, basic; light gray, polar OH/SH; green, amide; yellow, methionine; black, hydrophobic; orange, aromatic; purple, proline; gray, glycine.

named *AtRAD54*. None of the 40 *SWI2/SNF2* Arabidopsis genes is known to be involved in DNA recombination in plants except *AtINO80* (FRITSCH *et al.* 2004). Homologs of some of the identified genes, *e.g.*, *RAD54*, *RAD26*, and *RAD16*, were shown in yeast to be involved in diverse aspects of DNA recombination and/or repair (EISEN *et al.* 1995). Other genes have known chromatin, silencing, or development-related functions in plants, but have no known repair function (*e.g.*, *DDM1*, *MOM*, *DRD1*, *PICKLE/GYMNOS*, *PIE1*, *AtBRM*). Other genes have known homologs in humans, *e.g.*, *ATRX*, *Mi-2*, *MOT1*, and *CSB* (an *ERCC6/RAD26* homolog involved in the Cockayne syndrome B).

Nine domains that are conserved among the Arabidopsis Swi2/Snf2 proteins were identified by integrating results from three different methods (see MATERIALS AND METHODS and Figure 2). The linear order of the domains was conserved for all Arabidopsis proteins (with occasional duplications of one or two domains, as in At2g18760/CHR8). Eight of these domains were similar in sequence and in order to the yeast Rad54 protein (data not shown). Domain d is homologous to a DNA-dependent ATPase, with a very strong DEAH signature (EISEN *et al.* 1995). The other eight domains are homologous to the Snf2-helicase-like domain (Figure 2). Another functionally important domain in yeast is the region necessary for the interaction between Rad54 and Rad51 that was located within the NH2-terminal 115 residues (JIANG *et al.* 1996). This region was not conserved in any of the plant Rad54-like proteins (data not shown).

Expression of the Arabidopsis SWI2/SNF2 genes: We selected 14 *SWI2/SNF2* genes either because of their strong homology to *RAD54* or as representative of different *RAD54*-related clades or because they corresponded to well-known genes. We checked the expression of these selected genes, shown in boldface type in Figure 1, by using public data and by performing RT–PCR. First, we used the massively parallel signature sequences (MPSS) database (http://mpss.udel.edu/at/java.html).

