BRCC36A is epistatic to BRCA1 in DNA crosslink repair and homologous recombination in Arabidopsis thaliana

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ABSTRACT

BRCA1 is a well-known tumor suppressor protein in mammals, involved in multiple cellular processes such as DNA repair, chromosome segregation and chromatin remodeling. Interestingly, homologs of BRCA1 and several of its complex partners are also found in plants. As the respective mutants are viable, in contrast to mammalian mutants, detailed analyses of their biological role is possible. Here we demonstrate that the model plant Arabidopsis thaliana harbors two homologs of the mammalian BRCA1 interaction partner BRCC36, AtBRCC36A and AtBRCC36B. Mutants of both genes as well as the double mutants are fully fertile and show no defects in development. We were able to show that mutation of one of the homologs, AtBRCC36A, leads to a severe defect in intra- and interchromosomal homologous recombination (HR). A HR defect is also apparent in Atbrca1 mutants. As the Atbrcc36a/Atbrca1 double mutant behaves like the single mutants of AtBRCA1 and AtBRCC36A both proteins seem to be involved in a common pathway in the regulation of HR. AtBRCC36 is also epistatic to AtBRCA1 in DNA crosslink repair. Upon genotoxic stress, AtBRCC36A is transferred into the nucleus.

INTRODUCTION

Breast cancer is one of the most important diseases of women in the western world. Predisposition is correlated with mutations in genes that affect the stability and integrity of the genome. Breast cancer susceptibility proteins BRCA1 and BRCA2 belong to the most thoroughly analyzed human proteins of the last two decades (1). Mutations in BRCA1 were first linked to the development of breast cancer in 1990 (2). The protein functions as an E3 ubiquitin ligase together with its partner, BARD1 (3). The BRCA1/BARD1-heterodimer is involved in a number of processes such as cell-cycle control, DNA repair and chromatin remodeling (4–6). Homozygous mutations in these genes result in embryonic lethality in mice, which shows their developmental importance.

To fulfill its functions the heterodimer interacts with a sequence of partners in several different complexes (7). In mammals, phosphorylated BRCA1 is recruited to double-strand breaks (DSB) through its interaction with ABRA1 and RAP80 (8,9). The protein RAP80 binds K63-linked ubiquitin chains, made by the proteins UBC13, RNF8 and RNF168, on gammaH2AX and thereby guides BRCA1 and its partners to the DSB. In 2003 a complex called BRCC (BRCA1/BRCA2-containing complex), involved in repair of ionizing radiation induced damage, was described in mammals. It contained BRCA1, BARD1, BRCA2, RAD51, BRCC36 and BRCC45 in addition to other proteins (10). Chen et al. (11) showed that phosphorylation of BRCA1 by ATM and CHK2 depends on BRCC36 and that BRCA1 foci formation is impaired in BRCC36 depleted cells. Human BRCC36 has a deubiquitinating activity encoded by its N-terminal MPN-domain, which displays sequence homology with the human Poh1/Pad1 subunit of the 26S proteasome and with subunit 5 (Jab1) of the COP9 signalosome.

Beside the BRCC another BRCC36-containing complex, called BRISC (BRCC36 isopeptidase complex) exists in mammals. In this complex, BRCC36 works together with the proteins NBA1, ABRO1, ABRA1 and BRCC45 in cleaving K63-linked ubiquitin chains and therefore seems to antagonize the ubiquitinating function of UBC13, RNF8 and RNF168 (12,13). But beside the canonical role of deubiquitinating enzymes in antagonizing ubiquitin ligase activity, BRCC36 also enhances BRCA1/BARD1 E3 ubiquitin ligase activity (10). Dong et al. (10) could show that BRCC36 expression is increased in breast cancer tumors. So a possible scenario may involve the...
disruption of the normal function of the BRCA1-containing complex by overexpressed BRCC36 (10).

Indications that the BRCA1 function is widely conserved in multicellular organisms were described several years ago when orthologs of the gene were identified in other animal species, like Caenorhabditis elegans and Xenopus laevis (14,15). Surprisingly, BRCA1 as well as BRCA2 orthologs are also present in higher plants (16–18). Moreover, our group was able to identify a BARD1 ortholog in the model plant Arabidopsis thaliana (19). As the respective mutants are viable in plants, we could show that AtBRCA1 and AtBARD1 are epistatic in DNA crosslink repair. Mutation of AtBARD1 was correlated with severe defect in homologous recombination (HR). Now we were able to identify two BRCC36 orthologs in Arabidopsis and could address for the first time the questions of whether AtBRCC36 is involved in HR and crosslink repair and whether it is epistatic to AtBRCA1.

