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Inefficient double-strand DNA break repair is associated with increased fasciation in *Arabidopsis BRCA2* mutants

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Abstract

BRCA2 is a breast tumour susceptibility factor with functions in maintaining genome stability through ensuring efficient double-strand DNA break (DSB) repair via homologous recombination. Although best known in vertebrates, fungi, and higher plants also possess *BRCA2*-like genes. To investigate the role of *Arabidopsis BRCA2* genes in DNA repair in somatic cells, transposon insertion mutants of the *AtBRCA2a* and *AtBRCA2b* genes were identified and characterized. *atbrca2a-1* and *atbrca2b-1* mutant plants showed hypersensitivity to genotoxic stresses compared to wild-type plants. An *atbrca2a-1/atbrca2b-1* double mutant showed an additive increase in sensitivity to genotoxic stresses compared to each single mutant. In addition, it was found that *atbrca2* mutant plants displayed fasciation and abnormal phyllotaxy phenotypes with low incidence, and that the ratio of plants exhibiting these phenotypes is increased by γ-irradiation. Interestingly, these phenotypes were also induced by γ-irradiation in wild-type plants. Moreover, it was found that shoot apical meristems of the *atbrca2a-1/atbrca2b-1* double mutant show altered cell cycle progression. These data suggest that inefficient DSB repair in the *atbrca2a-1/atbrca2b-1* mutant leads to disorganization of the programmed cell cycle of apical meristems.

Key words: *Arabidopsis*, *BRCA2*, cell cycle, double-strand DNA breaks, fasciation, homologous recombination, γ-ray.

Introduction

Double-strand breaks (DSBs) are the most threatening type of DNA damage in living cells. There are two major DSB repair pathways: non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ repair is error-prone, and represents the predominant repair pathway during G_1 to early S-phase of the cell cycle (although this pathway is able to operate throughout the cell cycle). HR is important as an error-free repair pathway during late S to

G_2 phase of the cell cycle, when sister chromatids are available (Essers *et al.*, 2000; Couedel *et al.*, 2004; Bleuyard *et al.*, 2006).

HR processes are accomplished via the co-operation of a set of proteins known as RAD52 epistasis group proteins, of which MRE11/RAD50/XRS2 (NBS1) processes DSBs to generate 3′ single-strand DNA (ssDNA) tails, RAD52 and RAD51 paralogues assemble the eukaryotic recombinase,

RAD51 forms RAD51-single-strand DNA (ssDNA) nucleoprotein filaments with replication protein A, and RAD54 assists in D-loop formation of RAD51-dependent ssDNA and double-strand DNA (Thompson and Schild, 2001). In higher eukaryotic cells, RAD51 plays a dominant role in this pathway. Recent data indicate the important role of BRCA2 as well as RAD51 paralogues, which are co-factors of RAD51, in facilitating the assembly of RAD51 nucleoprotein filaments (Galkin *et al.*, 2005; van Veelen *et al.*, 2005).

The *BRCA2* gene was cloned based on an analysis of mutations in families predisposed to breast cancer showing that a large percentage of the kindred had alterations within this locus (Tavtigian *et al.*, 1996). BRCA2 is known to bind RAD51 directly through the eight conserved BRC repeats in BRCA2 (Wong *et al.*, 1997; Galkin *et al.*, 2005) as well as through the C-terminal region of BRCA2 (Esashi *et al.*, 2005). Using a gene knockout method to create mice with *BRCA2* mutations, homozygous mutant mice show embryo lethality associated with chromosomal rearrangements and breaks (Sharan *et al.*, 1997; Yu *et al.*, 2000). In addition, the formation of RAD51 foci following DNA damage is altered in *brca2* mutant cells (Yu *et al.*, 2000). These results suggest that BRCA2 is required to engage RAD51 in HR repair of DNA damage.

Siaud *et al.* (2004) reported the presence of two *BRCA2*-like genes in the *Arabidopsis* genome. The *Arabidopsis* BRCA2 proteins interact with RAD51, DMC1, and DSS1 *in vitro* (Siaud *et al.*, 2004; Dray *et al.*, 2006). Moreover, transgenic *Arabidopsis* plants transformed with a *BRCA2* RNA interference (RNAi) construct driven by the meiosis-specific *Arabidopsis DMC1* promoter were sterile and showed aberrant chromosomes in meiosis (Siaud *et al.*, 2004). However, the function of AtBRCA2 in HR repair, especially in somatic cells, i.e. during vegetative growth, was not determined.