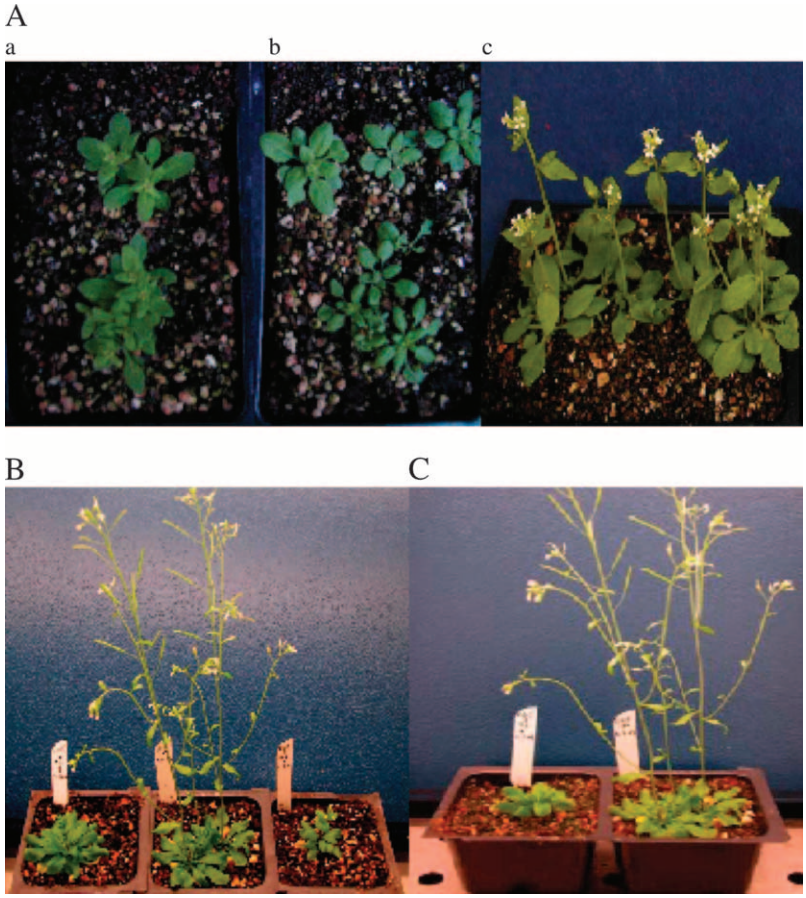

FIGURE 3.—Phenotypes of Arabidopsis plants transformed with RNAi constructs targeted to specific *SWI2/SNF2* members. (A) The phenotype of the *gymnos* mutant in the Landsberg background (a) is shown next to the corresponding RNAi line of *GYMNOS* (At2g25170/CHR6) in the Columbia background (b). Both show the same phenotype, namely growth inhibition and delay in flowering compared to the wild-type Landsberg phenotype (c). All plants were sown at the same time. (B) The plant in the middle is the Columbia wild type. It is flanked by two independent RNAi lines (in Columbia background) of At5g44800/CHR4, a gene homologous to the human *Mi-2* autoantigen. The phenotype of these plants is similar to that of the *gymnos* mutant (A, a). (C) The plant on the left is an RNAi line (in Columbia background) of *AtBRM* (At2g46020/CHR2) showing a retarded phenotype similar to *gymnos*; on the right is the wild-type Columbia.

MPSS quantitatively measures gene expression on the basis of the relative amount of 17- to 20-bp signatures within libraries containing 2–3 million signatures (BRENNER *et al.* 2000). This method was used in several species including Arabidopsis (HOTH *et al.* 2003). We found that some genes were relatively strongly expressed *e.g.*, *PICKLE/GYMNOS* (At2g25170/CHR6), while other genes, such as *AtRAD54*, were weakly expressed (see supplemental Table 1, http://www.genetics.org/supplemental/). Overall, genes were expressed in a housekeeping-like manner with no obvious organ specificity. In general, this conclusion was supported by an RT–PCR analysis (data not shown). Moreover, using the Affymetrix microarray data of MOLINIER *et al.* (2005), we found that of the 14 genes studied, only 1, At2g18760/CHR8, had increased RNA levels upon induction by genotoxic agents.

Disruption of the *SWI2/SNF2* genes activity in mutants and RNAi line: The role of some of the members of the *SWI2/SNF2* gene family in homologous recombination and DNA damage response was addressed using mutant analysis and/or gene silencing via RNAi targeted to a unique 3′ region of each gene. For *GYMNOS* and for *DDM1*, we had two independent mutant alleles, as well as RNAi plants. The two knockout alleles of *GYMNOS*, *gym-5* and *gym-6*, kindly provided by Yuval Eshed (ESHED *et al.* 1999), grew slower than wild type, flowered later, had reduced organ size, and displayed reduced apical dominance (ESHED *et al.* 1999). An identical phenotype was observed in the *GYMNOS* RNAi plants, At2g25170/CHR6 (Figure 3A). The phenotype of RNAi lines At5g44800/CHR4 (Mi-2-like) and *AtBRM* (At2g46020/CHR2) (Figure 3, B and C) was similar to that of *GYMNOS* (At2g25170/CHR6)—namely retarded in growth but fertile. This raised the possibility that RNAi designed for these genes also could silence *GYMNOS*. This possibility is unlikely for *AtBRM* (At2g46020/CHR2) because a mutant for this gene was described and shows the same phenotype as *GYMNOS* (PRYMAKOWSKA-BOSAK *et al.* 2003). For At5g44800/CHR4 (*Mi-2*-like), we tested the possibility of cross silencing by RT–PCR. We found that RNAi designed for *Mi-2* silenced *Mi-2* but not *GYMNOS*, and, conversely, RNAi designed for *GYMNOS* silenced *GYMNOS* but not *Mi-2* (Figure 4A).

