A homologue of the breast cancer-associated gene BARD1 is involved in DNA repair in plants

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hBRCA1 and hBARD1 are tumor suppressor proteins that are involved as heterodimer via ubiquitinylation in many cellular processes, such as DNA repair. Loss of BRCA1 or BARD1 results in early embryonic lethality and chromosomal instability. The *Arabidopsis* genome carries a BRCA1 homologue, and we were able to identify a BARD1 homologue. AtBRCA1 and the putative AtBARD1 protein are able to interact with each other as indicated by *in vitro* and *in planta* experiments. We have identified T-DNA insertion mutants for both genes, which show no visible phenotype under standard growth conditions and are fully fertile. Thus, in contrast to animals, both genes have no indispensable role during development and meiosis in plants. The two single as well as the double mutant are to a similar extent sensitive to mitomycin C, indicating an epistatic interaction in DNA crosslink repair. We could further demonstrate that in *Arabidopsis* BARD1 plays a prominent role in the regulation of homologous DNA repair in somatic cells.

Keywords: *Arabidopsis thaliana*; BARD1; breast cancer genes; DNA repair; homologous recombination

Introduction

Germline mutations of the *hBRCA1* (breast cancer susceptibility 1) gene are known to be responsible for about 50% of all inherited breast cancer cases (Miki *et al*, 1994). The human *BRCA1* gene codes for an 1863 amino acids (aa) long nuclear protein with two functionally important motifs. The first motif is located at the N-terminus of the protein and codes for a RING-finger domain, consisting of 40–60 aa. Many RING finger containing proteins function as ubiquitin E3 ligase (Wu *et al*, 1996; Joazeiro and Weissman, 2000). The second motif is located at the C-terminus and encodes two repeats of approximately 80 aa. These repeats were designated as *Br*east cancer *C-t*erminal repeats (BRCT; Callebaut and Mornon, 1997; Koonin *et al*, 1996). These BRCT domains are present in a large number of cell cycle checkpoint proteins ranging from bacteria to humans (Koonin *et al*, 1996; Callebaut and Mornon, 1997). Both RING and BRCT domains of hBRCA1 are well conserved and serve as common sites for missense mutations that predispose women to early-onset breast cancer (Ruffner *et al*, 2001; Rodriguez *et al*, 2004). Protein interaction studies using either the RING or BRCT domain of BRCA1 identified several interacting proteins (Jensen *et al*, 1998; Yarden and Brody, 2001). Interestingly, a protein found to interact with the N-terminal RING domain contained itself both a RING as well as two BRCT domains similar to BRCA1 (Wu *et al*, 1996). As further studies demonstrated that both proteins are able to form a heterodimer through their common N-terminal RING domain, this protein was designated BARD1, *breast cancer associated RING domain* (Meza *et al*, 1999; Joukov *et al*, 2001). This hBRCA1/hBARD1 heterodimer complex functions as an E3 ubiquitin ligase that catalyses the synthesis of polyubiquitin chains (reviewed by Baer and Ludwig, 2002).

DNA damage poses a continuous threat to genomic integrity in eukaryotic cells. A particularly lethal form of DNA damage is the DNA double-strand break (DSB). Cells have two major pathways for the repair of DSBs, homologous recombination (HR) and nonhomologous end joining (NHEJ) (reviewed by Puchta, 2005). Although NHEJ is a process in which the ends of a DSB might be modified, HR precisely restores the continuity of a broken DNA molecule using an intact and homologous DNA strand as template.

For a decade, multiple analyses have been performed to elucidate the biological role of BRCA1. Evidence for the involvement of BRCA1 in the repair of DSB originates from its association with hRAD51 (Scully *et al*, 1997a), and from the formation of foci at sites of DSBs after genotoxic stress (Scully *et al*, 1997b; Paull *et al*, 2000). Disruption of BRCA1 in mice results in embryonic lethality that is accompanied by growth retardation, apoptosis, cell cycle defects and genetic instability (Gowen *et al*, 2000). Taken together, these results demonstrate a very important role for BRCA1 in promoting HR and thus in maintaining genomic integrity.

In contrast to BRCA1 very few and partially indirect functional studies on BARD1 homologues were performed. Besides its function as E3 ubiquitin ligase in a complex with BRCA1, some studies indicated that the protein might also be involved in homologous DSB repair (Westermark *et al*, 2003; Stark *et al*, 2004). Recently, studies on a BARD1 homologue in *Caenorhabditis elegans* showed that depletion of the BARD1 protein resulted in germination defects and radiation sensitivity (Boulton *et al*, 2004).

Until 2003 orthologues of BRCA1 were only identified in other animal genomes, for example, *C. elegans* and *Xenopus laevis* (Joukov *et al*, 2001; Boulton *et al*, 2004). Surprisingly, Lafarge and Montane identified in 2003 a BRCA1 orthologue in the genome of the model plant *Arabidopsis*. Similar to its orthologue from humans, this protein has the characteristic RING and BRCT domains. Furthermore, it was shown that the

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transcription of the *AtBRCA1* gene was strongly induced by γ-irradiation (Lafarge and Montane, 2003). However, the study did not address the biological function of the protein in plants.

We have now been able to identify a hBARD1 homologue in *Arabidopsis* and in the following we characterise the biological role of AtBARD1 and AtBRCA1 in plants.

Results

Identification of a hBARD1 homologue in A. thaliana

The characteristic feature of both hBRCA1 and hBARD1 is the presence of a conserved RING as well as two BRCT domains. Orthologues of hBRCA1 and hBARD1 in *Mus musculus*, *C. elegans* or *X. laevis* display a similar domain structure (Szabo *et al*, 1996; Joukov *et al*, 2001; Boulton *et al*, 2004). To identify putative BARD1 homologues in the *Arabidopsis* genome, a database search was carried out in TAIR-BLASTP using hBARD1 as template (NP000456). This search resulted in two significant hits: At4g21070, which had previously been classified as the hBRCA1 homologue of *Arabidopsis* (Lafarge and Montane, 2003), and At1g04020. The homology of AtBRCA1 to the hBARD1 protein is restricted to the previously mentioned conserved RING and BRCT domains. However, At1g04020 has additional homology to hBARD1 outside the RING and BRCT domains, in total 22% amino-acid identity and 38% similarity (Figure 1A and B). We therefore assumed that At1g04020 might be the BARD1 homologue of *Arabidopsis*.

Using mRNA from *Arabidopsis* flowers as template, the cDNA from At1g04020 could be amplified by polymerase chain reaction (PCR). By RACE-PCR with nested gene-specific primers, 5′ and 3′ ends were obtained. The ORF of full-length *AtBARD1* has a total length of 2145 bp, contains 13 exons, and codes for a protein of 714 aa (Figure 1A). A cDNA clone (*BX815982*) from the GenBank confirmed this structure.

The ORF of *AtBRCA1* was also determined by RACE-PCR. In line with cDNA clones from SALK (*U24692*, *R24692* and *AF515728*), we identified the ORF of full-length *AtBRCA1* consisting of 2826 bp, containing 14 exons and coding for a protein of 941 aa. This is in contrast to the original report of Lafarge and Montane (2003) who identified the ORF of full-length *AtBRCA1* consisting of 4485 bp and 15 exons. The first exon postulated by Lafarge and Montane (2003) is part of another gene rather than the ORF of *AtBRCA1*.

