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The Rad17 homologue of *Arabidopsis* is involved in the regulation of DNA damage repair and homologous recombination

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Summary

Rad17 is involved in DNA checkpoint control in yeast and human cells. A homologue of this gene as well as other genes of the pathway (the 9-1-1 complex) are present in *Arabidopsis* and share conserved sequence domains with their yeast and human counterparts. DNA-damaging agents induce At*RAD*17 transcriptionally. At*RAD17* mutants show increased sensitivity to the DNA-damaging chemicals bleomycin and mitomycin C (MMC), which can be reversed by complementation, suggesting that the loss of function of Rad17 disturbs DNA repair in plant cells. Our results are further confirmed by the phenotype of a mutant of the 9-1-1 complex (Rad9), which is also sensitive to the same chemicals. At*RAD9* and At*RAD17* seem to be epistatic as the double mutant is not more sensitive to the chemicals than the single mutants. The mutants show a delay in the general repair of double-strand breaks (DSBs). However, frequencies of intrachromosomal homologous recombination (HR) are enhanced. Nevertheless, the mutants are proficient for a further induction of HR by genotoxic stresses. Our results indicate that a mutant Rad17 pathway is associated with a general deregulation of DNA repair, which seems to be correlated with a deficiency in non-homologous DSB repair.

Keywords: *Arabidopsis*, *RAD17*, *RAD9*, mutagens, double-strand break repair, comet assay.

Introduction

The progression of eukaryotic cells through the mitotic and meiotic cell cycle is tightly regulated by surveillance mechanisms known as checkpoint control systems (Carr, 1997; Elledge, 1996; Hartwell and Kastan, 1994). In response to DNA damages, checkpoint pathways mediate cell cycle arrest at G1, S or G2 phase and induce DNA repair networks to avoid mutations (Enoch and Norbury, 1995; Paulovich *et al.*, 1997). Much of our understanding of the factors implicated in the processes of checkpoint control derives from investigations of checkpoint-defective fission and budding yeast cells in which the detection of DNA lesions is disturbed. The DNA damage response is characterized by a slowdown or arrest of the cell cycle progression (Abraham, 2001; Lowndes and Murguia, 2000). The DNA damage response can cause changes in chromatin structure at the region of the damage and the transcriptional induction or post-translational modification of proteins, which participate in DNA repair (Bashkirov *et al.*, 2000; Morrison *et al.*, 2000). In budding yeast, a single persistent double strand break (DSB) can be detected and a response for repair be triggered (Lee *et al.*, 1998). Genetic studies in the fission yeast *Schizosaccharomyces pombe* identified key checkpoint molecules (Rad1, Hus (hydroxy urea sensitive)1 and Rad9; Al-Khodairy and Carr, 1992; Enoch *et al.*, 1992; Rowley *et al.*, 1992). Homologues of these proteins in other species have been identified (Freire *et al.*, 1998; Kostrub *et al.*, 1998; Lieberman *et al.*, 1996). They show sequence homology to the proliferating cell nuclear antigen (PCNA), and as a part of the Rad1/Hus1/Rad9-checkpoint complex (9-1-1 complex). they seem to function at the beginning of cell cycle checkpoint signal cascade (Caspari *et al.*, 2000; Thelen *et al.*, 1999; Venclovas and Thelen, 2000). The 9-1-1 complex associates with chromatin after treatment with DNA-damaging agents (Burtelow *et al.*, 2000) and Rad17 regulates this association (Kondo *et al.*, 2001; Melo *et al.*, 2001; Zou *et al.*, 2002). Rad17 revealed similarity to all five subunits of replication

factor C (Rfc; Griffiths *et al.*, 1995) and apparently acts as a PCNA clamp loader. *RAD1*, *RAD9*, *HUS1* and *RAD17* are necessary for S phase and G2/M arrest in response to both DNA damage and stalled DNA replication (Stewart and Enoch, 1996). The *Saccharomyces cerevisiae* Rad17 homologue Rad24 has been found in a complex with all Rfc subunits except for the largest one, the Rfc1 (Green *et al.*, 2000), indicating that Rad17 and Rfc1 compete for binding with the other four subunits. Both the Rad24/Rfc2-5 and the Rad17/Ddc (DNA damage checkpoint protein)1/Mec (mitosis entry checkpoint)3 complexes in *S. cerevisiae* (the latter is homologous to the Rad1/Rad9/Hus1 complex of *S. pombe* and *Homo sapiens*) translocate to sites of DNA damage (Kondo *et al.*, 2001; Melo *et al.*, 2001). The proteins have been conserved throughout eukaryotic evolution (Venclovas and Thelen, 2000). Comparative sequence analysis among plants, yeast and animals indicates that many factors involved in DNA repair and recombination are conserved between vertebrates and plants (*Arabidopsis* Genome Initiative, 2000; Britt and May, 2003; Hartung and Puchta, 2004). Although mutations of DSB repair genes are lethal in mammals, the mutated plants are usually viable, perhaps because of the absence of the apoptotic response by p53 in the latter (Frank *et al.*, 2000).

Recently, it was shown that uvh (UV-hypersensitivity) 1-plants defective in a homologue of the human repair endonuclease XPF (xeroderma pigmentosom, complementation group F) arrest in G2 after irradiation and that the arrest is abolished in mutant suppressor of gamma (*sog*)*1*. These plants show high levels of genomic instability and meiotic defects (Preuss and Britt, 2003). The *Arabidopsis* homologue of human *ATM* (ataxia telangiectasia mutated) was cloned and identified (Garcia *et al.*, 2000). *ATM* mutants are hypersensitive to mutagenes (Garcia *et al.*, 2003). In human cells, the Atm/Atr (ataxia telangiectasia and Rad3 related) and the Rad17 checkpoint complexes bind independently of each other to sites of DNA damage (Zou *et al.*, 2002). Treatment of human cells with genotoxic agents induced ATM/ATR-dependent phosphorylation of hRad17 (Bao *et al.*, 2001). ATM seems to be involved predominantly in the response of cells to DSBs (Abraham, 2001). DSBs present unique challenge to the cell. DSBs may be caused directly via replication of nicked templates or during repair of DNA damage such as adducts or cross-links. DSBs can be repaired by homologous recombination (HR) or by non-homologous end joining (NHEJ; Jeggo, 1998; Paques and Haber, 1999). HR requires regions highly similar or identical sequences for re-joning. NHEJ allows a sequence-independent direct reconnection of broken ends, a process that can be error prone.

Using the genomic sequence of *Arabidopsis*, we identified and characterized the gene structure of At*RAD17* and the three PCNA-like genes At*RAD9*, At*RAD1*-like and At*HUS1*. We use for the designation of these *Arabidopsis* genes the nomenclature derived from the *S. pombe* homologues, similar to the practice in vertebrates. Because of difficulties in the synchronization of cell cultures, a detailed functional analysis of cell cycle regulation is still not feasible in *Arabidopsis*, we concentrated the current study on the influence of the pathway on DNA repair. Mutants of At*RAD17* as well as At*RAD9* are more sensitive to the genotoxic agents bleomycin and mitomycin C (MMC). As the double mutant does not show an enhanced sensitivity, these genes seem to be in the same epistasis group. The repair of DSB is delayed in the mutants. Interestingly, HR is increased in the mutant background, indicating that the mutations do not lead to a general defect in DNA repair but mainly to a deficiency in non-homologous recombination.