MATERIALS AND METHODS

See Supplementary Data for details of the primers (Supplementary Table SI) and raw data of the sensitivity and HR-assays (Supplementary Table SII and III).

Analysis of T-DNA insertion lines

Seeds were obtained from the GABI and SALK collections (20,21). Seeds derived from heterozygous plants were cultivated in soil, and PCR assays with primers flanking the T-DNA insertions were used to screen 2- to 3-week-old plants. Plants homozygous for the T-DNA insertion were propagated further. The integration sites for the Atbrcc36a and b mutants were determined with primer combinations specific for the left or right border of the T-DNA and genomic sequences within the corresponding gene (Figure 2). PCR products were purified and sequenced (GATC Biotech, Konstanz, Germany).

RNA extraction and real-time–PCR

RNA from young Arabidopsis plantlets was isolated by using the RNEasy Plant Mini Kit from Qiagen (Hilden) according to the instructions of the manufacturer. Reverse transcription was performed according to the protocol of the RevertAid TM First Strand cDNA synthesis Kit from Fermentas (St Leon-Roti). The cDNA produced was used for PCRs to evaluate the mRNA level of the sequences in front of, spanning, and behind the T-DNA insertion sites in the interrupted genes. Moreover, the cDNA was used for quantitative Real-Time PCR (Figure 1B). Real-time PCR was performed as described in Chen et al. (22). For γ-irradiation the radioactive isotope cobalt-60 (60Co) was used (Institute of Toxicology and Genetics, Karlsruhe Institute of Technology).

HR-assay

Plants homozygous for the appropriate T-DNA insertion and the HR reporter line 651 or IC9C, respectively (23,24), were identified by PCR screening and used for the assays. As an internal control, wild-type plants segregating from the cross were used. Seeds were sterilized using 6% NaOCl solution and plated on GM. After 7 days, 15 seedlings for 651 and 20 for IC9C were transferred into halves of Petri dishes containing 10 ml of pure liquid GM. To induce HR 1 day later, bleomycin was added to a final concentration of 5 μg/ml. After five additional days for 651 and six for IC9C in liquid culture the seedlings were transferred into a staining solution (25). After two more days at 37°C, the seedlings were incubated in 70% ethanol for 16 h at 60°C, and subsequently, the number of blue sectors on each plant was determined by using a binocular microscope. The HR assays were repeated independently at least three times. The results were normalized to the appropriate wild-type, and the mean values and standard deviations were determined (Figures 3 and 4). To exclude artifacts due to reporter gene silencing, expression of the 5’ part of the GUS gene in the lines was verified by real-time PCR (data not shown).

Mutagen assays

Homozygous seeds from all lines, the double mutant and Col-0 wild-type were sterilized and plated on GM. After 7 days 10 seedlings were transferred into six-well plates containing 3 ml of pure liquid GM per well. One day later another 3 ml of liquid GM was added with the indicated concentration of the mutagen MMC. After 14 days the seedlings were taken out, pressed on paper towels to remove any excess liquid and weighed. For each experiment, the fresh weight of treated plants was normalized to that of the untreated plants of each line. All experiments were performed at least six times, and the mean value for each line was compared to the mean value for the wild-type for the corresponding mutagen concentration (Figure 6).

Cloning of fusion constructs

BRCC36A was fused to the N-terminus of YFP by first cloning YFP into the vector 35SpBARN (26) and then cloning the ORF of BRCC36A upstream of YFP. This construct was stably transformed by Agrobacteria transformation as described in Clough and Bent (27). Images were taken from epidermal cells of 2-week-old seedlings with a Zeiss Axio Cam MRm microscope with a Zeiss AxioCam MRm camera (Figure 5).