In plants it was demonstrated before that some genes coding for proteins involved in nucleotide metabolism and DNA repair can be induced by DNA damage, among them *AtBRCA1* (e.g. Chen *et al*, 2003; Lafarge and Montane, 2003). To characterise a possible correlation between the expression of *AtBRCA1* and *AtBARD1* 2-week-old seedlings were irradiated by γ-ray (100 Gy) and the transcript amount of both genes was measured after 1 h by quantitative real-time PCR (Figure 2A). As reported previously, a strong induction of the *AtBRCA1* transcript could be detected. In contrast, no significant change of the mRNA level of *AtBARD1* was found. Additionally, the expression of both genes in different tissues of 6–8-week-old *Arabidopsis* plants was analysed. RNA from roots, rosette leaves, inflorescence, young cauline leaves, flowers and siliques was isolated and the transcript amount of both genes was measured via real-time PCR. Higher amounts of mRNA of both *AtBRCA1* and *AtBARD1* could be detected in flowers and siliques. The expression in roots, rosette leaves, inflorescence and young cauline leaves was low (Figure 2B). Thus, in contrast to the application of genotoxic stress, the expression pattern of both genes in different organs correlated well, hinting to a functional interaction.

Protein–protein interaction between AtBARD1 and AtBRCA1

To test whether AtBRCA1 and the putative AtBARD1 protein are also able to interact directly, a two-hybrid analysis was performed.

First, it was tested whether AtBRCA1 and AtBARD1 contained an activation domain. It was previously demonstrated that this is the case for the hBRCA1 protein, whereas so far this has not been reported for the hBARD1 protein (Welcsh *et al* 2002). With the help of the LexA-based yeast two-hybrid system, we could clearly demonstrate that the full-length AtBARD1 protein contained an autoactivation domain (Figure 3A). Unfortunately, no consistent results were obtained using the full-length AtBRCA1 protein. This might reflect the presence of a weak transcriptional activation domain. Thus, in this assay it was only possible to use truncated versions of AtBRCA1 or AtBARD1 as bait. An N-terminal fragment of AtBRCA1 coding for the first 59 aa and containing the RING domain did not display any autoactivation and was used as bait. As prey the full-length AtBARD1 protein and a C-terminal AtBARD1 fragment containing the BRCT repeats but missing the RING domain were constructed. Indeed, an interaction of the RING domain of AtBRCA1 with the full-length AtBARD1 protein could be demonstrated (Figure 3A), whereas no interaction of the RING domain of AtBRCA1 with the C-terminal part of the AtBARD1 protein was found. Unfortunately, we failed to detect an interaction using the RING domain of AtBARD1 as bait and the complete AtBRCA1 protein (result not shown). However, it is not uncommon in two-hybrid analysis that only certain bait and prey combinations result in detectable interactions (Uetz *et al*, 2000).

Interaction of AtBARD1 and AtBRCA1 in planta

To further sustain our observation that AtBRCA1 and AtBARD1 interact, *in vivo* studies were carried out. We used a well-established method of bimolecular fluorescence complementation (BiFC; Hu *et al*, 2002) for the *in vivo* detection of protein–protein interactions, namely the split YFP system (Stolpe *et al*, 2005). Briefly, the assay is based on the observation that a N- (YN) and a C-terminal (YC) fragment of the yellow fluorescent protein (YFP) can only reconstitute a functional fluorophore when they are brought into tight contact. Two ORFs, driven by a double 35S promoter, are fused on separate plasmids to the respective YFP fragments; next, both constructs are brought into a plant cell for expression and the interaction of the fusion proteins can be monitored via epifluorescence microscopy.

To confirm the possible interaction between AtBRCA1 and AtBARD1, the full-length *AtBRCA1* ORF, the first N terminal 88 aa coding for the AtBRCA1 RING domain and the last 797 C-terminal aa of the ORF of *AtBRCA1* were fused to the C-terminal part of the YFP protein. The full-length *AtBARD1* ORF was fused to the N-terminal part of the YFP protein. Next, the different constructs, together with a plasmid

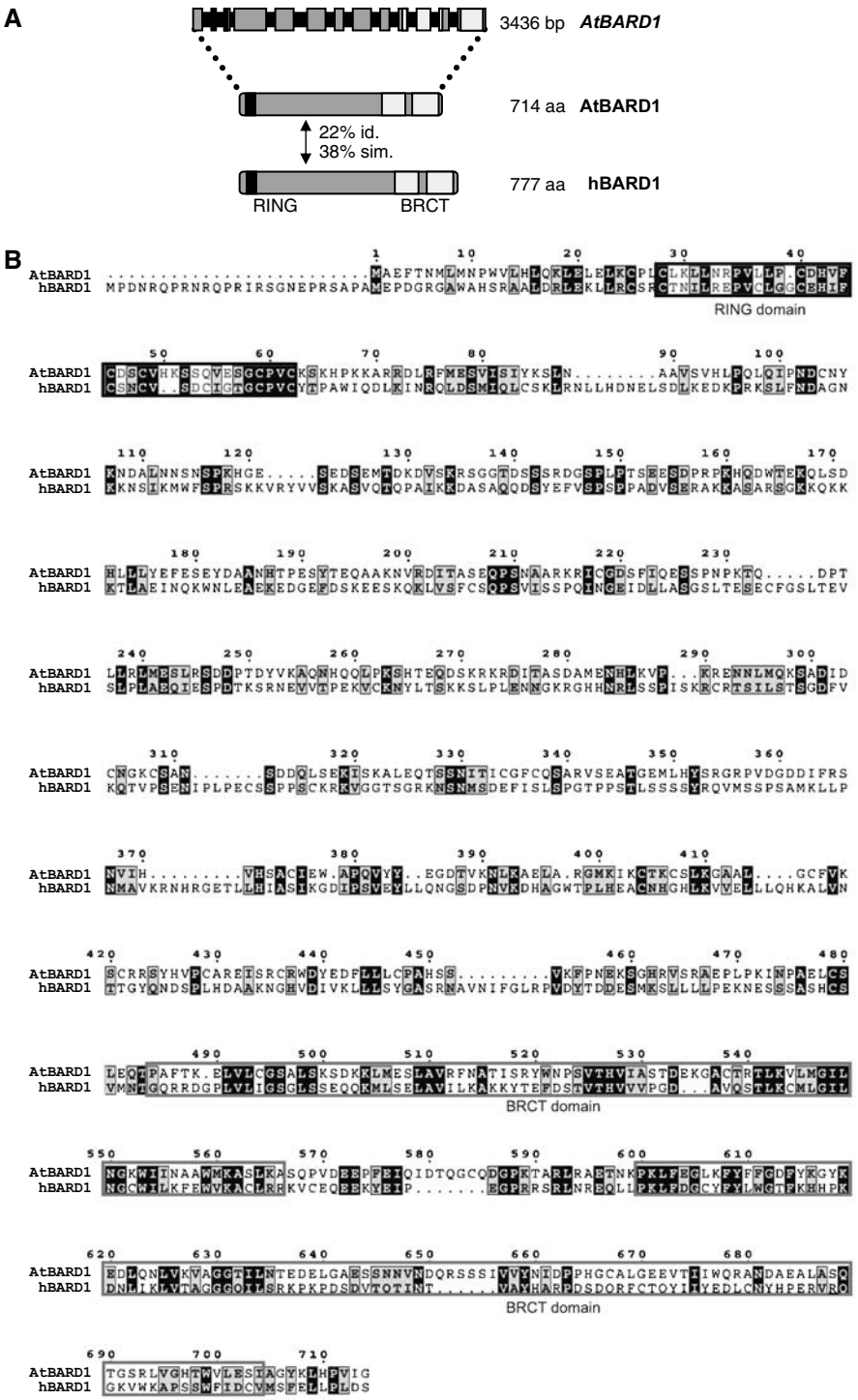

Figure 1 Gene structure of *AtBARD1* and comparison of the AtBARD1 and hBARD1 proteins. (**A**) A schematic representation of the AtBARD1 intron exon structure. Exons are represented by grey boxes, introns by black bars. In total, the *AtBARD1* gene counts 13 exons, the gene has a length of 3436 bp encoding a protein of 714 aa. The AtBARD1 protein has a similar structure as the hBARD1 protein, also containing a conserved RING domain (black regions) and two BRCT domains (light grey regions). Both proteins have an identity of 22% and a similarity of 38%. (**B**) Protein sequence alignment of AtBARD1 against hBARD1. Identical amino acids are shaded black whereas similar amino acids are shown in grey. Conserved RING and BRCT domain structures are indicated by black and light grey frames, respectively.