Results

*Identification of homologues of the four checkpoint genes At*RAD17*, At*RAD1-like*, At*RAD9 *and At*HUS1 *in* Arabidopsis thaliana *and cDNA analysis*

Database search using TAIR-TBLASTN with the *S. cerevisiae*, *S. pombe* and *H. sapiens* checkpoint proteins as probe resulted in significant hits (At*RAD17* At5g66130, At*RAD1-like* At4g17760, At*RAD9* At3g05480 and At*HUS1* At1g52530). As the abbreviation At*RAD1* was used before for the plant homologue of *S. cerevisiae RAD1*, *S. pombe* RAD16 and human XPF subunit of an endonuclease complex, implicated in nucleotide excision repair and interstrand cross-link repair (Gallego *et al.*, 2000), we refer to At4g17760 as At*RAD1-like*. Using mRNA from *Arabidopsis* flowers as template, the four cDNAs could be amplified via PCR. By RACE (rapid amplification of cDNA ends) (Matz *et al.*, 1999) with nested gene-specific primers, 5′ and 3′ ends were obtained. The schematic structure of the four *Arabidopsis* genes is shown in Figure 1(a). The open-reading frame (ORF) of full-length AtRAD17 (11 exons) has a total length of 1800 bp, coding for a putative protein of 599 amino acids (aa). The sequence is in accordance with the prediction of TAIR (The Arabidopsis Information Resource) and the reported sequence submitted to the GenBank (Sugiyama, H., Oguchi, K., Tamura, K and Takahashi, H. GenBank Accession AB030250). For At*RAD1-like*, two different cDNAs resulting from alternative splicing could be detected (Accession AJ608271: splice variant 1; and AJ608272: splice variant 2). Both contain seven exons and differ only in the 5′ end of exon 2. The ORF of the longer transcript contains 903 bp (300 aa), and the putative ORF of the shorter form contains 558 bp (185 aa) corresponding to the last 558 bp of the longer form. The longer variant is in accordance with the prediction of TAIR and the

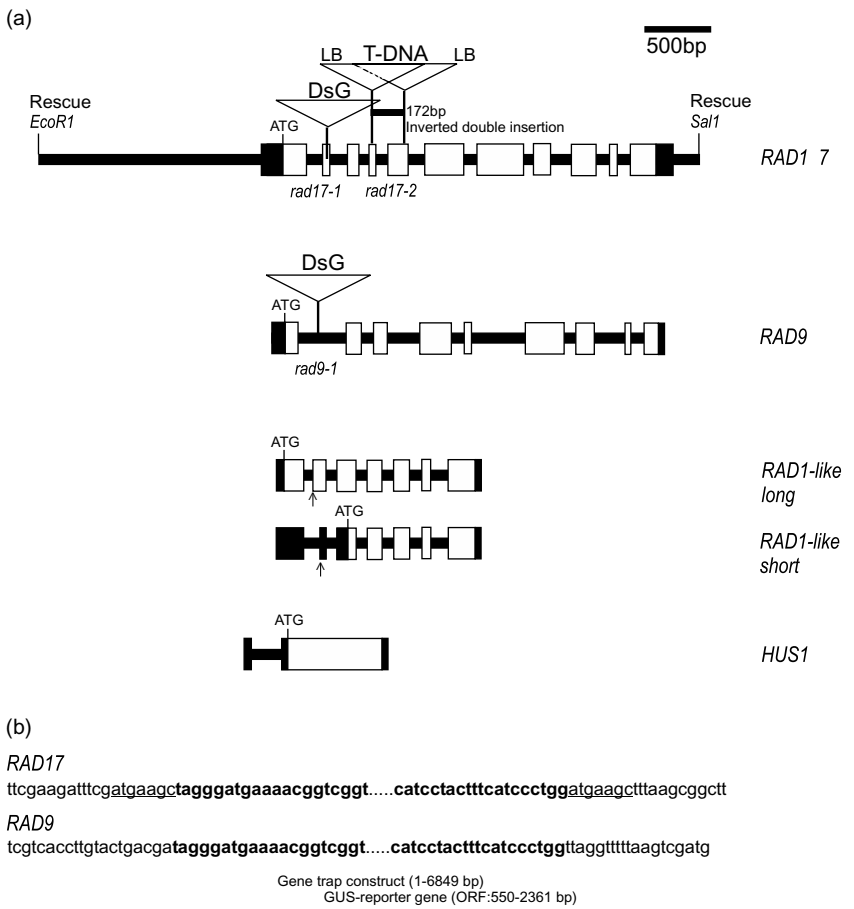


Figure 1. The At*RAD17*, At*RAD9*, At*RAD1-like* and At*HUS1* homologues of *A. thaliana*.
(a) Genomic structure of four putative DNA checkpoint control genes of *A. thaliana* with the sites of transposon and T-DNA integrations (DsG, gene trap transposon; and T-DNA, transfer DNA). Exons (boxes) were deduced from the cDNA sequence, and black boxes indicate the 5′ and 3′ UTRs. *RAD1*-like: arrows indicate the different 5′ splice sites in exon 2. The position of the insertions in At*RAD9* and At*RAD17* is indicated by triangles, respectively. At*RAD9*: The transposon insertion is located in intron 1. At*RAD17*: The transposon insertion is located in exon 2 and has caused a duplication of 7 bp. In both cases, the orientation of the GUS gene is in the same orientation as the mutated gene. The T-DNA double insertion has caused a 172-bp deletion comprising a part of exon 4, intron 4 and a part of exon 5. The restriction sites of At*RAD17* mark the cloning sites of the DNA fragment used for complementation.
(b) Sequence and position of the gene trap insertion sites. The transposon sequence is shown in bold letters and has a multiple splice acceptor fused to the GUS gene (http://spot. cshl.org/genetrap_database/traps.html). The At*Rad17* sequence duplication is underlined.

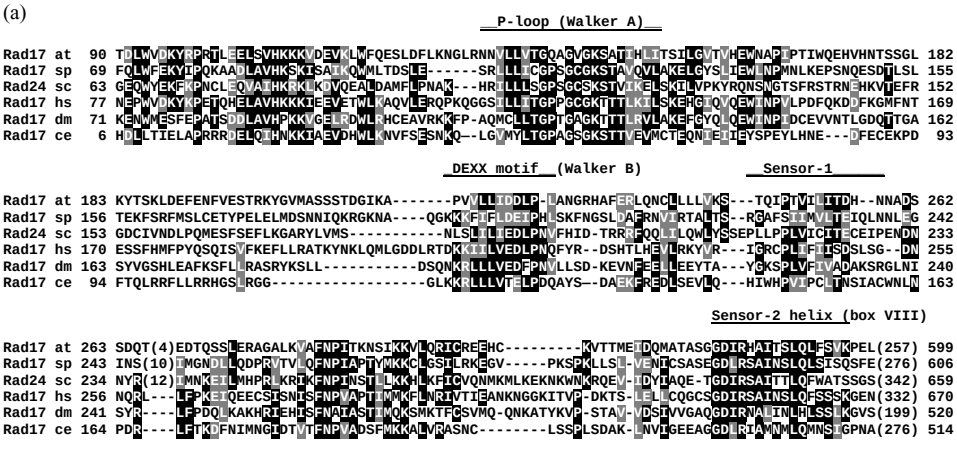
reported sequence submitted to the GenBank, and the shorter one has 49 bp deleted at the 5′ end of exon 2, causing a frameshift. We presume that translation of a putative protein might start with a methionine in exon 3, thus resulting in a truncated protein with a deletion of the first 115 aa at the N-terminus (Figure 2b). Analysis of the full-length cDNA of the At*RAD9* gene (Accession AJ608269) revealed an ORF of 1263 bp (420 aa) with nine exons (in accordance with MIPS (Munich Information Center for Protein Sequences) prediction). The full-length coding sequence of At*HUS1* (Accession AJ608270) consists of 960 bp (319 aa) and contains two exons. The first exon is part of the 5′ untranslated region (UTR). The intron/exon pattern differs from the TAIR and MIPS predictions. We isolated an AtHus1-cDNA with a 38-bp longer first exon at the 3′ end than predicted. Because of the frameshift in the second exon, the resulting cDNA encodes a protein 25 aa shorter than the predicted one.

Relation of AtRad17, AtRad1-like, AtRad9 and AtHus1 to homologues of other species

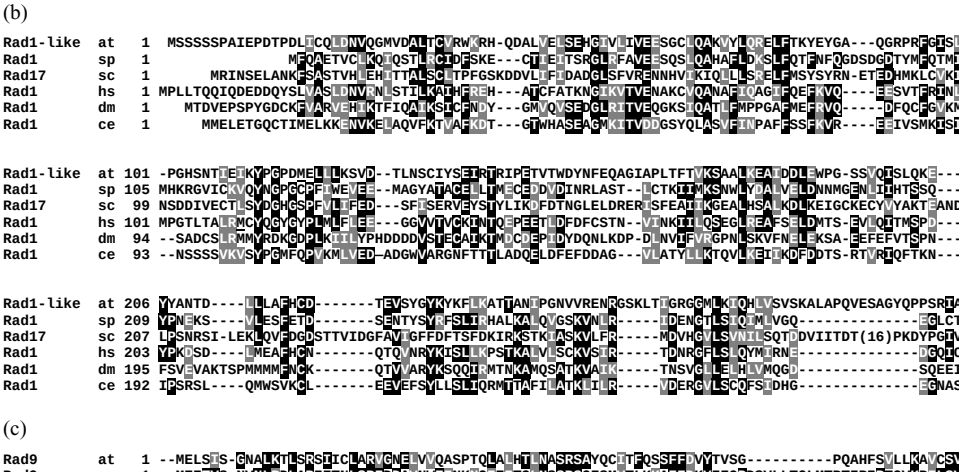
We aligned the four putative proteins from *Arabidopsis thaliana* to the homologous proteins of *H. sapiens*, *S. pombe*, *S. cerevisiae*, *Drosophila melanogaster* and *Caenorhabditis elegans*, to characterize conserved domains (Figure 2a–d). AtRad17 protein shares 28% of amino acid with human Rad17 between amino acid positions 75 and 508 and 23% with *S. pombe* Rad17 between positions 92 and 519. It exhibits a higher degree of sequence identity in the conserved replication factor-C-like domains and involves the Walker A motif between positions 132 and 152. Walker A is characterized as a phosphate-binding P-loop. It has been shown by sequence searches and alignment that human Rad17, *S. pombe* Rad17 and the clamp loaders belong to a large group of ATPases group described as AAA+ class (Neuwald *et al.*, 1999). Besides Walker A and