RESULTS

Two BRCC36 homologs are present in A. thaliana

To identify BRCC36 homologs, a database search was done with tblastn using human BRCC36 as the template (NP_077308). Two significant hits were found on Chromosome 1 and 3: At1g80210 (BRCC36A) and At3g06820 (BRCC36B). The genes consist of 2921 bp (At1g80210) and 2594 bp (At3g06820), respectively, including UTRs. They each contain 10 exons. The corresponding proteins have a length of 406 amino acids and 405 amino acids, respectively. In comparison to human BRCC36 the proteins show an overall identity of 31.3% (At1g80210) and 30.1% (At3g06820). Both proteins,
BRCC36A and BRCC36B, contain a domain called MPN that is also present in the human protein. Sequence comparison of BRCC36A and BRCC36B revealed that the two proteins are closely related, showing an identity of 90.9%, and apparently arose by a recent gene duplication (Figure 1A). For further analysis we searched the Phytozome V5.0 database for homologs in other plant species. We could find one homolog in each annotated species, except Vitis vinifera, Medicago truncatula and Glycine max where also two homologs were recorded. After analyzing the duplications in V. vinifera and M. truncatula in more detail we found annotation failures as in both organisms the single copy BRCC36 gene was annotated in two parts. In G. max also two nearly identical homologs were annotated, which arose through a tetraploidization event an estimated 10–15 million years ago. The duplication in A. thaliana presumably arose only in Brassicaceae as we could also find it in Arabidopsis lyrata, but not in other families of the order Brassicales, like the Caryophyllaceae (http://www.phytozome.net/results.php). Interestingly most plant proteins have a stretch of ~60–90 amino acids in the middle part of BRCC36, which can not be found in the animal homologs.

Figure 1. (A) Comparison of the amino acid sequence of HsBRCC36 with the sequences of AtBRCC36A and B. Identical amino acids are marked with black boxes, similar amino acids are shaded. (B) Expression of AtBRCC36A and B compared to the expression of AtBRCA1 after γ-irradiation. The transcription level is given in relation to actin mRNA and the mRNA of the corresponding untreated seedlings, and is the mean of two different qRT–PCR reactions. Bars represent the standard deviation. Black bar, AtBRCA1; grey bar, AtBRCC36A; light grey bar, AtBRCC36B.
BRCC36A and BRCC36B are expressed constitutively

In plants, induction of expression after DNA damage can be observed for several genes involved in DNA repair, like BRCA1 or RAD51 (16,22,30). To find a possible correlation in transcriptional regulation between BRCA1 and the BRCC36 homologs, 2-week-old seedlings were irradiated with γ-rays (75 Gy) and the amount of transcript was measured 1 h after irradiation by quantitative real-time PCR. As previously reported, BRCA1 was highly induced; whereas no significant change in transcript amount was observed for either of the BRCC36 homologs (Figure 1B). A similar observation has been reported previously for BARD1 (19). Both homologs were expressed in all the organs tested (roots, rosette leaves, leaves, flowers, siliques). Expression of BRCC36A in siliques and BRCC36B in flowers was slightly higher than in the other organs tested. BRCA1 expression was increased in both organs (data not shown).

BRCC36 is dispensable for vegetative growth and fertility

To elucidate the biological function of BRCC36A and BRCC36B in plants, mutants of both genes were characterized. The gene loci, At1g80210 and At3g06820, were used to screen the sequence database of T-DNA insertion mutants on the SIGnAL website [Salk Institute Genomic Analysis Laboratory, (20)], and two mutant lines were identified for each gene. For BRCC36A the insertions are located in the middle of the gene in intron 4 (GABI_195B04, Atbrcc36a-1) and at the end in exon 8 (SALK_130227, Atbrcc36a-2). For BRCC36B they are located in exon 4 (GABI_773H02, Atbrcc36b-1) and in intron 4 (SALK_086443, Atbrcc36b-2) (Figure 2).

To test the expression level of the genes in the mutant lines a reverse transcriptase PCR (RT–PCR) was performed with primer pairs binding upstream, across and downstream from the T-DNA insertion. For all lines, expression upstream of the T-DNA could be demonstrated, but no expression was observed with the primer pair
spanning the insertion. Downstream from the T-DNA insertion expression was detected for all lines except Atbrcc36a-2. In this line, T-DNA integration was coupled with a deletion spanning the rest of the ORF and parts of the 3'UTR of the gene. In the Atbrcc36b-1 line T-DNA insertion was coupled with a duplication of 789 bp of the gene (data not shown). Thus, no full-length BRCC36A or BRCC36B mRNAs could be detected in the corresponding mutants.

None of the single mutants showed any obvious deviation from normal growth. The plants were fertile and produced seeds in numbers similar to the wild-type. We also obtained Atbrcc36a-1/b-2 double mutants by crossing, but the absence of both homologs had no visible effect either, as the resulting plants exhibited normal growth and fertility.