containing the CPRF2 protein (common plant regulatory factor 2) fused to CFP as nuclear marker (e.g. Figure 3B′ and C′), were transiently expressed after particle bombardment in etiolated mustard seedlings. As positive control, the ASK1 protein fused to the N-terminus of YFP, and an EID1-YFP-C-terminal fusion was used (Figure 3H). EID1 and ASK1 are interacting proteins of the Skp1-Cullin-F-box-protein ubiquitin ligase that targets proteins for degradation and functions as a negative regulator in phytochrome A-specific light signalling. The negative control was a deleted version of the EID1 (EID1ΔF) protein not able to interact with ASK1 (Figure 3I; Stolpe *et al*, 2005). As further controls AtBRCA1

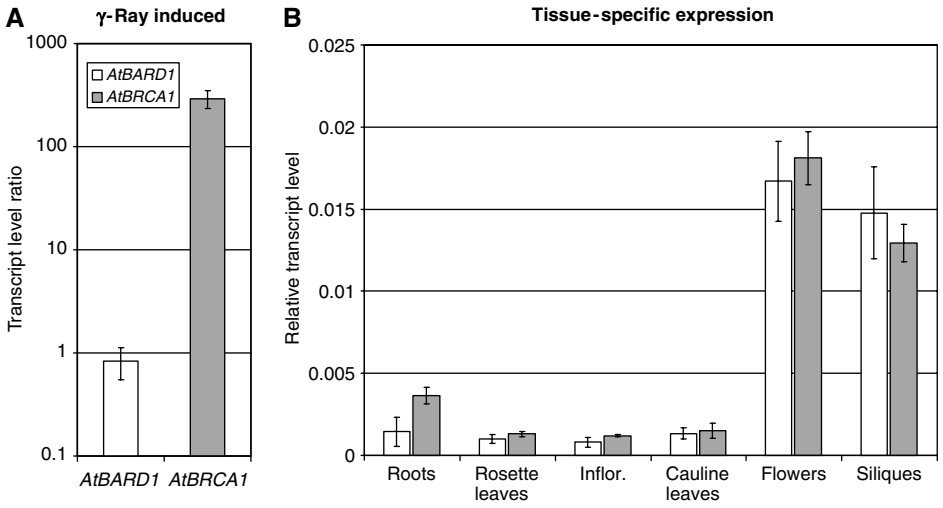

Figure 2 Expression analysis of *AtBARD1* and *AtBRCA1* in *Arabidopsis*. (**A**) The expression of *AtBARD1* and *AtBRCA1* was analysed by relative quantification using real time PCR 1 h after irradiation by γ ray. Transcription level ratio of *AtBARD1* and *AtBRCA1* is given in relation to actin mRNA and the mRNA of the respective untreated seedlings, and is the mean of six different reactions ±s.d. White bars, *AtBARD1;* grey bars, *AtBRCA1*. (**B**) The expression pattern of *AtBARD1* and *AtBRCA1* in different plant tissues was analysed by relative quantification using real time PCR. RNA from roots, rosette leaves, inflorescence, cauline leaves, flowers and siliques of soil grown plants was analysed. Expression of *AtBARD1*/*AtBRCA1* is given relative to actin mRNA levels and is the mean of six different reactions ±s.d. Similar results were obtained in independent experiments.

or AtBARD1 constructs fused with the N- or C-terminal part of the YFP protein, respectively, were used together with the respective pMAV-GW-YN and pMAV-GW-YC empty vectors. After an overnight incubation period, the seedlings were screened for the presence of an YFP signal. Routinely, 1–5 transfected cells per seedling were obtained. The results are based on at least two independent experiments using four mustard seedlings for each transfection. For each single combination, the results were uniform, that is, besides the CFP signal, either in all or in none of the transfected cells an YFP signal could be detected.

Not only in case of the full-length ORF of *AtBRCA1* combined with the complete AtBARD1 protein an YFP signal could be detected (Figure 3B), but also in the AtBRCA1 RING domain and the AtBARD1 protein (Figure 3D). No YFP signal was observed when the C-terminus of AtBRCA1 was coexpressed with the AtBARD1 protein (Figure 3F). No YFP signal could be obtained when combinations of the single constructs of AtBRCA1 and AtBARD1 with the pMAV-GW-YN and pMAV-GW-YC empty vectors were used (Figure 3C, E and G). Taken together, our experiments clearly demonstrate that AtBARD1 and AtBRCA1 are able to interact, and that this interaction is mediated by the AtBRCA1 RING domain. This is in line with our experiments from the two-hybrid system.

Mutant atbard1 plants are phenotypically normal but sensitive to mitomycin C

Functional studies were necessary in order to elucidate the biological role of the BARD1 homologue in plants. The putative *AtBARD1* gene sequence was used to screen the sequence database of T-DNA insertion mutants on the SIGnAL webpage (Salk Institute Genomic Analysis Laboratory; Alonso *et al*, 2003). Two *atbard1* T-DNA mutant lines were identified. The respective plants were obtained, propagated, and homozygous individuals of the respective insertions could be identified. The insertion sites were determined in detail by PCR. Figure 4 provides a detailed characterisation of the T-DNA insertions of *AtBARD1*.

The two *atbard1* T-DNA insertions are located at the beginning of the gene. Both insertions carry left T-DNA borders at their ends, indicating the integration of a double T-DNA insert in inverted orientation. The first insertion, *SALK 097601*, *atbard1-1*, is present in the first intron and results in a deletion of 18 nucleotides. The second insertion, *SALK 031862, atbard1-2*, is located in the third exon, which codes for the N-terminal RING domain and leads to a deletion of 5 nucleotides within the coding sequence (Figure 4B).

In order to assess the expression level of *AtBARD1* in the homozygous T-DNA lines, reverse transcription–polymerase chain reaction (RT–PCR) experiments were performed with homozygous mutants using primer pairs binding in front, across and after the insertions (Figure 4A and C). In case of both lines, expression of an mRNA before the insertion could be demonstrated (Figure 4C). With primers spanning across the insertions, we were not able to amplify any product for both alleles. An expression after the insertion was detected for *atbard1-1*. In contrast, no expression could be found for *atbard1-2,* indicating that this allele most probably represents a 'true' null *atbard1* mutation.

In comparison to wild-type plants, all plant lines homozygous for the respective insertions did not differ in their phenotypes when grown under standard conditions. However, when challenged with the DNA crosslinking agent mitomycin C (MMC), the T-DNA insertion mutants showed a more sensitive phenotype as compared to wild-type seedlings (Figure 5). The mutant seedlings were smaller and less viable. Interestingly, the line *atbard1-2* showed a slightly stronger phenotype after treatment with MMC than *atbard1-1*. Other mutagenic treatments with bleomycin or UV radiation did not display an increased sensitivity in the mutant background (results not shown).