Figure 2. Sequence alignment of the proteins Rad17, Rad1-like, Rad9 and Hus1.
Multiple alignment of conserved domains of the putative checkpoint control proteins from *A. thaliana* (At), *S. pombe* (Sp), *S. cerevisiae* (Sc), *H. sapiens* (Hs), *D. melanogaster* (Dm) and *C. elegans* (Ce). (a) Rad17, (b) Rad1, (c) Rad9 and (d) Hus1. Gaps are indicated by dashes. Conserved amino acids are black shaded and similar amino acids are grey shaded. The putative methionine start of the truncated AtRad1-like (amino acid position: 116) and HsRad1 (amino acid position: 110) splice form are underlined. The positions of the amino acids in each protein are shown at left and right. Amino acid sequences were aligned using CLUSTALW.

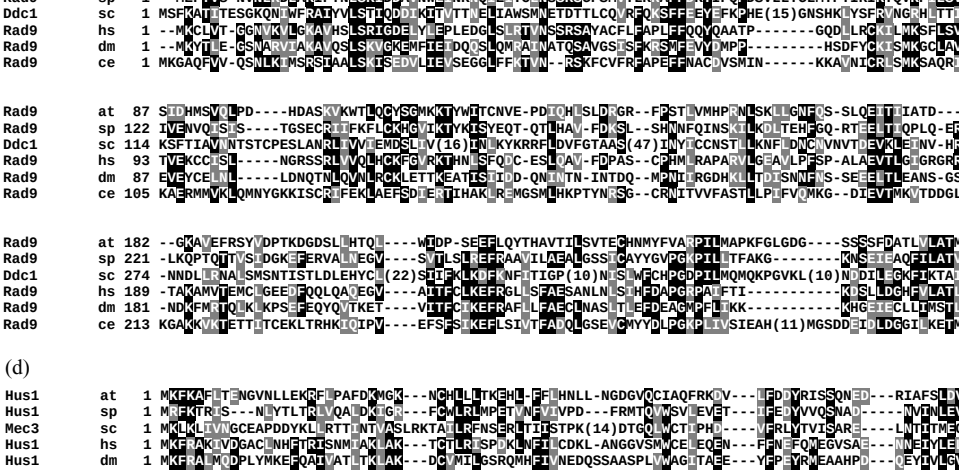
(a)


P-loop (Walker A)
DEXX motif (Walker B)
Sensor-1
Sensor-2 helix (box VIII)

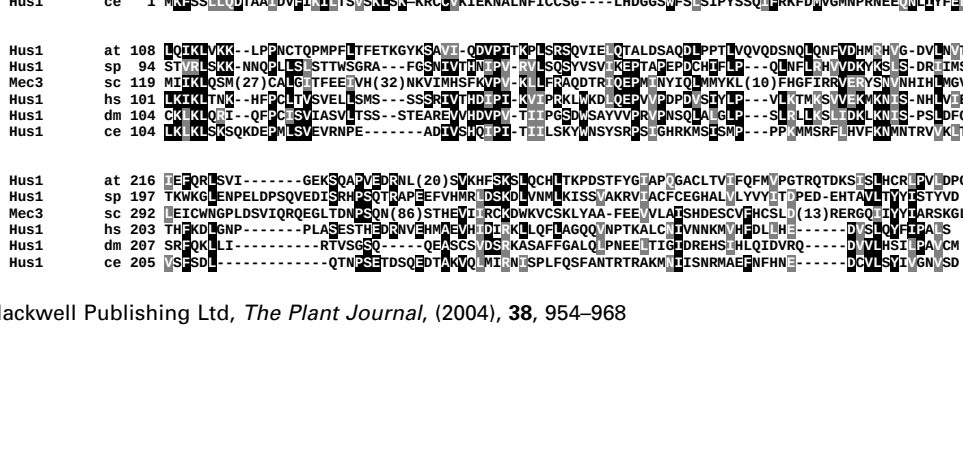
(b)



(c)



(d)



another nucleotide-binding motif (Walker B or DEXX motif), Sensor-1 and Sensor-2 helices share several conserved sequences that distinguish them from other NTPases and contain boxes previously defined for RFC-related proteins (Cullmann *et al.*, 1995). The Walker B motif of AtRad17 is located between amino acid positions 215 and 225, the putative Sensor-1 motif extends from positions 248 to 260 and Sensor-2 (RFC-boxVIII) from positions 324 to 339 (Figure 2a).

Structural comparisons of Rad checkpoint proteins with PCNA proteins based on fold recognition and comparative modelling revealed that these proteins are conserved (Venclovas and Thelen, 2000). Short amino acid domains were found to be conserved between the PCNA proteins, and the Rad9 proteins of eukaryotes including *Arabidopsis* share some of these domains. The putative AtRad9 shows domains, which are present in other Rad9 proteins but absent in PCNA proteins, indicating that the identified gene may indeed have similar functions as Rad9 in *S. pombe* and human. The AtRad9 amino acid sequence shares 28% of identical amino acid between positions 1 and 164 and 40% between positions 377 and 415 with human Rad9 and with *S. pombe* Rad9 31% of amino acid between positions 1 and 61 and 19% between positions 88 and 270. As for At*RAD1-like*, a long and a short cDNA have been identified for human *RAD1*. Also the short form of human Rad1 is characterized by an N-terminal truncation, comprising 109 aa (Parker *et al.*, 1998). The long AtRad1-like form shares 25% identity with the long one of human Rad1 between positions 10 and 297. The presumed AtHus1 protein shows 22% identity with the human Hus1 protein between amino acid poistions 1 and 241.

*At*RAD17 *is induced by DNA damage*

Previous experiments indicated that the At*RAD17* gene is inducible by genotoxic stress (Chen *et al.*, 2003). To sustain this finding and to test whether members of the 9-1-1 complex can be induced by genotoxic agents, real-time PCR experiments were performed. Treatment of seedlings with bleomycin yielded (1 μg ml^{-1}, 6 h) a sevenfold induction, and treatment with MMC (22 μg ml^{-1}, 6 h) yielded a 3-fold gamma irradiation (100 Gy) and a 17-fold induction of At*RAD17* mRNA. However, the members of the 9-1-1 complex (At*Rad9*, At*Hus1* and At*Rad1-like*) did not show significant transcriptional change (Table 1). Steady state expression of these four genes was compared and no significant difference at the basal expression level among the genes was observed (with At*RAD17* as the highest and At*RAD9* as the weakest expressed genes within a range of fivefold difference; data not shown). As a positive control of transcriptional induction, expression of At*Rad51* was also determined and yielded a 10- to 30-fold induction upon genotoxin treatment.

Table 1 Effect of genotoxin treatments on expression of At*RAD17*, At*HUS1*, At*RAD1* and At*RAD9* as determined by real-time PCR

	At*RAD17*	At*HUS1*	At*RAD1*	At*RAD9*	*60S RP L27A*
Bleomycin	7.4	1.3	1.5	1.0	1.0
MMC	3.1	−1.2	1.1	1.1	1.0
Gamma	16.9	1.0	1.8	1.0	1.0

60S RP L27A (ribosomal protein 27) is used as reference gene (gamma, gamma-irradiation; and − indicates down regulation).

rad17 *and* rad9 *mutants*

To analyse the function of the putative checkpoint homologues *in vivo*, we mainly concentrated our analysis on the At*RAD17* gene using two independent mutants. To further sustain our findings, a mutant of At*RAD9* was used. We used mutant lines with gene trap transposons insertions (Cold Spring Harbour (CSH), USA) in the At*RAD17* and the At*RAD9* genes, respectively, and a line with a T-DNA integration (TAIR) in the At*RAD17* gene. Both the transposon construct (Ds transposable element) and the T-DNA contain a neomycin phosphotransferase (NPTII) gene (conferring resistance to kanamycin) as a selectable marker.