To determine the function of AtBrca2a and AtBrca2b in somatic cells, mutants in the genes encoding each of these proteins were isolated and characterized: *atbrca2a-1* and *atbrca2b-1*. Each single mutant exhibited hypersensitivity to genotoxic stresses compared to wild-type plants (Nossen), and the *atbrca2a-1/atbrca2b-1* double mutant showed an additive increase in sensitivity to genotoxic stresses compared to each single mutant. Interestingly, *atbrca2* mutant plants displayed fasciation and abnormal phyllotaxy phenotypes with low incidence, with the proportion of plants exhibiting these phenotypes being increased by γ-irradiation. Moreover, cell cycle regulation in the *atbrca2* mutant was investigated. The relationship between inefficient DSB repair, the abnormal phyllotaxy and/or fasciation phenotype, and cell cycle progression is discussed.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana ecotypes Nossen and Col. were used in this study. The *atbrca2a-1* and *atbrca2b-1* mutants were found by searching the *Ds* mutant collection (Nossen background) established by the RIKEN Institute (Fedoroff and Smith, 1993; Kuromori *et al.*, 2004; Ito *et al.*, 2005). Plants were grown on a soil mixture of equal parts of vermiculite and commercial soil (Sakata Super Mix, Sakata Seed Co., Tokyo, Japan) in a growth chamber at 22 °C under 12/12 h (light/dark) cycle conditions. Sterile plants were cultured on Murashige–Skoog medium (Murashige and Skoog, 1962) solidified with 0.25% gelrite (Wako, Tokyo, Japan) (MS gelrite plate) or 0.8% bacto-agar (Difco, Detroit, MI) (MS agar medium) in a growth chamber under 12/12 h (light/dark) cycle conditions at 22 °C.

Isolation of DNA and RNA

Genomic DNA was isolated from 2–4-week-old sterile plants using a DNeasy Plant Maxi kit (Qiagen, Hilden, Germany). Total RNA was isolated from 2-week-old plants and young flower buds using the RNeasy Plant Mini kit (Qiagen) according to the manufacturer's instructions.

DNA sequencing

Sequencing reactions were performed with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). The reaction products were analysed with an automatic DNA sequencer (ABI PRISM 3100-Avant Genetic Analyzer, Applied Biosystems).

PCR genotyping assay

Plant genotypes of *AtBRCA2a*(–/–) and *AtBRCA2b*(–/–) mutations were identified by PCR. The wild-type *AtBRCA2a* locus was identified by PCR with the primer combination F6N15-1 (5′-CAAATCGTTTTCAACTTTCCCGCCGTCT-3′) and F6N15-2 (5′-CATTTGGGGAATTGAGCAATTTGTGTTCC-3′). The mutant locus of *AtBRCA2a* was identified by PCR with Ds3′-4 (5′-CCGTCCCGCAAGTTAAATATG-3′) and F6N15-2. The wild-type *AtBRCA2b* locus was identified by PCR with the primer combination F7A7-1 (5′-GGCTTCCCCCGTGTAAATTATAGTTCTCAG-3′) and F7A7-2 (5′-CGTTTGGGGAATTGAGCAATTTGTGTTCT-3′). The mutant locus of *AtBRCA2b* was identified by PCR with Ds5′-3m (5′-ACCTCGGGTTCGAAATCGATCGG-3′) and F7A7-1. PCR products were examined by direct sequencing.

Production of BRCA2–RNAi plants

To produce a hairpin RNAi construct, the Gateway Cloning System (Invitrogen, USA) was used; *AtBRCA2* gene-specific primers fused to attB1 and attB2 sequences, Br2-attB1-F1 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGGCATTTATTTTCCGATTCCAGC-3′) and Br2-attB2-R1 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTGAAAAAGACTGTTGGGAACACC-3′) were used to amplify a 502 bp fragment of the *AtBRCA2b* gene by PCR. A BP clonase reaction was carried out to clone the PCR product into the donor vector pDONR221

(Invitrogen, USA). An LR clonase reaction was carried out to transfer DNA fragments from this intermediate clone to the destination vector [pB7GWIWG2(II)] (Karimi *et al.*, 2002). The resulting plasmid, pB7GWIWG2(II)-BRCA2 (BRCA2-RNAi vector) was introduced into *Agrobacterium tumefaciens* strain EHA105, and the resultant strain (Col. background) was used in RNAi silencing experiments.

Cisplatin treatment and γ-irradiation

Sensitivity of wild-type (Nossen and Col.), mutant plants (Nossen background) and RNAi plants (Col. background) to genotoxic stresses was scored based on the number of true leaves produced after genotoxic stress treatment according to our previous study (Osakabe *et al.*, 2005). For the assay of cisplatin sensitivity, seeds in lots of 50 were imbibed by soaking in 200 μl water for 4 d at 4 °C. Presoaked seeds were plated on MS agar medium containing the appropriate concentration of cisplatin. At 14 d after plating on MS agar plates, plants were scored for the production of true leaves. For the assay of γ-ray sensitivity, imbibed seeds were irradiated and immediately plated on MS agar medium. Ten days after planting on MS agar medium, plants were scored for the production of true leaves.