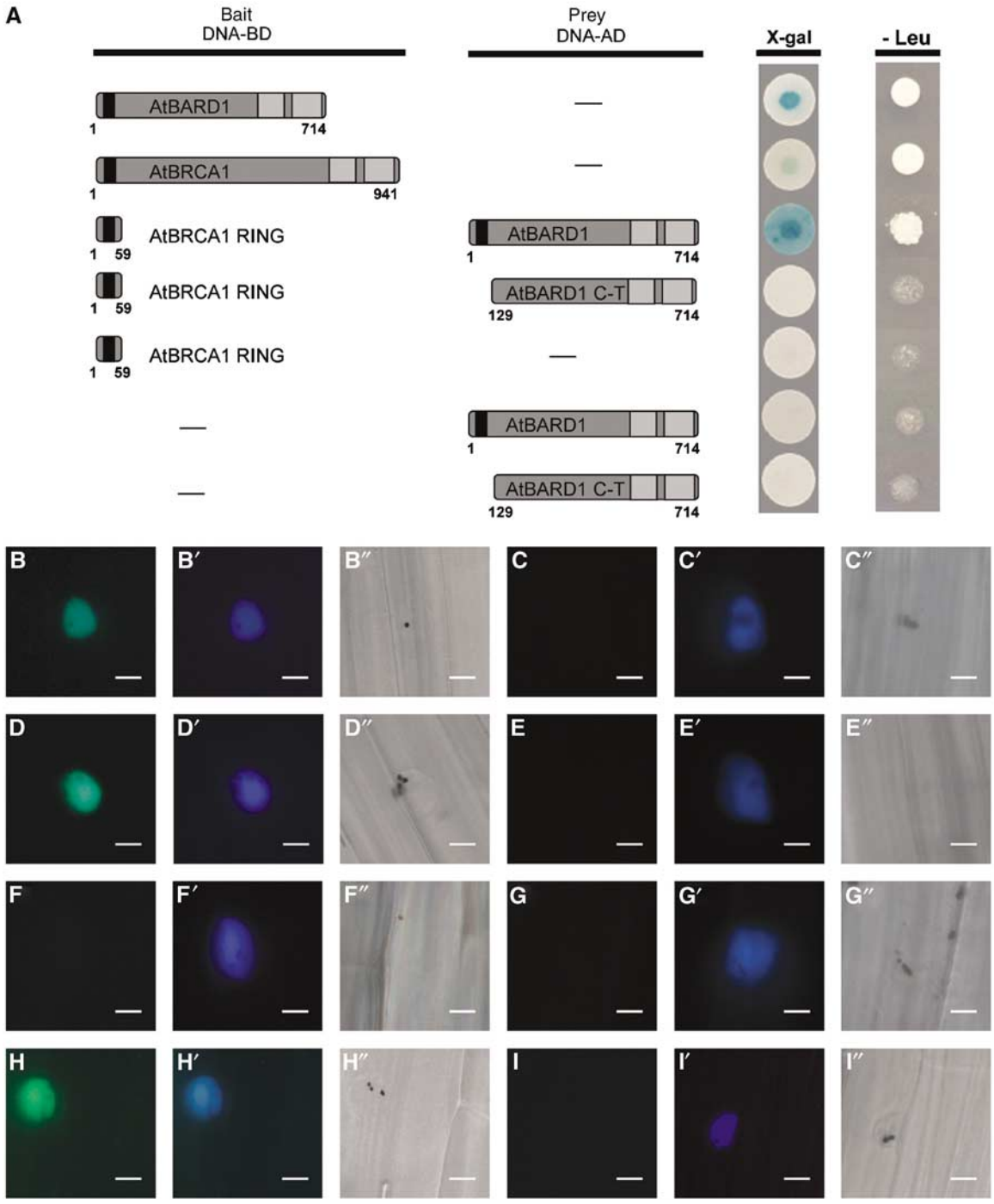

Figure 3 Characterisation of the AtBARD1 and AtBRCA1 interaction by yeast two hybrid assay and by BiFC in transiently transfected mustard seedlings. (**A**) Yeast two hybrid assay. Different constructs of AtBRCA1 or AtBARD1 were used either as bait (DNA BD) or prey (DNA AD) and tested for their ability to activate the *lacZ* reporter gene (X gal) and the nutritional marker gene leucine (Leu). The full length protein AtBARD1 was, when fused to a DNA binding domain (DNA BD), able to activate the *lacZ* reporter gene as well as the leucine reporter gene. The use of AtBRCA1 as bait led to inconsistent results. A truncated version of AtBRCA1, containing the first 59 N terminal aa (representing the RING domain) interacted with the complete AtBARD1 protein fused to the activation domain and resulted in the activation of the *lacZ* reporter gene and the leucine reporter gene. No interaction could be demonstrated between the AtBRCA1 RING domain and a C terminal part of AtBARD1 (AtBARD1 C T). Furthermore, none of the single used constructs was able to activate transcription. Blue staining of the yeast colonies appeared within 30 min for the AtBARD1 protein, to up to 2 h for the AtBRCA1 AtBARD1 interaction, whereas the growth of yeast colonies on LEU lacking medium was determined after 2 days. **(B I)** (B′ I′) and (B″ I″) BiFC analysis in transiently transfected mustard seedlings. The pictures B I show an YFP signal in case of a protein interaction, in the nucleus of a representative cell, owing to the restoration of the YFP complex. The pictures B′ I′ show a CFP signal from a cotransfected nuclear marker. The pictures B″ I″ show the same cells as in (B I) and (B′ I′), respectively, by bright field microscopy. Bars 20 µm. B, B′ and B″ AtBRCA1 (YC) and AtBARD1 (YN); C, C′ and C″ AtBRCA1 (YC) and empty vector pMAV GW YN; D, D′ and D″ AtBRCA1 RING (YC) and AtBARD1 (YN); E, E′ and E″ AtBRCA1 RING (YC) and empty vector pMAV GW YN; F, F′ and F″ AtBRCA1 C terminus (YC) and AtBARD1 (YN); G, G′ and G″ AtBARD1 (YN) and empty vector pMAV GW YC; H, H′ and H″ ASK1 (YN) and EID1 (YC), positive control; I, I′ and I″ ASK1 (YN) and EID1ΔF (YC), negative control.

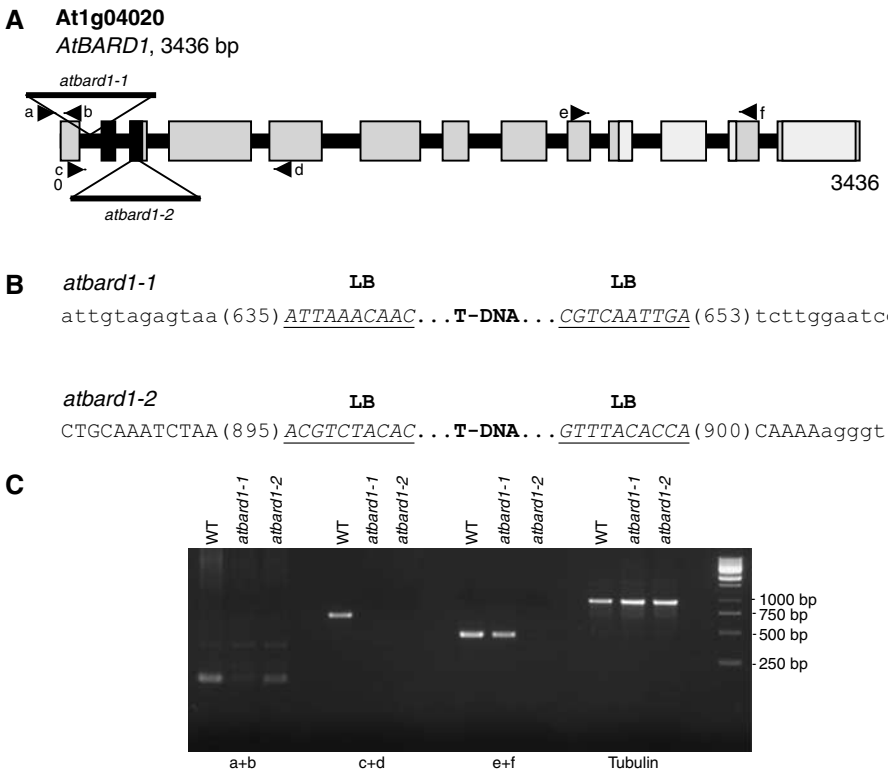

Figure 4 Schematic structure of the *AtBARD1* gene and its T DNA insertions. (**A**) The *AtBARD1* gene consists of 13 exons. Regions coding for the RING and BRCT domain are indicated in black and light grey, respectively. Two T DNA insertions were identified. One insertion is located in the first intron, and denominated *atbard1 1* whereas the second insertion is located in the third exon, and denominated *atbard1 2*. (**B**) An overview of the precise locations of the T DNA inserts in the *AtBARD1* gene. Intron sequences are displayed as lower case letters, exon sequences as capital letters, and T DNA border sequences are underlined (LB: left border). (**C**) Semiquantitative RT PCR on different regions of the *AtBARD1* gene. Primer pairs were used that bind in front of (a + b), across (c + d) and after (e + f) the T DNA insertions. The β tubulin gene was taken as control. WT: wild type.