For *DDM1*, the two mutant alleles, *ddm1-2* and *ddm1-5*, were kindly provided by Eric Richards. The gene *DDM1* has a typical mutant phenotype of demethylation (JEDDELOH *et al.* 1999). The same phenotype was found for the RNAi targeted to *DDM1* (see supplemental Figure 1, http://www.genetics.org/supplemental/). This phenocopy indicates specific silencing in this line. The lack of cross silencing of *DDM1* by RNAi from related genes is shown in Figure 4B. For locus At5g63950/CHR24, only one knockout mutant was available and a

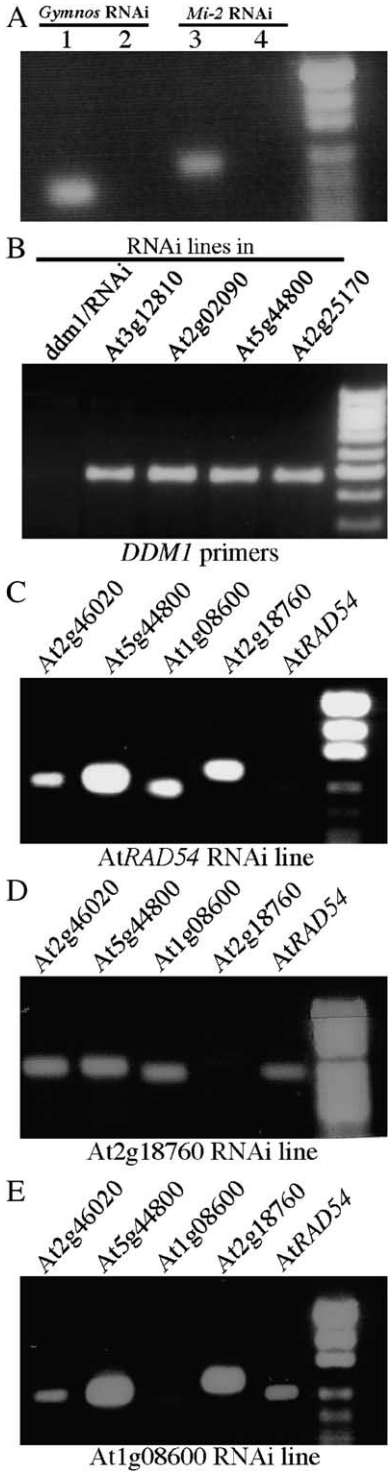

Figure 4.—RT–PCR analysis of gene silencing in RNAi lines. (A) Analysis of two closely related genes, *GYMNOS* and *the Mi-2*-like gene, silenced by RNAi vectors. Lanes 1 and 2 show the analysis of *GYMNOS* RNAi plant, with *Mi-2*-specific primers and *GYMNOS*-specific primers, respectively. Lanes 3 and 4 show the analysis of an *Mi-2* RNAi plant, with *GYMNOS*-specific primers and *Mi-2*-specific primers, respectively. (B) Activity of DDM1: it is silenced in an RNAi line for *DDM1*, but not in closely related genes. C–E show silencing of the target genes for AtRAD54/CHR25, At2g18760/CHR8, and At1g08600/CHR20 RNAi lines, respectively, but not of the nontarget genes indicated for each lane. In A–E the right lane is a size marker.

homozygote line was isolated from T-DNA insertion line SALK_007071. This mutant contains a T-DNA insertion in exon1 and produced no transcript as determined by RT–PCR (data not shown) and had no visible phenotype.

For other genes, where knockout mutants were not available, gene disruption relied only on RNAi silencing. In all cases studied here, RNAi lines did silence their target genes as determined by RT–PCR (see supplemental Figure 2, http://www.genetics.org/supplemental/ and examples in Figure 4). The specificity of RNAi silencing, *i.e.*, the lack of spread of silencing to related genes, was tested and is described below for At3g19210/CHR25 (*AtRAD54*). The RNAi line targeted to *AtRAD54* did not silence its closely related genes At2g18760/CHR8 or At1g08600/CHR20 but did silence its target (Figure 4C). Conversely, RNAi in At2g18760/CHR8 or At1g08600/CHR20 silenced the target genes but not *AtRAD54* (Figure 4, D and E). We did not check systematically all the possible combinations of RNAi lines and of the genes that they could silence. However, the lack of cross silencing between the closely related genes described above suggests that spreading of silencing to the more distant genes is unlikely. Moreover, the phenocopy of the mutants by the RNAi lines described above for *GYMNOS*, *DDM1*, and *AtBRM* further supports silencing specificity. Finally, it should be noted that the *SWI2/SNF2* family is ancient and that although there is conservation at the protein level, the DNA sequences of the different members are quite different; therefore, cross silencing of divergent genes is not very likely. For example, the putative *RAD54* ortholog *AtRAD54* is closer to the yeast *RAD54* gene than to any other plant homolog.