BRCC36A is required for intra- and interchromosomal HR

Reidt et al. (19) showed that Atbard1 has a defect in HR. To analyze the role of BRCC36 and BRCA1 in HR, we crossed the reporter line 651 into the mutant backgrounds. This recombination substrate consists of two overlapping fragments of the β-glucuronidase gene (uidA) interrupted by a hygromycin resistance marker. These separated uidA sequences share a 618-bp overlap in inverted orientation. A functional uidA gene can be restored by HR (Figure 3A). Thus, after histochemical staining each recombination event is represented by a blue sector on the plant. Plants homozygous for the T-DNA insertion and the recombination substrate were used for the assays. Seedlings were incubated in liquid germination medium (GM) with bleomycin (5 μg/ml), which induces single- and double-strand breaks (31). After staining, recombination events were counted on the seedlings.

The Atbrcc36a mutants showed a strong defect in HR: in case of Atbrcc36a-1 recombination is reduced to about a third of the wild-type level and in case of Atbrcc36a-2 to about a 10th. In contrast, the Atbrcc36b mutants showed no significant reduction in recombination efficiency (Figure 3B): Although Atbrcc36b-2 seems to be less efficient in HR than Atbrcc36b-1, standard deviations overlap with wild-type for both mutants. Thus, despite their high homology, the two homologs seem to play different roles in HR. We also tested whether the Atbrcc36a-1 allele in its hemizygous state is able to reduce HR. This was not the case (data not shown) making it unlikely that the detected reduction of HR is due to negative complementation of a truncated BRCC36A protein.

As the main mechanism for the restoration of the reporter gene in the line 651 is intrachromosomal recombination (23), we were also interested in the role of BRCC36 in other HR reactions. Therefore, we crossed all four single mutants with the IC9C line and induced DSBs by bleomycin treatment. In this case, restoration of the β-glucuronidase gene is only possible through interchromosomal recombination (Figure 3C, (24)). Interestingly all mutants behaved in this background in the same manner as in case of the recombination line 651. The HR frequency of Atbrcc36a-1 was reduced to about a third in comparison to wild-type, whereas in

Figure 3. HR efficiencies in Atbrcc36a-1/-2 and b-1/-2 (651 and IC9C) compared to wild-type. Figures show the recombination efficiencies in percent, normalized to the wild-type control, in bleomycin treated plants. (A) Recombination trap harbored by the 651 line. (B) HR efficiencies in the 651 background. (C) Recombination trap harbored by the IC9C line. (D) HR efficiencies in the IC9C background. Bars represent the standard deviation.
case of Atbrcc36a-2 the reduction was more drastic to less than a 10th. Both Atbrcc36b mutants did not show any reduction in HR (Figure 3D).

AtBRCC36A is epistatic to AtBRCA1 in HR

To characterize the role of BRCC36A in HR in more detail, we wanted to elucidate its relation to BRCA1. We have been able to show before the BRCA1 is involved in DNA crosslink repair in plants (19), but no data on its involvement in HR have been published. Therefore, we first tested whether BRCA1 is, as its homolog in mammals (32), indeed involved in HR. Plants homozygous for the T-DNA insertion and the recombination substrate 651 were used for the assays. The BRCA1 insertion line used in this study was already characterized by Reidt et al. (19) (SALK_014731, Atbrca1-1). We found that the Atbrca1-1 mutant revealed a defect in HR after bleomycin treatment similar to the Atbrcc36a mutants (Figure 4A). Finally, to test a putative epistasis between BRCC36A and BRCA1, we analyzed the recombination behavior of the Atbrcc36a-1/Atbrca1-1 double mutant and the corresponding single mutants. We found that the Atbrcc36a-1/Atbrca1-1 double mutant behaved like both single mutants (Figure 4A). Thus, BRCA1 and BRCC36A are indeed epistatic in HR. As a control, we analyzed a Atbrcc36a-1/b-2 double mutant obtained in the 651 reporter background. The double mutant behaved like the Atbrcc36a mutants, indicating again that BRCC36B is dispensable for bleomycin-induced HR (Figure 4B).

BRCC36A is localized in the nucleus after genotoxic stress

We previously demonstrated that BRCA1, together with its partner BARD1, can be found in the plant nucleus (19). To test whether BRCC36A is also located in the nucleus, a C-terminal fusion of the ORF with the yellow fluorescent protein (YFP) was cloned and stably transformed into Arabidopsis. Several homozygous single locus lines were subsequently analyzed. Surprisingly, in these lines the protein was localized mainly in the cytoplasm under standard growth conditions (Figure 5A). However, 1 h after the application of bleomycin, fluorescence was also observed in the nucleus (Figure 5B). This observation demonstrates that BRCC36A is transferred into the nucleus upon genotoxic stress.