We characterized the insertions molecularly in detail. The transposon in the *rad9-1* mutant (line GT8083) inserted in intron 1, the transposon in the *rad17-1* mutant (line GT534) inserted in exon 2 causing a 7-bp duplication (Figure 1a,b). The insertion of the *rad17-2* mutant (line SALK_009384) in exon 4 consists of two T-DNAs in inverted orientation with the left borders joined to the plant DNA (Figure 1a) and is combined with a deletion of 172 bp, containing the complete intron 4 and a part of exon 5. Transgenic plants with a single integration were identified by segregation analysis and Southern blotting of DNA from *rad17-1* and *rad9-1*. A unique band appeared on Southern blots after digestion with three different restriction enzymes and hybridization with transgene DNA probe (data not shown). Plants homozygous for the integration were selected by PCR. Homozygous transgenic plants did not differ phenotypically from wild-type plants. By RT-PCR with primers designed to cover the entire ORF, At*RAD17* and At*RAD9* transcripts were found in 10-day-old seedlings and in flowers of wild-type plants. No At*RAD17* mRNA was detectable in *rad17-1* and *rad17-2* plants and no At*RAD9* mRNA in *rad9-1* plants. However, RT-PCR analysis (data not shown) indicated that parts of the genes downstream of the insertion are transcribed in the mutants, as has been reported for other insertion mutants before (e.g. Friesner and Britt, 2003). Our results allow the conclusion, that the inserted transposons prevent the synthesis of the full-length mRNAs of At*RAD17* and At*RAD9*, respectively. At*RAD9* mRNA levels in the *rad17-1* line and *RAD17* mRNA levels in the *rad9-1* line were comparable to those of the wild type.

rad17 *and* rad9 *mutant lines show increased sensitivity to DNA-damaging agents*

Schizosaccharomyces pombe and mammalian *rad17* and *rad9* cells (as the respective mutant cells *rad24* and *ddc1* in *S. cerevisiae*) are hypersensitive to DNA-damaging agents and irradiation (e.g. Eckardt-Schupp *et al.*, 1987; Griffiths *et al.*, 1995; Li *et al.*, 1999; Longhese *et al.*, 1997; Roos-Mattjus *et al.*, 2003). Seedlings of *rad17-1*, *rad17-2*, *rad9-1* and the wild type were incubated 8 days after germination with different concentrations of the mutagens bleomycin and MMC, in liquid germination medium (GM). MMC

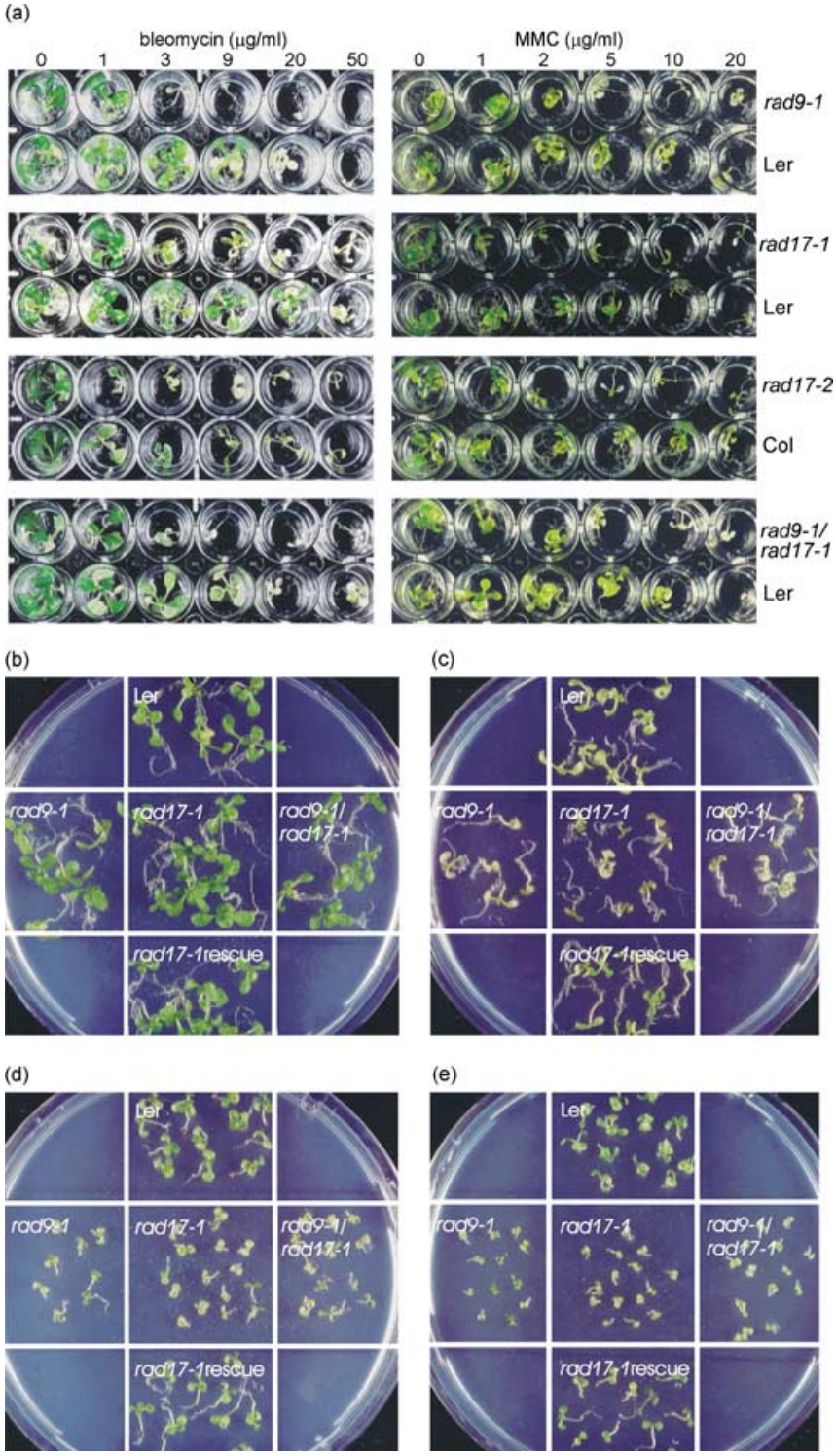


Figure 3. Bleomycin and MMC sensitivity of *rad9-1*, *rad17-1*, *rad17-2*, and *rad9-1/rad17-1* seedlings.
(a) Comparison of seedlings after a 2-week-long treatment with different concentrations of bleomycin and MMC.
(b–e) Bleomycin and MMC sensitivity of *rad9-1*, *rad17-1*, *rad9-1/rad17-1* and *rad17-1* rescue seedlings. Two-week-old plants grown in Petri dishes on GM containing following concentrations of mutagens: (b) no mutagen; (c) 3 μg ml^{-1} bleomycin; (d) 15 μg ml^{-1} MMC; and (e) 30 μg ml^{-1} MMC.

causes DNA interstrand cross-links (Friedberg *et al.*, 1995) and bleomycin is a true radiomimeticum causing single-strand break and DSB. After treatment for 14 days, the survival rate was checked (Figure 3a). Both homozygous *rad17* mutant lines as well as the *rad9-1* mutants die after treatment with 3–9 μg ml^{-1} of bleomycin, while wild-type plants tolerate a concentration as high as 9 μg ml^{-1}. MMC concentrations of 5–10 μg ml^{-1} had lethal effects on the mutants, whereas wild-type plants survived those concentrations (Figure 3a). To test whether the two genes are part of a common or of independent pathways responsible for the mutagene sensitivity, a double mutant was produced by crossing. Seedlings homozygous for both *rad17-1* and *rad9-1* are not more sensitive to bleomycin and MMC than the single homozygous mutants (Figure 3a), indicating that the proteins Rad17 and Rad9 act epistatically, i.e. are involved in a common pathway. We confirmed the hypersensitivity of *rad17-1*, *rad9-1* and *rad17-1/rad9-1* lines by incubating the seeds in GM containing bleomycin and MMC. The seeds were spread on bleomycin- and MMC-containing agar medium and their growth was analysed 12 days after germination. In the absence of the mutagenes, both single mutants and the double mutant had grown to similar stage and extent as the wild type (Figure 3b), whereas the three mutant lines show a strong hypersensitivity to bleomycin (Figure 3c) and MMC (Figure 3d,e) as compared to the wild type. In this experiment, bleomycin treatment caused bleaching of leaves, and after MMC treatment, shorter roots and reduced shoot growth were typical mutant phenotypes.