Radiation sensitivity: Sensitivity to γ-irradiation has often been associated with alterations in DNA repair/recombination. We therefore checked the response to doses of 30 krad in the RNAi lines, in pools of 10 independent transformants per line and in the mutants. An example is shown in Figure 5A for the two *ddm1* mutant alleles (*ddm1-2* and *ddm1-5*) compared to wild type. Seedlings that developed two or more true leaves were considered as resistant, while seedlings with cotyledons only or with only one true leaf were considered as sensitive. We found that ~7% of the irradiated seeds developed two or more true leaves in the wild type while in the RNAi plants, five lines were totally sensitive (did not develop true leaves and eventually died), three other lines were partially affected (Figure 5B and Table 1), and four lines had the same response as wild type (Figure 5B). The hypersensitive RNAi lines included RNAi of *GYMNOS* (At2g25170/CHR6), human *Mi-2* (At5g44800/CHR4), *DDM1* (At5g66750/CHR1), *SNF2* subfamily global transcription activator *AtBRM* (At2g46020/CHR2), and another gene related to this family (At1g03750/CHR9). The knockout mutants present in the experiment, namely *gymnos* (alleles *gym-5* and *gym-6*), *ddm1* (alleles *ddm1-2* and *ddm1-5*), and the

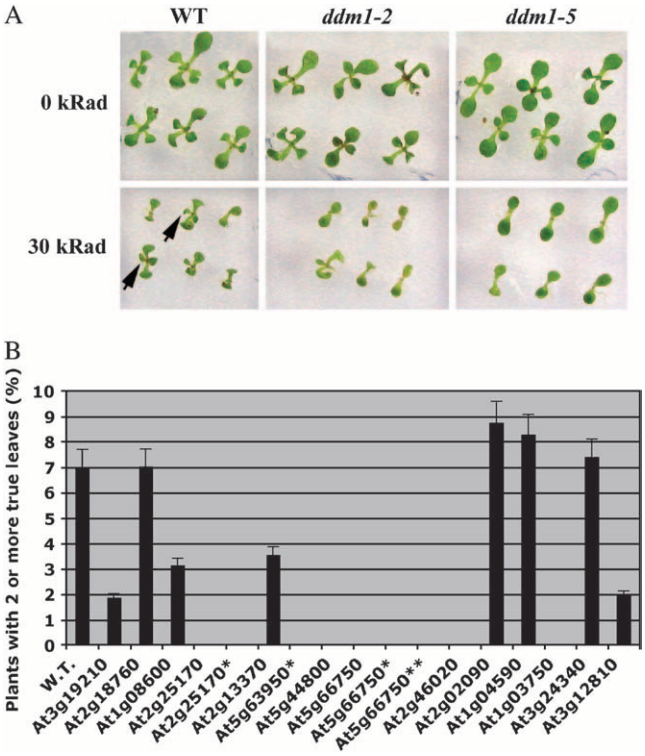

FIGURE 5.—Response of Arabidopsis RNAi lines and mutants in *SWI2/SNF2* genes to γ-irradiation. An example of γ-sensitive seedlings is shown in A for two different alleles of *ddm1* in the Columbia background, namely *ddm1-2* and *ddm1-5*, by comparison to wild type (ecotype Columbia). Arrows point to seedlings that were resistant to a 30-krad dose of γ-irradiation, *i.e.*, that developed two true leaves 10 days after irradiation. Sensitive seedlings were arrested in their growth and had only two cotyledons or only one true leaf. The RNAi lines and mutants studied in this work were all tested for response to γ-irradiation (B). Radiation response is expressed as the percentage of seedlings that developed two or more true leaves following γ-irradiation with a 30-krad dose. Loci marked with an asterisk (*) correspond to homozygous mutants: *gym5* allele for At2g25170/CHR6, SALK_007071 for At5g63950/CHR24, and *ddm1-2* for At5g66750/CHR1. The second *ddm1* allele, *ddm1-5*, is marked with two asterisks (**). The wild-type column corresponds to the ecotype Columbia. Bars, SEM. A total of 400–500 seedlings were monitored for each line. These seedlings were derived from the progeny seeds of a pool of at least 10 independent RNAi transformants.