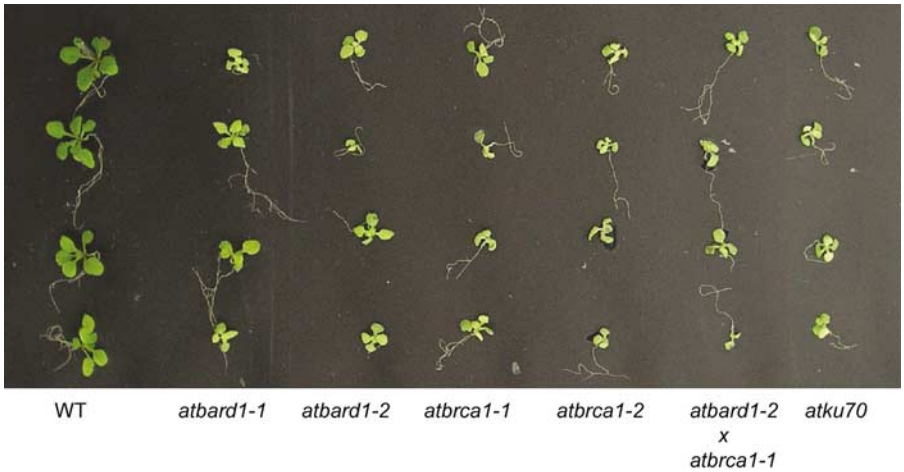

Figure 5 Hypersensitivity of different *Arabidopsis* mutants to the DNA damaging agent MMC. *Arabidopsis* seeds from the mutant lines *atbard1 1, atbard1 2, atbrca1 1, atbrca1 2* as well as the double mutant *atbard1 2/atbrca1 1* were tested for their sensitivity to MMC. Wild type seeds (Columbia) and *atku70* (a sensitive control line; Bundock *et al*, 2002) were used as controls. Seeds were plated on GM medium containing 30 μg MMC/ml, 17 days later seedlings were analysed for their sensitivity. WT: wild type.

AtBARD1 is dispensable for meiosis

We checked whether the selfed progeny of the T-DNA mutant *atbard1* plants was fertile in order to test whether the AtBARD1 protein plays a role during meiosis. Both *atbard1* T-DNA insertion mutants produced viable seeds at similar numbers as the wild-type plants. As minor meiotic defects are often correlated with reduced viability of male gametes, pollen of both mutants were analysed with Alexander (1969) staining. However, a similar number of viable pollen could be detected in wild-type and mutant anthers, indicating that AtBARD1 is not necessarily required for the progression of meiosis in plants (data not shown).

Intrachromosomal HR is reduced in atbard1 mutant plants and less inducible by genotoxic stress

To test the frequency of somatic HR *in planta*, a well-established recombination assay using the transgenic line 651 was performed (Swoboda *et al*, 1994). The recombination substrate within the transgene consists of two overlapping fragments of the β-glucuronidase gene (*GUS*; uidA) interrupted by a hygromycin selectable marker gene. The separated uidA sequences share a common overlap of 566 bp in inverted orientation. HR between the two overlapping DNA sequences produces a functional *uidA* gene. Cell clusters expressing β-glucuronidase activity can be detected as blue sectors after histochemical staining, and it was shown before that these sectors indeed arise from recombination events (Swoboda *et al*, 1994). The homozygous *atbard1-1* and *1-2* mutants were crossed with a transgenic line carrying the *651* transgene and selfed again to obtain plants homozygous with respect to the *atbard1* insertion as well as the 651 transgene. Seedlings were incubated in liquid germination medium (GM) with and without bleomycin (10 μg/ml). Bleomycin is a radiomimeticum causing single-stranded breaks (SSB) and DSB (Harsch *et al*, 2000). Next, recombination events were counted in 12-day-old seedlings. For both mutant *atbard1* lines, the distribution and frequency of recombination events were determined. Figure 6 shows a representative individual experiment for each mutant line. The significance of the differences of the HR events between mutants and segregated control plants was confirmed by the pair-wise nonparametric Mann–Whitney *U*-test. The experiment was repeated three times for each line (Table I). In all three independent experiments, a significant reduction of HR was found in the mutant backgrounds, either with or without genotoxic stress. A comparison between the untreated segregated control plants and the untreated *atbard1-1* and *atbard1-2* homozygous plants showed that the frequency of recombination events in the mutant plants was 2–3 times and about 10 times lower, respectively, as compared to the control line (Figure 6A and B; Table I).

When both mutant *atbard1* lines were challenged with bleomycin (10 μg/ml), the frequency of recombination events increased by about two orders of magnitude in the control lines, whereas the induction was significantly lower in both *atbard1-1* and *atbard1-2* lines (Figure 6C and D; Table I). This is also demonstrated by the fact that in all cases the relation