Complementation of the rad17-1 *mutant*

To confirm that the phenotype of the *rad17-1* mutant is because of a loss of function, we cloned the genomic wild-type At*RAD17* region harbouring 1724 bp upstream of the transcription start, the 3078-bp transcription region and the downstream region 194 bp of the transcription stop, in the binary vector pZP221. After agrobacteria-mediated transformation, we selected plants carrying one copy of the transferred At*RAD17* wild-type gene and in the next generation the seedlings homozygous for the transferred At*RAD17* gene. The germination of wild-type *rad17-1*, with At*RAD17*-transformed *rad17-1* seedlings (named *rad17-1* rescue) on GM plates with bleomycin (3 μg ml^{-1}) and MMC (10 and 15 μg ml^{-1}), showed clearly that the rescued plants tolerate these concentrations like or almost like the wild type (Figure 3b–e). Thus, the hypersensitivity of the *rad17-1* line is indeed because of the mutated At*RAD17* gene.

Analysis of DSB repair with the comet assay

To test whether the sensitivity to genotoxic agents is correlated with a deficient repair of DSBs, we performed comet assays with nuclei of the *rad17-1* and *rad9-1* mutants and wild-type plants of the respective background (Landsberg *erecta* (L*er*)). Having a different background (Columbia (Col)-0), the mutant *rad9-2* was not included in the analysis. Plants were treated with bleomycin, and the percentage of DNA in comet tails indicative for DSBs was determined at different times after application of the genotoxin. The kinetics of DSB repair is biphasic as portrayed in Figure 4. Less DSBs are repaired in the mutants after shorter time periods (10 min). Apparently, both mutants have a defect in this first, very rapid mechanism of DSB repair. Nevertheless, repair of DSB induced by bleomycin in wild-type *Arabidopsis* as well as in *rad* mutants was completed within 1 h. This indicates that the defect in the rapid DSB repair pathway can be compensated by a slower pathway for which Rad17 and Rad9 are not required in *Arabidopsis*.

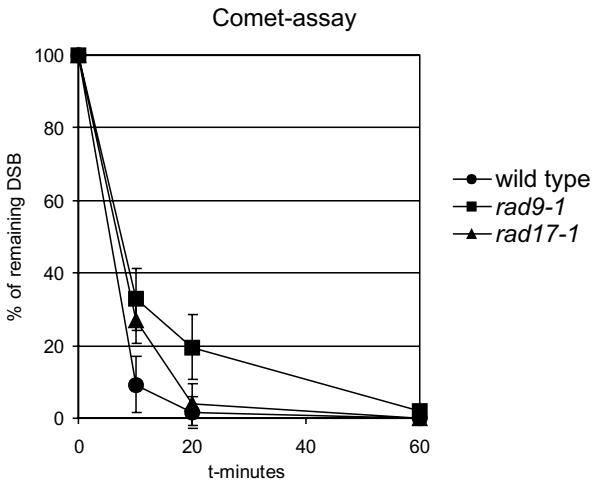


Figure 4. Analysis of DSB repair by the comet assay. Mean percentage of DSB remaining (±SE) in wild-type *Arabidopsis* L*er* plants and mutants *rad9-1* and *rad17-1* at different time points after treatment with 30 μg ml^{-1} bleomycin as determined by the comet assay.

The frequency of intrachromosomal homologous recombination with and without genotoxin treatment is higher in the rad17 *and* rad9 *mutants than in the wild type*

To determine whether the defect of rapid DSB repair is linked to homologous recombination, we tested the influence of the mutated At*RAD17* and At*RAD9* genes on the frequency of HR by a well-established *in planta* recombination assay (Orel and Puchta, 2003; Schmidt-Puchta *et al.*, 2004; Swoboda *et al.*, 1994). The recombination substrate and reporter transgene consists of two overlapping fragments of the β-glucuronidase (GUS; *uidA*) gene interrupted by a hygromycin selectable marker gene. The separated GUS sequences share a common overlap of 566 bp in inverted orientation. Homologous recombination between

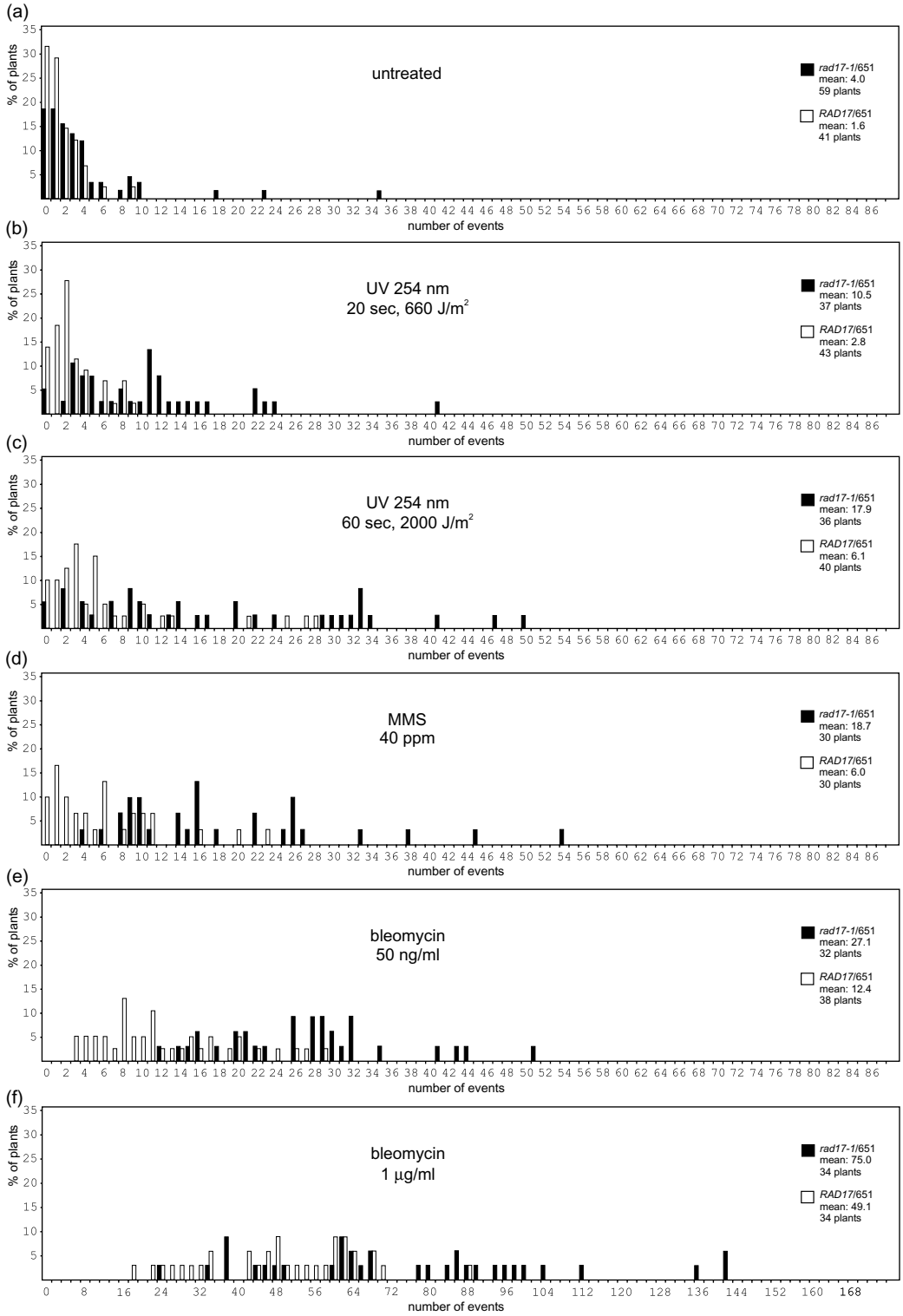


Figure 5. Homologous recombination events in 651/*rad17-1* seedlings.
Diagram showing the proportions of seedlings with a given number of blue spots. (a) Untreated, (b) 20 sec UV (254 nm), 660 J m^{-2}, (c) 60 sec UV (254 nm), 2000 J m^{-2}, (d) 40 p.p.m. MMS, (e) 50 ng ml^{-1} bleomycin and (f) 1 µg ml^{-1} bleomycin. Homozygous *rad17-1* mutants are indicated in black bars and control plants homozygous for *RAD17* genes in white bars.