T-DNA insertion line in At5g63950/CHR24, a *RAD26*-like gene, were all γ-irradiation sensitive: irradiation of the homozygous mutant resulted in seedlings with no true leaves 10 days after irradiation, followed by death of the seedlings within a few days (Figure 5B). Sensitivity to UV was determined visually on the basis of the appearance of typical symptoms such as curled and yellowish leaves. Those lines that clearly showed these symptoms were defined as UV sensitive, as summarized in Table 1.

Intrachromosomal recombination: The RNAi plasmids were transformed into *Arabidopsis thaliana* plants of line N1IC4-651, which is homozygous for a single copy of a T-DNA insertion, containing an intrachromosomal inverted repeat recombination assay construct, kindly provided by Holger Puchta (PUCHTA *et al.* 1995). In this assay, the activity of a GUS reporter gene is restored upon recombination between the two inverted repeats in *cis* and can be detected in whole plants as blue sectors upon histochemical staining (JEFFERSON 1987). The average spots number per wild type was ~1.5/plant (Figure 6), in the same range as determined in previous studies (SWOBODA *et al.* 1994; PUCHTA *et al.* 1995). The RNAi transformants that showed the strongest decrease in intrachromosomal recombination (ICR) rates corresponded to the *Mi-2*-like gene (At5g44800/CHR4) and to the RNAi targeted at *AtBRM* (At2g46020/CHR2) (Figure 6). RNAi in both genes gave an average spots number of 0.3/plant (Figure 6). This significant decrease in the ICR rates was found in two (of two) independent RNAi transformants for both the *Mi-2*-like gene (At5g44800/CHR4) and *AtBRM* (At2g46020/CHR2) (Figure 6). Although these two RNAi lines showed a retarded phenotype, when spots were counted, seedling size was not different from that of other RNAi lines or wild type. Therefore, size difference could not account for the difference in the number of spots. For the other 11 genes there were no significant alterations in ICR rates, as determined from the average number of blue sectors in two to four independent RNAi transformants (see summary in Table 1).

DISCUSSION

We have addressed the role of the Arabidopsis *SWI2/SNF2-RAD54* homologous genes in genome maintenance. In this study we found a wide implication of the *SWI2/SNF2* members in DNA damage response and in recombination, suggesting the importance of chromatin remodeling in plant genome maintenance and implying relatively low redundancy in this large and ancient gene family (Table 1).

The role of Arabidopsis *SWI2/SNF2* genes in DNA damage response: Sequence homology was a good predictor of gene function with regard to the role of Swi2/Snf2 proteins in DNA damage response. Disruption of gene activity lead to sensitivity to γ- and/or UV irradiation for most of the studied genes (Table 1). Interestingly, mutants or RNAi lines in genes annotated as *ERCC6/RAD26* homologs, with a predicted excision repair and/or transcription-repair coupling function (At2g18760/CHR8 and At5g63950/CHR24), were sensitive to UV irradiation as their yeast homolog or as UV-sensitive humans afflicted with Cockayne B syndrome, a defect in *ERCC6/RAD26* homolog (TROELSTRA *et al.* 1992; CITTERIO *et al.* 2000). RNAi lines in the putative ortholog of *RAD54* (At3g19210/CHR25) were sensitive to both UV and γ-irradiation, as expected from similar phenotypes found in mutants of yeast (BUDD and MORTIMER 1982) or of *RAD54* orthologous genes in

TABLE 1

Summary of radiation sensitivity and homologous recombination in RNAi lines or mutants of 14 *SWI2/SNF2* genes