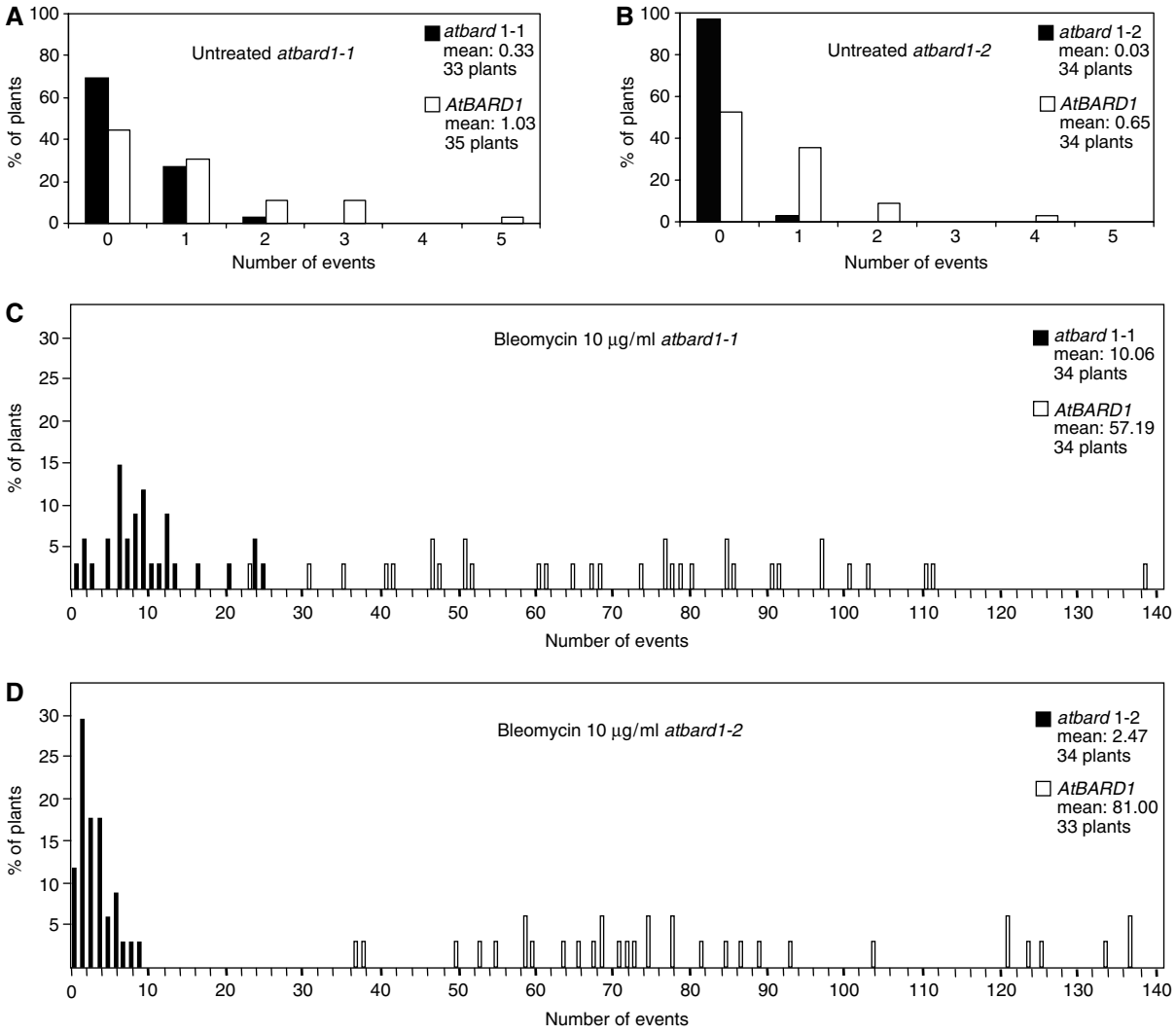

Figure 6 HR events in 651/*atbard1* seedlings. The diagrams show the percentage of seedlings with a given number of blue spots. (**A**) Untreated *atbard1 1*, (**B**) untreated *atbard1 2*, (**C**) bleomycin treated *atbard1 1* and (**D**) bleomycin treated *atbard1 2*. *atbard1* seedlings are displayed as black bars, segregated control plants homozygous for *AtBARD1* are shown as white bars.

between the mean recombination frequencies of mutant (m2) and segregated control plants (m1) was lower with than without application of genotoxic stress (see Table I last column m2/m1). Taking into account the enhanced sensitivity to MMC of *atbard1-2* in comparison to *atbard1-1,* the differences between the two mutants in HR can be taken as a hint that only in case of *atbard1-2* the insertion of the T-DNA into the gene resulted in a 'true' null mutation.

Independent of the different degrees of deficiency found in the two mutant lines, our results clearly demonstrate that AtBARD1 is not only required for the repair of DSBs by HR under standard growth conditions, but also for the regulation of HR induction after application of genotoxic stress.

AtBRCA1 and AtBARD1 are epistatic for cross-link repair

Our two-hybrid data as well as *in planta* experiments indicated that AtBARD1 and AtBRCA1 physically interact. To demonstrate a genetic interaction we screened the sequence databases of T-DNA insertion mutants on the SIGnAL (Salk Institute Genomic Analysis Laboratory; Alonso *et al*, 2003) and Garlic (Syngenta) webpages (Sessions *et al*, 2002). Two *atbrca1* T-DNA mutant lines were identified. The insertion sites were determined in detail by PCR. Figure 7 provides a precise characterisation of the T-DNA insertions in the *AtBRCA1* gene. The first insertion in the *AtBRCA1* gene, *SALK 014731, atbrca1-1*, is located in the fourth exon. Left borders of T-DNA were found at both ends of the insert, indicating the integration of T-DNAs in tandem inverted orientation. The integration led to the deletion of 18 nucleotides of the fourth exon (Figure 7A and B). The second insertion, *GARLIC 916 C09, atbrca1-2*, was located in the fifth intron more to the middle of the gene, and the insert is flanked by a right and a left T-DNA border. This insertion led to the deletion of eight nucleotides of the fifth intron (Figure 7A and B).

Table I Somatic HR in *atbard1 1* and segregated control plants (A), and in *atbard1 2* and segregated control plants (B)

Control			atbard1			Relation
n	*N*	m1	*n*	*N*	m2	m2/m1
(A) No genotoxic stress						
35	16	0.46	34	7	0.21	0.46
36	22	0.61	33	6	0.18	0.30
35	36	1.03	33	11	0.33	0.32
Mean		0.70±0.20			0.24±0.08	0.36*
Bleomycin induction (10 μg/ml)						
33	2733	77.97	34	803	23.61	0.30
33	1947	61.97	34	393	11.56	0.19
34	2431	57.19	34	342	10.06	0.18
Mean		65.71±10.88			15.08±7.43	0.22*
(B) No genotoxic stress						
33	16	0.48	34	2	0.06	0.13
31	20	0.65	32	3	0.09	0.14
34	22	0.65	34	1	0.03	0.04
Mean		0.59±0.10			0.06±0.03	0.10*
Bleomycin induction (10 μg/ml)						
34	1422	41.82	34	28	0.82	0.02
34	3063	90.09	34	65	1.91	0.02
33	2673	81.00	34	84	2.47	0.03
Mean		70.97±25.65			1.73±0.84	0.22*

Data are numbers of plants tested (*n*), total blue stained recombination spots (*N*), and the mean number of spots per plant per chromosomal recombination assay (m1: control; m2: *atbard1*) in three different experiments (*calculated from the means of the three experiments).

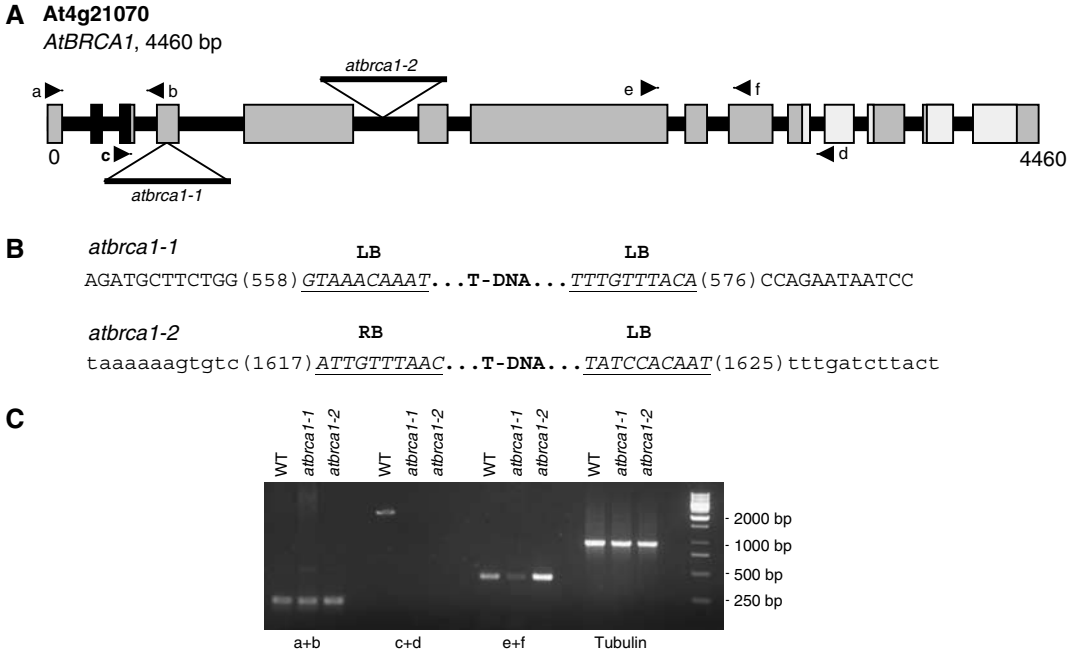

Figure 7 Schematic structure of the *AtBRCA1* gene and its T DNA insertions. (**A**) The *AtBRCA1* gene consists of 14 exons. The RING and BRCT domains are indicated as black and light grey regions, respectively. Two T DNA insertions were identified. One insertion is located in the fourth exon and denominated *atbrca1 1*, whereas the second insertion is located in the fifth intron and denominated *atbrca1 2*. (**B**) An overview of the precise locations of the T DNA inserts in the *AtBRCA1* gene. Intron sequences are displayed as lower case letters, exon sequences as capital letters, and T DNA border sequences are underlined. (LB: left border; RB: right border). (**C**) Semi quantitative RT PCR on different regions of the *AtBRCA1* gene. Primer pairs were used in front of (a + b), across (c + d) and after (e + f) the T DNA insertion. The β tubulin gene was taken as control. WT: wild type.