the two overlapping DNA sequences produces a functional GUS gene. Cell cluster expressing GUS activity can be detected as blue sectors after histochemical staining, and it was shown before that these sectors indeed rise from recombination events (Swoboda *et al.*, 1994). Mutant lines were crossed with the GUS recombination line 651 (Puchta *et al.*, 1995), and in the F_3 generation, the seedlings homozygous for the GUS recombination substrate and homozygous for the *rad* mutations or the wild type were used to determine the number of HR events. Homozygous 20-day-old seedlings of 651/*rad17-1* and 651/*RAD17* genotypes were histochemically stained. The distribution and frequency of recombination events determined per individual are shown in Figure 5(a) and Table 2. The 651/*rad17-1* seedlings showed an increase of blue sectors (up to 35 per individual) compared to wild-type control (not more than four per individual), the mean was 2.5-fold higher than that in the control. Furthermore, we tested the frequency of recombination events after treating the seedlings with DNA-damaging agents (Figure 5b–f; Table 2). Ten-day-old plants were treated with methylmethanesulphonate (MMS) (40 p.p.m.) or with different concentrations of bleomycin (50 ng ml^{-1} and 1 $\mu g\ ml^{-1}$) or irradiated with UV light (20-sec pulse with 254 nm, 660 J m^{-2} and 60-sec pulse with 254 nm, 2000 J m^{-2}). Recombination events were counted in 20-day-old seedlings. A clear increase of blue sectors in the GUS/*rad17-1* mutant and control lines indicated an induction of HR in all cases (Figure 5b–f; Table 2). After treatments with MMS, bleomycin and UV light, the GUS/*rad17-1* showed a medial 1.5-fold (bleomycin: 1 $\mu g\ ml^{-1}$) to 3.8-fold (UV: 20 sec) increase of blue spots compared to the 651/*RAD17*. The significance of the differences of HR events between mutant and the wild type was confirmed by the pairwise non-parametric Mann–Whitney *U*-test (Table 2, see also Experimental procedures). We tested 651/rad17-2 under the same conditions and also observed an increased frequency without (2.4-fold) and with genotoxic agents (bleomycin 50 ng ml^{-1}, 2.6-fold; bleomycin 1 $\mu g\ ml^{-1}$, 1.5-fold; and MMS 40 p.p.m., 4.8-fold, data not shown), clearly sustaining our findings with the other *rad17* mutant. Furthermore, we tested the 651/*rad9-1* line by the recombination assay and found the same kind of reaction in this mutant as well (Figure 6; Table 2). The strongest increase compared to the control siblings was found after MMS induction (5.4-fold) but taken together the rates of increase between the mutant lines and their respective control lines are similar. Whereas after genotoxin treatment a mean 2.7 ± 0.8 (5) enhancement was found in the mutant *rad17-1* background in comparison to the wild type, the enhancement was 2.5 without such a treatment. In case of the *rad9-1* mutant, it was 3.5 ± 1.1 (5) with and 3.8 without genotoxin treatment in comparison to the wild type. Thus, although frequencies of HR are enhanced in mutant background, the induction of HR by genotoxic stress is still functional in these plants.

Discussion

Arabidopsis encodes putative homologues of the DNA checkpoint factors AtRad17, AtRad1-like, AtRad9 and AtHus1. Each of the putative *Arabidopsis* proteins shows more sequence similarities to the respective homologous protein in man and yeast than to any other *Arabidopsis* ORF, suggesting that these proteins are indeed the respective checkpoint homologues. We were able to find two different *RAD1*-like splice variants in *Arabidopsis*. The longer form of human Rad1-like seems to code for a 3′- to 5′-exonuclease (Parker *et al.*, 1998). We did not test AtRad1-like for exonuclease activity because the amino acids, which were found to be essential for this activity in human Rad1, are not conserved in AtRad1-like. *rad9* and *rad17* mutant plants are viable but hypersensitive to DNA-damaging agents. DNA damage sensitivity may have different reasons, such as defective checkpoint mechanisms, failure to induce transcription of DNA damage-inducible genes or insufficient DNA repair. Because of the homology to the *RAD17* and *RAD9* genes in *S. pombe* and mammals and the fact that also other genes homologous to genes

Table 2 Medium recombination frequencies in *rad17-1* and *rad9-1* seedlings

	rad17-1		*RAD17*				*rad9-1*		*RAD9*			
Treatment	*n*	*X*	*n*	*X*	Enhancement	*P*-value	*n*	*X*	*n*	*X*	Enhancement	*P*-value
Not treated	59	4	41	1.6	2.5	<0.01	48	4.5	43	1.2	3.8	<0.01
UV 254 nm (20 sec)	37	10.5	43	2.8	3.8	<0.01	39	7.4	36	2.7	2.7	<0.01
UV 254 nm (60 sec)	36	17.9	40	6.1	2.9	<0.01	33	17	33	6.1	2.8	<0.01
MMS (40 p.p.m.)	30	18.7	30	6	3.1	<0.01	32	27.7	45	5.1	5.4	<0.01
Bleomycin (50 ng ml^{-1})	32	27.1	38	12.4	2.2	<0.01	48	15.7	49	5.2	3	<0.01
Bleomycin (1 $\mu g\ ml^{-1}$)	34	75	34	49.1	1.5	<0.01	34	86.5	33	25	3.5	<0.01

n, number of plants analysed; *X*, mean number of blue sectors (recombination events); enhancement, stimulation of recombination in the mutant background in comparison to the wild type; *P*-value (Mann–Whitney test), probability that the measured recombination events between mutant and the wild type are identical. A value of <0.05 indicates a significant statistical difference.