Gene	CHR[a]	Homolog[b]	Predicted function	Observed function[c]		
				γ-IR	UV-C	ICR Rate
At3g19210	CHR25	*RAD54*	DNA repair and homologous recombination	S	S	WT
At2g18760	CHR8	*ERCC6/RAD26*	Excision repair and/or transcription-repair coupling	WT	S	WT
At1g08600	CHR20	*ATRX*	Transcriptional regulator	S	S	WT
At2g25170	CHR6	*PICKLE/GYMNOS*	Regulation of multiple gene families	*S*	*S*	WT
At2g13370	CHR5	*ND*	Unknown	WT	S	WT
At5g63950	CHR24	*ERCC6/RAD26*	Excision repair and/or transcription-repair coupling	*S*	*S*	ND
At5g44800	CHR4	*hMi-2-LIKE*	Human mi-2 autoantigen-like for dermatomyositis	S	R	Reduced
At5g66750	CHR1	*DDM1*	Maintenance of DNA methylation 1	*S*	S	WT
At2g46020	CHR2	*AtBRM*	Controls shoot and flower development	S	ND	Reduced
At2g02090	CHR19	*ETL1*	Transcriptional regulation and DNA repair	WT	WT	WT
At1g05490	CHR31	ND	Unknown	WT	R	WT
At1g03750	CHR9	ND	Unknown	S	WT	WT
At3g24340	CHR40	ND	Unknown	WT	WT	WT
At3g12810	CHR13	*PIE1/SRCAP*	Required for *FLC* activation and floral repression	S	R	WT

[a] The CHR number corresponds to the chromatin remodeling gene number given in the Plant Chromatin Database (http://www.chromdb.org).

[b] The name of the homolog is given or of the gene itself (underlined) whenever it has been previously characterized.

[c] The response of the RNAi or both RNAi and mutant (in italics) plants to γ-irradiation (γ-IR) or UV-C (254 nm) is indicated as sensitive (S), resistant (R), similar to wild type (WT), or not determined (ND). The intrachromosomal recombination rate (ICR) is indicated as similar or reduced compared to WT. For *GYMNOS*, two mutant alleles and one RNAi line were tested and gave the same results. For At5g63950, only the mutant line was tested.

chicken (Bezzubova *et al.* 1997), mice (Essers *et al.* 1997), fission yeast (Muris *et al.* 1997), and Drosophila (Kooistra *et al.* 1997). Altogether, this suggests a strong conservation of gene sequence and DNA damage response across the three eukaryotic kingdoms (plants, fungi, and animals). Another interesting finding of this study is the radiation sensitivity of mutants in genes previously studied for their involvement in development (*AtBRM*, *PICKLE/GYMNOS*, *PIE1*) or in silencing (*DDM1*) but not known as playing a role in DNA damage response.

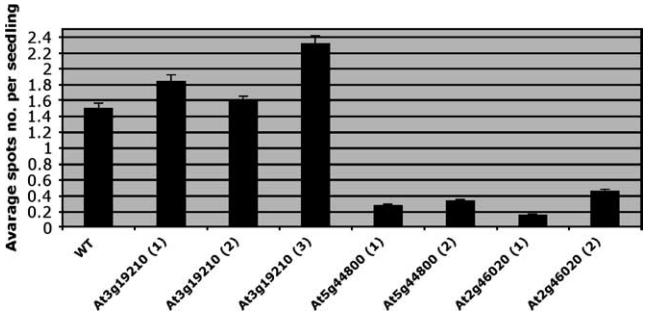

Figure 6.—Intrachromosomal recombination (ICR) in RNAi lines derived from the Arabidospsis *SWI2/SNF2* genes. The 13 RNAi lines studied in this work were tested. Only two loci showed a significant reduction in ICR rates (At5g44800/CHR4 and At2g46020/CHR2). This reduction was observed in two (of two) independent RNAi transformants. In the remaining 11 lines there was no significant alteration in ICR rates compared to wild type. An example is shown for three (of three) independent RNAi lines for At3g19210 (AtRAD54). The ICR rate is expressed as the number of spots/plant seen after histochemical staining of 3-week-old seedlings as previously described (Puchta *et al.* 1995). The wild-type column corresponds to the ecotype Columbia. Bars, SEM. A total of 100–150 seedlings were tested for each line.