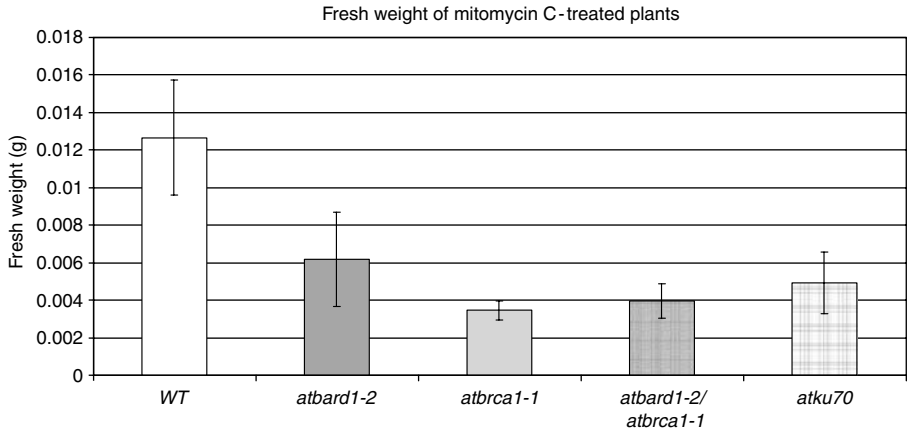

Figure 8 Fresh weight determination of different *atbard1* and *atbrca1* mutants treated with the DNA damaging agent MMC. Fresh weight of *atbard1 2, atbrca1 1,* as well as the double mutant line *atbard1 2/atbrca1 1* and *atku70* (a sensitive control line) mutant seedlings grown on GM medium containing 30 μg MMC/ml for 17 days. Values shown are means from 10 seedlings (±s.d.). WT: wild type.

A precise analysis of the *AtBRCA1* expression level in the *atbrca1* mutant lines demonstrated that the expression level of the mRNA in front of the T-DNA inserts was unchanged (Figure 7C). With primers spanning the insertion sites, we were not able to amplify any product, neither from the *atbrca1-1* nor from the *atbrca1-2* allele. Primers downstream of the insertion demonstrated that the expression in the *atbrca1-1* line is drastically reduced as compared to the wild-type AtBRCA1 expression, whereas the expression in the *atbrca1-2* line is moderately increased.

Both insertion lines were viable and fully fertile. Moreover, both lines were sensitive to MMC (Figure 5). The *atbrca1-1* mutant line was crossed with the mutant *atbard1-2* line to create an *atbard1-2/atbrca1-1* double mutant. To elucidate whether both proteins act in the same DNA repair pathway, we quantified in repeated experiments the degree of MMC sensitivity by fresh weight determination of the double mutant in comparison with both single mutants (Figure 8). Indeed, the double mutant was not more sensitive than each of the single mutants, indicating that AtBRCA1 and AtBARD1 are epistatic for DNA cross-link repair.

Discussion

Sequence analysis has revealed that *Arabidopsis* contains orthologues of genes involved in human genetic diseases as well as in cancer (the *Arabidopsis* genome initiative). Interestingly, two genes, the mutations of which were found to be frequently associated with breast cancer, *BRCA1* and *BRCA2*, are present in plant genomes, too. BRCA2 is involved in HR and seems to nucleate RAD51 filament formation at dsDNA–ssDNA junctions (e.g. Yang *et al*, 2005). In *Arabidopsis*, two recently duplicated BRCA2 homologues are present. An RNAi approach demonstrated that knocking down the expression of the genes strongly impairs meiosis (Siaud *et al*, 2004). It has been reported before that *Arabidopsis* contains a BRCA1 homologue, the expression of which is induced after DNA damage (Lafarge and Montane, 2003). We have now also been able to detect an ORF for a BARD1 homologue in the *Arabidopsis* genome and to characterise its function and the relation to AtBRCA1 in plants.

AtBARD1 is not induced by genotoxic stress

The expression levels of *AtBARD1* and *AtBRCA1* were similar in most investigated plant tissues (Figure 2), consistent with the hypothesis of their functional interaction. However, under genotoxic stress, the *AtBARD1* transcript level remained constant, whereas *AtBRCA1* was strongly induced. Interestingly, in mice the BARD1 expression also correlated with the BRCA1 expression. However, during the ovulatory cycle, BRCA1 and BARD1 are modulated differently in the uterus (Irminger-Finger *et al*, 1998). Furthermore, upon genotoxic stress both *BRCA1* and *BARD1* mRNAs are induced (Aunoble *et al*, 2001; Irminger-Finger *et al*, 2001). Therefore, it seems that the regulatory mechanism of AtBARD1 and AtBRCA1 in plants differs from humans.

AtBARD1 and AtBRCA1 interact and are involved in cross-link repair

hBARD1 has been reported to form a dimer with hBRCA1 (Wu *et al*, 1996). If the two ORFs in plants had similar functions as in mammals, we would expect the respective proteins to interact. Indeed, we were able to demonstrate a specific interaction by the use of the split YFP system in *planta* and the yeast two-hybrid assay (Figure 3). This result can be taken as a strong hint that both proteins function in a common complex in a similar way as the E3 ubiquitin ligase in humans. Further support for a genetic association of the two proteins could be obtained by mutant analysis. Various studies in mammals have shown that mutations in genes important for the DNA repair lead to chromosomal instabilities and increased sensitivity to DNA-damaging agents, such as radiation, or cross-linking agents, like MMC (e.g. Cui *et al*, 1999). Similar effects were also reported for *brca1* and *bard1* mutant cells (Mamon *et al*, 2003; Westermark *et al*, 2003). To decipher whether the putative AtBARD1 and AtBRCA1 homologues were involved in DNA repair, we investigated the sensitivity of the insertion mutants to DNA damage-inducing chemicals. When challenged with the cross-linking agent MMC, we could observe an increased sensitivity for *atbard1* and *atbrca1* mutants. The fact that the *atbard1-2/atbrca1-1* double mutant was not more sensitive than the single mutants indicates that both factors are involved in the same pathway of DNA cross-link repair in plants (Figure 8). Interestingly, the fact that the mutants are not sensitive to

bleomycin is reminiscent to the behaviour of certain plant mutants involved in HR. It has been reported that the RAD51 paralogues AtRAD51B, AtRAD51C and AtXRCC3 are sensitive to MMC but not to γ-rays (Bleuyard and White, 2004; Abe *et al*, 2005; Osakabe *et al*, 2005).

BARD1 might be involved in transcriptional regulation

Recent studies showed that hBRCA1 associates with the upstream stimulatory factor 2 and is a component of a DNA-binding complex (Cable *et al*, 2003). These findings suggest a role for the endogenous BRCA1 protein complex in transcription through a defined DNA-binding sequence and indicate that one function of BRCA1 is to coregulate the expression of genes involved in various cellular processes. It is noteworthy to state that in our two-hybrid experiments, it was demonstrated that the complete AtBARD1 protein contains an activation domain (Figure 3A). In contrast to BRCA1 (Monteiro *et al*, 1996), a similar finding has not been reported before for other eukaryotic BARD1 ORFs. The activation domain of AtBARD1 could be taken as a hint that similar to hBRCA1 BARD1 might also be involved in transcriptional regulation in eukaryotes in general. It will be interesting to determine to what extent changes in the transcriptome of *Arabidopsis* occur in the *atbard1* mutants.

The role of AtBARD1 and AtBRCA1 in meiosis

Although key factors in the mechanisms and in the regulation of HR are conserved between different eukaryotes, very important differences can be detected between yeast, plants and animals (Hartung and Puchta, 2004). *Saccharomyces cerevisiae* does not contain a number of factors involved in the regulation of HR, such as BRCA1, BARD1, BRCA2 and p53 that are present in animals. Interestingly, plants do contain both BRCA homologues and BARD1, although no indication for a structural or functional p53 homologue exists. Nevertheless, indications for an UV-induced apoptosis pathway present in *Arabidopsis* were recently reported (Danon *et al*, 2004).