BRCC36A is epistatic to BRCA1 in DNA crosslink repair

We showed that loss of BRCA1 and BARD1 in Arabidopsis results in a mild sensitivity to the DNA-crosslinking agent Mitomycin C [MMC; (19)]. We therefore tested whether the same holds true for the Atbrcc36a and Atbrcc36b mutants. Surprisingly, not only the Atbrcc36a mutants, but also the Atbrcc36b mutants showed MMC sensitivity similar to Atbrca1-1 (Figure 6A). This indicates that in contrast to HR both homologs play a role in crosslink repair. To define the role of the proteins in detail, we obtained double mutants for further testing. The sensitivity of the Atbrcc36a-1/b-2 double mutant was comparable to that of the single mutants (Figure 6B). To define the role of BRCC36A in relation to BRCA1 in crosslink repair, we combined the Atbrcc36a-1 mutation with the Atbrca1-1 mutation (Figure 6C). The double mutant displayed no enhanced sensitivity against MMC compared to the single mutants, indicating that, as in the case of HR, BRCC36A might be epistatic to BRCA1 in this repair pathway, too. However, as the applied MMC concentration is quite high and the growth defects are minor in comparison to other mutants with defects in DNA repair and recombination like Atmus81 (33) this result should only be taken as hint that both proteins act in the same repair pathway. Treatment with other agents like bleomycin or methyl methane sulfonate did not cause any response different from that of wild-type.

DISCUSSION

Many DNA repair mutants show embryonic lethality in mammals, but are viable in plants (34). This holds also true for the BRCA1 and BRCA2 mutants. Using A. thaliana as a model organism it could be demonstrated that besides a defect in double strand break repair in somatic cells (17) BRCA2 is required for meiotic recombination (18). We were able to show that BRCA1 is required for DNA crosslink repair (19) and as reported...
in this communication for somatic HR. This result was expected, as a defect in HR has been reported after a knockdown of BRCA1 expression in mammalian cells (32) and we reported previously that a knockout of the heterodimeric partner of BRCA1, BARD1, also leads to a defect in HR in Arabidopsis (19). However, neither BRCA1 mutants nor BARD1 mutants had a defect in fertility (19).

The main purpose of the current work was to characterize the role of two BRCC36 homologs in DNA repair.

Although it has been reported previously that BRCC36 depletion leads to radiation sensitivity in mammalian cells (10), nothing was known about the involvement of the protein in HR in any eukaryote. We were able to show that knockouts of either or both BRCC36 homologs have no visible effects on development or fertility of A. thaliana. This indicates that BRCC36 like BRCA1 (19) and in contrast to BRCA2 (18) has no essential role during meiosis. In contrast, BRCC36A is required for intra- and interchromosomal HR and DNA crosslink repair in
somatic cells. This question has not been addressed for BRCC36 homologs in animal systems before. Furthermore, we demonstrated for the first time that a BRCC36 homolog is epistatic to BRCA1 in respect to HR. These data, obtained in a plant, extend observations from mammals, which show that BRCA1 and BRCC36 localize to radiation induced DNA damage foci (11). Moreover, we could show that in Arabidopsis BRCC36A is not permanently present in the nucleus, but localizes there after application of genotoxic stress. It is tempting to speculate that its transport is dependent on DNA damage recognition and it will be interesting to define the factors involved in the underlying mechanism by transforming the BRCC36A-YFP fusion construct into Arabidopsis mutants that have a defect in DNA damage recognition.

The fact that the two BRCC36 homologs in Arabidopsis are more closely related to each other than to their human homolog indicates that they result from a recent gene duplication. Intriguingly, both homologs differ in their biological function. Both proteins are identical at their N-Terminus, but no BRCA1 and BARD1 homologs in its genome can be taken as further hint that BRCA1-mediated repair. Indeed, the association of BRCC36 with a DSB might be a prerequisite for BRCA1-mediated repair. Moreover, the release of BRCA1 and its partners from DNA after repair might be favored by BRISC-mediated deubiquitination. Beside the two BRCC36 homologs, another complex partner, BRCC45, can be found as single copy gene in Arabidopsis. It will be interesting to define its biological function in planta, too.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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