Role of the Arabidopsis *SWI2/SNF2* genes in homologous recombination: Unlike for radiation sensitivity, in which the alteration of most genes affected the response to UV or γ-irradiation, only 2 of the 13 tested genes (Table 1) were involved in homologous recombination as determined by the inverted repeat recombination assay that we used. This assay measures essentially crossover between the repeats (that leads to an inversion) (Prado *et al.* 2003). These two cases (At5g44800/CHR4 and At2g46020/CHR2) add to *AtINO80*, the first *SWI2/SNF2* plant gene that was shown to be involved in homologous recombination between repeats (Fritsch *et al.* 2004). We cannot rule out that the 11 remaining *SWI2/SNF2* genes, which did not affect HR between inverted repeats, might also be involved in other types of HR because different genes may affect HR differently depending on the nature of the partners (Haber 2000). For example, in yeast, *RAD54* affects mostly mitotic recombinational repair between sister chromatids, while its homolog *TID1* affects recombination between homologs during meiosis (Arbel *et al.* 1999). A thorough analysis should therefore assay the role of the Arabidopsis *SWI2/SNF2* genes on HR between a broad range of partners, *e.g.*, interchromatid or interhomolog somatic recombination (Molinier *et al.* 2004), between direct repeats (Swoboda *et al.* 1994), in gene targeting,

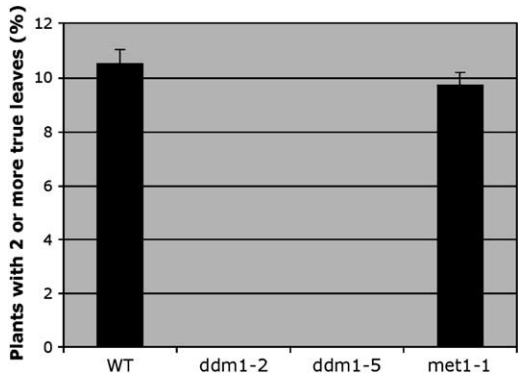

FIGURE 7.—Response of Arabidopsis *ddm1-2 and met1-1* mutants to γ-irradiation. The percentage of seedlings that developed two or more true leaves following γ-irradiation with a 30-krad dose is shown for the wild type (ecotype Columbia) and for the *ddm1-2* and *met1-1* homozygote mutants. Bars, SEM. A total of 400–500 seedlings were monitored for each line.

and between homologs during meiosis. In support for this recombination substrate specificity, we showed recently that expression of the yeast *RAD54* gene in Arabidopsis enhanced gene targeting frequencies (SHAKED *et al.* 2005) while it did not affect intrachromosomal recombination rates (our unpublished data).

The comparison of *DDM1 vs. MET1* in DNA damage response—chromatin remodeling or cytosine methylation? Mutations in both *DDM1* and *MET1* cause demethylation of the genome, with Ddm1 affecting mostly heterochromatin regions and more gradually low copy sequences (JEDDELOH *et al.* 1999), while Met1 acts throughout the genome (KANKEL *et al.* 2003). A notable difference between these two proteins is that Ddm1 is a nucleosome remodeling protein (BRZESKI and JERZMANOWSKI 2003) while Met1 is a cytosine methyltransferase enzyme. The response to DNA damage of the *ddm1 vs. met1* mutants provides insight as to whether the radiation sensitivity of *ddm1* is caused by disruption of chromatin-remodeling functions or by alterations in cytosine methylation. We found that the *met1* mutant, kindly provided by Eric Richards, has the same DNA damage response as wild type, while *ddm1* mutant alleles are sensitive (Figure 7). The *met1-1* mutant allele used here retains 30% of its cytosine methylation compared to wild type (KANKEL *et al.* 2003). Therefore, although one cannot rule out the importance of cytosine methylation in DNA damage response, the strong reduction in cytosine methylation was not associated with an altered response to DNA damage, suggesting a nonessential role of cytosine methylation in γ-irradiation response. In *ddm1*, in addition to a reduction in cytosine methylation, a strong alteration in nuclear organization and chromatin structure, particularly in the centromeric and pericentromeric regions, was found (PROBST *et al.* 2003). It is possible that this alteration in chromatin is the cause for radiation sensitivity of the *ddm1* mutants.

A link between chromatin structure, gene silencing, and genome maintenance had been previously proposed for the *MIM* gene (HANIN *et al.* 2000) and for *BRU1*, an Arabidopsis gene linking heterochromatin stability to gene silencing as well as to DNA repair (TAKEDA *et al.* 2004). Our results on *DDM1* provide a new example of this link for a chromatin remodeling *SWI2/SNF2* gene and further support the link between genetic and epigenetic stability.

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