A row of viable mutant defects in certain steps of HR could be isolated in plants in contrast to other higher eukaryotes owing to embryo lethality. Among these genes are *AtRAD51*, *AtMRE11*, *AtRAD50* and the *RAD51* paralogues. Owing to the fact that plant mutants are viable, *Arabidopsis* is an ideal object to analyse the role of these factors in meiosis. Indeed, mutations of *AtRAD51*, *AtMRE11*, *AtRAD50*, *AtXRCC3* and *AtRAD51C* resulted in sterility owing to aberrant meiosis (Bleuyard and White, 2004; Bleuyard *et al*, 2004, 2005; Li *et al*, 2004, 2005; Puizina *et al*, 2004; Abe *et al*, 2005). A sterile phenotype was also reported for a mouse *BRCA1* mutant with a deletion of intron 11 that was obtained in a p53 + / background (Xu *et al*, 2003). To our knowledge, no studies have been published on the role of BARD1 in meiosis of mammals. In *C. elegans* depletion of BRCA1 or BARD1 by a RNAi approach, which does not lead to a complete depletion of the protein, resulted in a reduction of germ cell viability of about 20% (Boulton *et al*, 2004). Thus, the fact that *atbrca1* and *atbard1* plants seem to be as fertile as wild types was surprising to us. Alexander staining of the pollen from both mutants showed no differences to wild-type pollen, demonstrating that both proteins do not have an indispensable role in meiosis. However, we cannot, of course, exclude minor effects on pollen viability in the percent range. In principle, AtBRCA1 and AtBARD1 could have an influence on meiotic recombination. However, as no functional p53 homologue seems to be present in plants, it might well be that the respective meiocytes survive, and completion of meiotic recombination is achieved by other factors. It will be interesting to test with a recently developed assay system if crossover rates are changed in the mutants (Melamed-Bessudo *et al*, 2005).

The role of AtBARD1 in homologous DNA recombination in somatic cells

The data presented in this study are, to our knowledge, the first direct proof using bard1$^{-/-}$ mutants that BARD1 is involved in homologous DSB repair in somatic eukaryotic cells. *bard1*-null mice generated by targeted mutagenesis display a phenotype of early embryonic lethality (McCarthy *et al*, 2003), eliminating the possibility of a detailed study to the effect on HR. Experiments performed with mouse cells expressing truncated mouse or human BARD1 peptides, capable of interacting with BRCA1, indeed resulted in a deficiency of homologous DNA repair. Repair was mildly reduced in BRCA1 wild-type cells and severely reduced in cells that harbour a BRCA1 splice product deleted for exon 11 (Westermark *et al*, 2003; Stark *et al*, 2004). However, strictly speaking this approach of negative complementation disturbed the function of the BRCA1/BARD1 heterodimer and not by directly blocking the function of the BARD1 protein.

Interestingly, both *atbard1* mutants show a defect in HR with and without induction of genotoxic stress. This is reminiscent to studies on *AtERCC1* (Dubest *et al*, 2004) and *AtRAD51C* (Abe *et al*, 2005). AtERCC1 is part of an exonuclease that is involved in removing nonhomologous ends from DSBs during HR in somatic cells (Dubest *et al*, 2004). AtRAD51C is most probably involved in the resolution of intermediates arising during DSB repair by HR. Whereas these proteins are directly involved in the recombination process by their enzymatic activities, BARD1 as part of an ubitiquin ligase might be more indirectly involved in the process by regulating the activity of respective factor(s) involved in the mechanisms of the reaction. Recently, a defect in the induction of recombination after Flagelin and H_2O_2 activity was correlated with a mutation in the *AtSNM1* gene (Molinier *et al*, 2004). However, no defect of induction could be observed with bleomycin, so that there must be at least two different regulation cascades involved in the induction of HR after stress, one induced by a pathogen attack, and the other by DNA damage. It will be a challenge of future experiments to define the cascades in detail.

Materials and methods

Characterisation of the A. thaliana insertion mutants

The *atbard1* T DNA insertion lines (*SALK 097601* and *SALK 031862*) as well as the *atbrca1* insertion line *SALK 014731* were obtained from the *Arabidopsis* T DNA collection in Nottingham. The *atbrca1* insertion line, *Garlic line 916 C09*, was obtained from the T DNA collection of Syngenta Biotechnology Inc. (SBI).

To obtain *651/atbard1* plants, homozygous lines for *atbard1* were crossed with plants homozygous for the transgene *651*, carrying a scorable recombination substrate (Puchta *et al* (1995) in C24 background). Siblings homozygous for *651* transgene and homo zygous for the *AtBARD1* wild type alleles were used as control lines.

Growth conditions and mutagen test

Seeds of *A. thaliana* were surface sterilised in 6% sodium hypochlorite for 8 min and rinsed several times with sterile water. Plants were grown in growth chambers at 23°C under white light (16 h light/8 h dark). Sterilised seeds were spread on GM agar

containing different concentrations of MMC. Two weeks later, plants were screened for their sensitivity.

Detection of recombination events and calculation of recombination frequency in wild-type and mutant Arabidopsis seedlings

One week old seedlings were transferred to Petri dishes containing liquid GM medium. The next day bleomycin was added (Duchefa) to a concentration of 10 μg/ml, 5 days later the seedlings were used for GUS staining. Histochemical staining was performed as described by Swoboda *et al* (1994). Plants were destained in 70% ethanol. Blue spots were counted under a binocular.

Two-hybrid analysis

Yeast two hybrid experiments were performed with the yeast strain *EGY48* (*MATα his3, trp1, ura3 LexAop*6 LEU2)*, which had been transformed by integration of the linearised reporter plasmid p8op lacZ into the genome (carrying a *lacZ* reporter gene under the control of eight LexA operators) (Estojak *et al*, 1995).

Arabidopsis AtBRCA1 and *AtBARD1* sequences, either full length or truncated, were amplified by PCR as *EcoR*1 and *Xho*1/*Sal*1 fragments and cloned into the *EcoR*1 *Xho*1 sites of the bait and prey vectors pGildaBD and pB42AD in order to create an in frame fusion protein. Primer pairs are listed in the Supplementary data.

Two hybrid assays were performed as described by Estojak *et al* (1995) and according to the manufacturer's instructions (Clontech).

BiFC analysis

AtBRCA1 or fragments of *AtBRCA1* were fused with the C terminal part of the YFP protein whereas *AtBARD1* was fused to the N terminal part of YFP. *AtBRCA1* full length, RING and C terminal fragments as well as the *AtBARD1* full length fragment were cloned into the pMAV GW YN and pMAV GW YC vectors, respectively, via two step Gateway cloning (Invitrogen). Primer pairs are listed in the Supplementary data. Constructs were introduced by biolistic transformation into mustard seedlings as described by Holweg *et al* (2004). Vector combinations (samples and controls) were used (1 2 μg of each plasmid) as described by Stolpe *et al* (2005). Images were captured on a Zeiss Axioscope 2 microscope with a Zeiss Axiocam video camera and enhanced using Adobe Photoshop 6.0 Software.

RNA isolation and real-time PCR analysis

Total RNA was isolated with the help of the Qiagen Total RNA isolation kit. Next, RNA was treated with RNAse free DNAse, followed by a reverse transcription with *MuMLV* reverse transcrip tase and polydT as first strand synthesis primer. Real time PCR analysis was performed as described (Chen *et al*, 2003). Primers for quantitative RT PCR are listed in the Supplementary data.

Database screening

Sequence searches were performed using TAIR BLASTP 2.0. Protein sequences were aligned by pileup. Sequence files were exported to ESPript 2.0 at http://prodes.toulouse.inra.fr/ESPript/cgi bin/nph ESPript exe.cgi for box shading analysis.

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