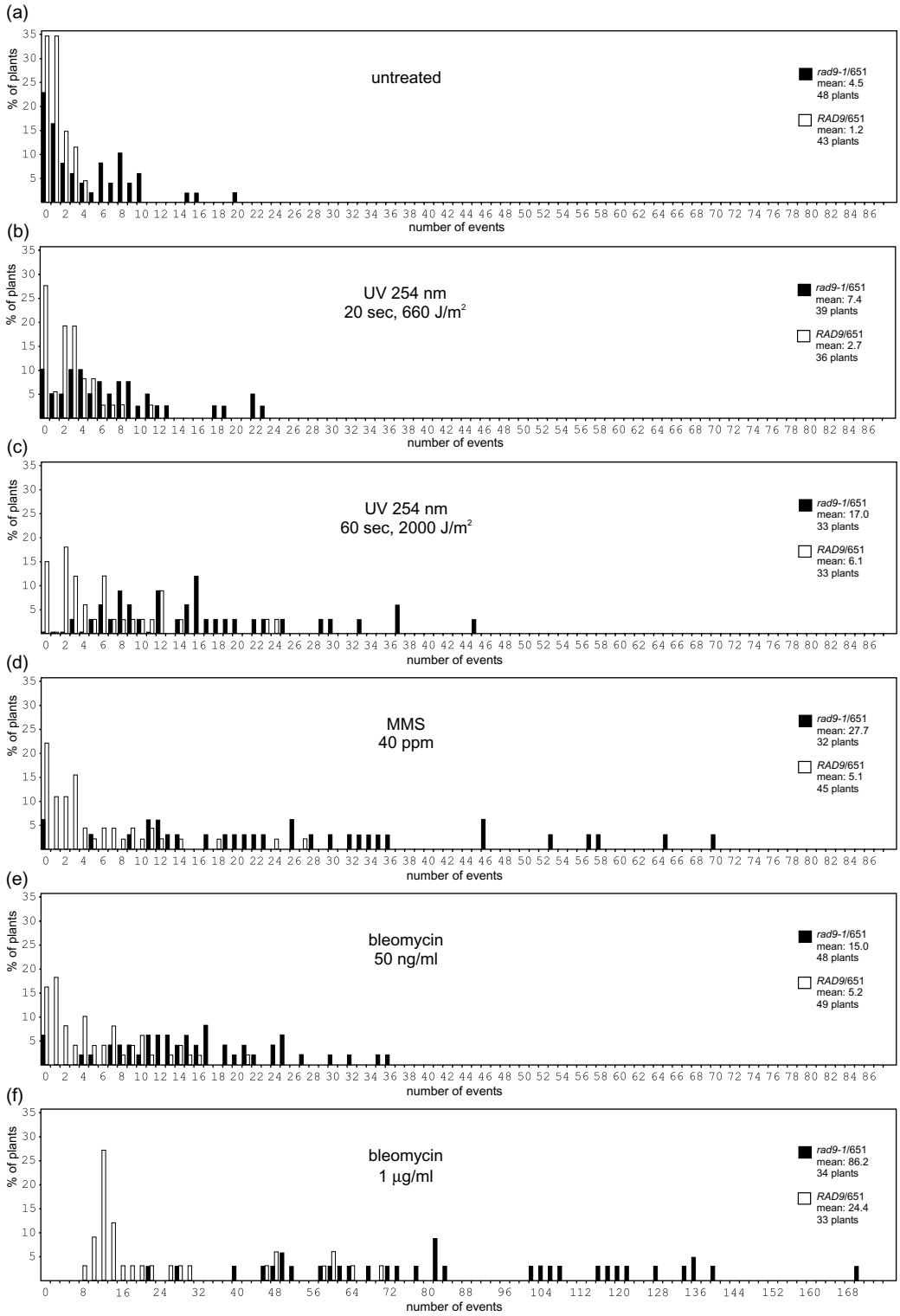


Figure 6. Homologous recombination events in 651/*rad9-1* seedlings.
Diagram showing the proportions of seedlings with a given number of blue spots. (a) Untreated, (b) 20 sec UV (254 nm), 660 J m^{-2}, (c) 60 sec UV (254 nm), 2000 J m^{-2}, (d) 40 p.p.m. MMS, (e) 50 ng ml^{-1} bleomycin, and (f) 1 µg ml^{-1} bleomycin. Homozygous *rad9-1* mutants are indicated in black bars and control plants homozygous for *RAD9* in white bars.

involved in the checkpoint pathway in these organisms could be found in the *Arabidopsis* genome, it is likely that these two genes function in a DNA damage checkpoint pathway in *Arabidopsis* too. Unfortunately, because of difficulties in the synchronization of cell cultures, a detailed direct functional analysis of cell cycle regulation is still not feasible in *Arabidopsis*. Therefore, we concentrated in the current study our efforts on the influence of the *RAD17* pathway on DNA repair. Both *rad17* and *rad9* mutants of *Arabidopsis* are sensitive to bleomycin and MMC, indicating a similar phenotype as was observed for mutations in these genes in yeast and human cells. These mutant cells die at lower mutagene concentrations than those lethal for wild-type cells. We clearly could show by two different alleles and complementation that the loss of function of At*RAD17* increases the sensitivity for DNA-damaging agents. Although only one mutated *rad9* allele was used in this study, the fact that in the mutant, the unique Ds insertion located in the At*RAD9* gene is correlated with similar sensitivities and a hyper-recombinogenic phenotype like the *rad17* mutants is for us a very strong indication that phenotype is indeed because of the loss of the At*RAD9* function. Indeed, the fact that the homozygous *rad9-1*/*rad17-1* double mutant shows a similar sensitivity to bleomycin and MMC as the single mutants suggests that both proteins are also involved in the same pathway in *Arabidopsis*.

We found a clear increase of HR events in the *rad9* and *rad17* mutants, both in untreated and bleomycin-, MMS- and UV-treated seedlings. How can we explain the enhancement of HR in the mutant background? In *Arabidopsis*, wild-type DSBs are mainly repaired by NHEJ, whereas in the yeast wild type, such lesions are primarily repaired by HR. Using a plasmid-based DSB repair assay in *S. cerevisiae*, the efficiency of error-free NHEJ was found to be four- to fivefold decreased in the DNA damage checkpoint mutant *RAD24* (homologous to *S. pombe RAD17*; de la Torre-Ruiz and Lowndes, 2000). In the case that a loss of function of *RAD17* also impairs the error-free NHEJ in plant cells, a partial switch of repair from NHEJ to HR would be plausible. The comet assay indicated a slowdown of DSB repair. We can only speculate whether or not the slower DSB repair mechanism represents a specific subset of NHEJ or even HR. Taking the hyper-recombination phenotype of the mutants into account, the disturbed faster DSB repair pathway seems to be homology independent. Different *Arabidopsis* mutants altered in HR have been identified (Masson and Paszkowski, 1997). HR is also increased in *Arabidopsis rad50* mutant (Gherbi *et al.*, 2001). There are indications that HR and NHEJ compete in mammals (Allen *et al.*, 2002), and also in plants, this competition is discussed (Gherbi *et al.*, 2001; Puchta *et al.*, 1996; Siebert and Puchta, 2002). It was shown for *S. cerevisiae* that quickly repaired lesions do not trigger the DNA damage response (Rouse and Jackson, 2002). By contrast, slower repair results in persisting lesions. Only if DNA repair cannot be completed, the RFC-like (Rad24/Rfc2–5) and PCNA-like (Rad17/Ddc1/Mec3) complexes of *S. cerevisiae* are recruited to the sites of damage and trigger global responses such as cell cycle arrest, chromatin modulation and increased repair capacity (Rouse and Jackson, 2002). NHEJ may occur in all phases of cell cycle, but it is most important in G0/G1 when a sister chromatid is not available. HR is most efficient between aligned sister chromatids, hence from late S until metaphase. If *RAD17* is required for the NHEJ pathway in *Arabidopsis* similarly as shown for the respective gene *RAD24* in *S. cerevisiae*, errors will accumulate during the cell cycle in the mutant background until G2, when an increased repair by HR that is not impaired in the *rad9* and *rad17* mutants is possible. Induction of HR by genotoxic stress is also not disturbed in the *rad9* and *rad17* mutants. Obviously, AtRad9 and AtRad17 are essential for neither activation nor performance of the HR machinery.

However, the view that because of the deficiency in checkpoint control, the cells are just not able to repair all breaks by NHEJ in time before they enter late S and G2 phases might be too simplistic. Mechanistic insight into how the DNA damage checkpoint might more directly control the efficiency of end joining has been provided by the observation that SIR and KU proteins are re-localized from telomeres to the sites of DSBs. For this process, an intact DNA damage checkpoint pathway is required (Martin *et al.*, 1999; McAinsh *et al.*, 1999; Mills *et al.*, 1999). However, convincing models on a direct role of the DNA damage checkpoint proteins in NHEJ have yet not been provided.

RAD17 seems to be a multifunctional gene. In *S. cerevisiae*, *Rad24* was found to interact with not only Ddc1 (Rad9 homologous of *S. pombe*) but also recombination proteins Sae1, Sae2 and Msh5 in vegetative and meiotic cells (Hong and Roeder, 2002). Other indications that the checkpoint proteins influence proteins involved in HR in yeast come from mutants of the SRS2 gene, encoding a DNA helicase involved in HR that are lethal in the background of *rad54* or *rad50* mutants. But these double mutants can be rescued by mutations in *RAD24* (*RAD17* homologous of *S. pombe*), *RAD17* (*RAD1* homologous of *S. pombe*) and *MEC3* (*HUS1* homologous of *S. pombe*) (Klein, 2001). On the other hand, *RAD24* is required for meiotic recombination in yeast. In the mutant, obviously the synapsis is aberrant and the levels of ectopic recombinations are elevated (Grushcow *et al.*, 1999). However, the *rad17* mutation itself in *Arabidopsis* has obviously no strong effect on meiosis because the progeny of the *rad17* mutants were fertile and produced similar numbers of seeds as wild-type plants.

Interestingly, we found that At*RAD17* expression is induced by various genotoxic agents. In contrast, no

induction was found for the members of the putative 9-1-1 complex in *Arabidopsis*. Thus, the activity of the complex might be regulated post-transcriptionally. Indeed, *in vivo*, the human Rad9 protein is phosphorylated in response to DNA damage, suggesting that it participates in a DNA damage-inducible signalling pathway (Volkmer and Karnitz, 1999). Biochemical data predict that in human cells, the 9-1-1 complex is loaded on chromatin by the Rad17 complex in response to DNA damage (Roos-Mattjus *et al.*, 2002). As Rad17 is a sensor of DNA damage itself, other factors might be involved in its transcriptional regulation. This points to complex regular circuits in the response of plants to DNA damage. Only a further detailed analysis of the response of *Arabidopsis* to genotoxic agents will help to elucidate this puzzle.

Experimental procedures

Database screening

Sequence searches were performed using TAIR BLAST 2.0. Multiple alignments were carried out on the internet using CLUSTALW and sequence analysis was carried out with the help of DNASTAR (DNASTAR, Inc., Madison, WI, USA).

RNA isolation

Seedlings and flowers were homogenized in liquid N_2 with a mortar and pestle. Total RNA was isolated using Trizol reagent following the manufacturer's instructions (TriFast, peqLab, Erlangen, Germany), and poly(A)+ RNA was purified from total RNA using an mRNA enrichment kit (peqLab).

cDNA synthesis and RACE

cDNA synthesis was performed according to a SMART-protocol from Clontech (Heidelberg, Germany) using 15 ng of mRNA from seedlings and flowers and RACE reactions according to an improved SMART-protocol (Matz *et al.*, 1999).

DNA isolation and Southern analysis

Total DNA was isolated from *Arabidopsis* leaves as described by Salomon and Puchta (1998). For Southern analysis, 5 μg of genomic DNA was digested with different restriction enzymes overnight, fractionated on a 1% agarose gel and transferred to a nylon hybridization membrane 'Hybond N' (Amersham, UK). The DNA probe was labelled using a random priming labelling kit (Amersham) and [α-^{32}]dCTP (Amersham). Hybridization was performed according to Church and Gilbert (1984).

*Expression analysis of At*RAD17, *At*RAD1-like, *At*HUS1 *and At*RAD9 *by real-time PCR*

Arabidopsis seedlings were grown in GM medium for 2 weeks and then treated with 1.5 μg ml^{-1} bleomycin or 22 μg ml^{-1} MMC for 6 h (Chen *et al.*, 2003), and RNA was isolated immediately after treatment. For the gamma-irradiation, *Arabidopsis* seedlings (2 weeks old) were irradiated with 100 Gy gamma-ray. After the seedlings were transferred into the growth chamber and incubated for 5 h, RNA was isolated. Real-time PCR analysis was performed according to Chen *et al.* (2003). Primers used were: RAD17-F (5′-dCAGTGATATTTCTGAAGATG-3′), RAD17-R (5′-dAGAGAAGTGACTCTCTCCAT-3′), HUS1-F (5′-dAAGAGCCTTCAATGTCAC-3′), HUS1-R (5′-dGGAAGTCGGCAATGCAAA-3′), RAD1-F (5′-dCGTAGTGAGAGAAAACAG-3′), RAD1-R (5′-dGAGGCTTCACAAAGAACTC-3′), RAD9-F (5′-dTCCTAAGTCCCAACGTCC-3′), and RAD9-R (5′-dTTCATTGTGAGGTGGTGT-3′). The relative template concentrations were evaluated based on the standard curve for the 60S ribosomal protein gene L27A. For the normalization, the relative amount of the reference gene (60S ribosomal protein gene L27A) was determined. Each PCR run was carried out in triplicate. The results are mean values of at least two PCR runs.

Characterization of the Arabidopsis *insertion mutants*

The *rad9-1* (GT8083) and *rad17-1* (GT534) transposon lines (L*er* background) obtained from Cold Spring Harbor Laboratory, USA (http://genetrap.cshl.org/traps.html) contain the reporter gene GUS without promoter and NPTII gene (conferring resistance to kanamycin) as a selectable marker and have been constructed using the Ac/Ds transposable elements (Martienssen and Springer, 1998; Sundaresan *et al.*, 1995). Although the GUS construct is oriented in the direction same as that for At*RAD9* and At*RAD17*, no blue staining could de detected in both lines after histochemical treatment. The *rad17-2* line (in Col-0 background) has been obtained from the *Arabidopsis* T-DNA collection (Donor: Joseph R. Ecker) in Nottingham, UK.

To generate *rad9-1*/*rad17-1* double mutants, plants homozygous for *rad9-1* (linked to chromosome 3) were crossed to plants homozygous for *rad17-1* (linked to chromosome 5). Double mutants segregated in the F_2 progeny in the expected 1 : 15 ratio. To construct 651/*rad9-1* and 651/*rad17-1*, plants homozygous for *rad9-1* or *rad17-1* were crossed with homozygous GUS651 plants (in C24 background). Mutants homozygous for *rad17-1* and GUS segregated in the F_2 approximately in the 1 : 15 ratio, but the homozygous 651/*rad9-1* mutants segregated at a lower frequency. Siblings homozygous for GUS and for the *RAD9* and *RAD17* wild-type alleles were used as control lines. A homozygous 651/*rad17-2* line and its control line were constructed in the same way.

Complementation of the rad17-1 *mutant*

A 4996-bp genomic fragment containing the complete At*RAD17* transcribed region (3078 bp) with 1724 bp upstream and 194 bp downstream was amplified using the oligonucleotides 5′-dAATGGAATTCTCCTGCCATTCAAAG-3′ (1896 bp upstream of the ORF) and 5′-dTCTGAGTCGACCTTCTTCGCTGCTTC-3′ (245 bp behind ORF). After purification, the fragment was digested with *Eco*RI and *Sal*I, cloned in the binary vector pPZP221 (Hajdukiewicz *et al.*, 1994) conferring gentamycine resistance and sequenced. The plasmid was transformed via *Agrobacterium tumefaciens* (strain C58) into *Arabidopsis* applying the floral dip method (Clough and Bent, 1998).

Growth conditions and mutagene tests

Seeds of *A. thaliana* were sterilized in 3% sodium hypochlorite for 10 min, and rinsed several times with sterile water. Plants were grown in chambers at 21°C under white light (16 h light/8 h dark).

For mutagene tests, sterilized seeds were spread over GM agar containing different mutagene concentrations. Alternatively, seedlings were grown on GM without mutagenes for 8 days and then transferred in liquid GM containing different mutagene concentrations for 14 days.

Comet assay

Induction and repair of DSBs in 2-week-old bleomycin-treated seedlings was monitored using the comet assay in 2-week-old seedlings treated for 1 h with 30 μg bleomycin ml^{-1}. Nuclei released from tissue by chopping with a razor blade were imbedded in agarose on glass slides and lysed with sarcosine and 2.5 M NaCl to remove cytoplasm and most nuclear proteins, leaving supercoiled DNA as 'nucleoids'. During electrophoresis in Tris–acetate buffer (pH approximately 7), broken DNA migrates towards the anode and forms a 'comet tail' of DNA extending from the nucleoid. This enables to quantify DNA damage as percentage of DNA in comet tails (%T-DNA) after propidium iodide staining of DNA under the epifluorescence microscope using the Comet module of LUCIA software (http://www.lim.cz).

Each experimental point is represented by the mean value (±standard error = SE) from at least three independent experiments in which 100 comets from four gels were evaluated, based on the median values of the percentage of migrated DNA (%T-DNA). The percentage of DSBs remaining after a given repair time t_x is defined as:

$$\%\ \text{of DSBs remaining}\ (t_x) = \frac{\%\text{T DNA}\ (t_x) - \%\text{T DNA (control)}}{\%\text{T DNA}\ (t_0) - \%\text{T DNA (control)}} \times 100$$

Induction of recombination by bleomycin, MMS and UV

Ten-day-old seedlings were grown in liquid GM medium containing different concentrations of mutagenes as bleomycin (50 ng ml^{-1} and 1 μg ml^{-1}) and MMS (40 p.p.m.) or irradiated with UV light (20-sec pulse with 254 nm, 660 J m^{-2}; and 60-sec pulse with 254 nm, 2000 J m^{-2}) using a UV 1800 Stratalinker (Stratagene, La Jolla, CA, USA). Ten days later the seedlings were used for GUS staining.

Histochemical staining procedure and detection of recombination events

Histochemical staining, as described by Schmidt-Puchta *et al.* (2004), was performed with 20-day-old plants. The plants were vacuum infiltrated for 5 min with staining buffer containing 0.05% 5-bromo-4-chloro-3-indolyl glucuronide (X-Glu) substrate (Duchefa, the Netherlands) in 100 mM phosphate buffer (pH 7.0), 0.05% NaN_3. Then plants were incubated for 96 h at 37°C. By subsequent incubation in ethanol for 1 h at 65°C, the plants were destained. Blue spots were counted using a binocular.

Statistical analysis

The address the statistical significance of the differences in the homologous recombination frequencies (mutant versus wild type) the pairwise Mann–Whitney *U*-test, a non-parametric (distribution-free) test was performed, to compare two independent groups of sampled data whose regularities of distribution are unknown (no normal distribution). In this test the number of blue spots of the respective mutants and the wild type are converted to rank order data. The probability (*P*)-values calculated by the Mann–Whitney formula (http://eatworms.swmed.edu/~leon/stats/utest.html) are given in Table 2. A *P*-value of 0.01 indicates a high statistical significance that the recombination events of mutant and the wild type are different.

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