In situ hybridization analysis

In situ hybridization was performed essentially as described by Kouchi and Hata (1993). Plant material was fixed in 4% (w/v) paraformaldehyde in 50 mM sodium phosphate buffer (pH 7.2) for 6 h at 4 °C, dehydrated through a graded ethanol series and *tert*-butanol series, and finally embedded in Paraplast Plus (Sherwood Medical, St Louis, MO). Microtome sections (6–10 μm thick) were applied to glass slides treated with silane. The cDNA for *AtWUS* was a kind gift from Dr T Araki. The antisense and sense RNAs of *AtWUS* were labelled with digoxigenin by *in vitro* transcription of linearized pBluescript KS⁺ carrying a fragment of the entire coding sequence of the *AtWUS* cDNA. Hybridization and immunological detection were conducted according to the methods of Kouchi and Hata (1993).

Histochemical assay of AtCYCB1;1::GUS reporter

Arabidopsis thaliana (ecotype Col.) plants transformed with AtCYCB1;1::GUS (Colon-Carmona *et al.*, 1999) were crossed with *atbrca2* mutant and wild-type plants (Nossen). Sterile 1-week-old plants containing the AtCYCB1;1::GUS reporter gene and homozygous for the *AtBRCA2a* and *AtBRCA2b* mutation (*AtBRCA2a-1⁻/⁻/AtBRCA2b-1⁻/⁻*) were used for histochemical GUS staining. On the other hand, the *AtBRCA2*–RNAi construct was transformed to *Arabidopsis* Col. plants with *AtCYCB1;1::GUS*. Seeds were imbibed by soaking in 200 μl water for 4 d at 4 °C. Presoaked seeds were plated on MS agar medium. At 7 d after plating on MS agar plates, plants were treated with aphidicolin or cisplatin (3 d). After treatment with aphidicolin or cisplatin, plant materials were soaked in GUS

staining buffer containing 100 mM sodium phosphate (pH 7.0), 10 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X100, 0.5 mg ml⁻¹ X-Gluc, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 100 μg ml⁻¹ chloramphenicol, and 5% methanol. The enzymatic reaction was performed over 1 d at 37 °C. Tissues were cleared with 70% ethanol to remove pigments. Plants were observed under a dissecting microscope.

Results

Identification of Arabidopsis Ds *insertional mutants in* AtBRCA2 *genes*

To investigate the function of the *AtBRCA2a* and *AtBRCA2b* genes in somatic cells, a search was made for loss-of-function mutants using a reverse genetic approach. Single mutant lines were identified in the RIKEN *Ds* insertion collection (Nossen background) (Fedoroff and Smith, 1993; Kuromori *et al.*, 2004; Ito *et al.*, 2005), (http://rarge.gsc.riken.go.jp/dsmutant/), and the corresponding alleles were named *atbrca2a-1* and *atbrca2b-1*. The *AtBRCA2a/Ds* and *AtBRCA2b/Ds* junctions were amplified by PCR and sequenced to determine the position of the *Ds* insertion (see Supplementary Fig. S1A, B at *JXB* online). In the *atbrca2a-1* allele, the transposon is inserted in the first exon of the *AtBRCA2a* gene (45 bp downstream from the ATG start codon), whereas in the *atbrca2b-1* allele of the *AtBRCA2b* gene, the transposon is inserted within the 5' untranslated region of the first exon of the *AtBRCA2b* gene (91 bp upstream of the ATG; see Supplementary Fig. S1A, B at *JXB* online). Direct sequence analysis of PCR products revealed that the *Ds* insertions had not introduced any deletions or other modifications in the *AtBRCA2a* and *AtBRCA2b* genes.

Since *AtBRCA2a* and *AtBRCA2b* encode proteins that are 94.5% identical to each other, and these genes are probably the result of a recent duplication (Siaud *et al.*, 2004), it is possible that the two proteins play a redundant role in *Arabidopsis*. To investigate redundancy of the two *AtBRCA2* genes, genetic crosses between the two homozygous single mutants were performed to generate double mutants.

atbrca2 mutants are associated with hypersensitivity to DNA damaging agents

Previous studies in vertebrates have indicated that hypersensitivity to genotoxic regents such as cisplatin and γ-irradiation is a consistent feature of mutants deficient in HR repair (Liu *et al.*, 1998; Takata *et al.*, 2000; Sasaki *et al.*, 2004). Therefore, it was thought likely that *atbrca2* mutants would be more sensitive than wild-type plants to genotoxic agents. To test this hypothesis, sensitivity to cisplatin and γ-irradiation in *atbrca2* mutants was examined. γ-Irradiation is known to induce DSBs directly (Dizdaroglu and Bergtold, 1986). Cisplatin (*cis*-diaminedichloroplatinum-II) is a cross-linking reagent that forms DNA intra- and

inter-strand cross-links (Zamble and Lippard, 1995). Inter-strand cross-links are repaired exclusively by HR, and not by NHEJ (Essers *et al.*, 2000; De Silva *et al.*, 2002; Sasaki *et al.*, 2004). In this context, these sensitivity tests can provide evidence for the role of AtBRCA2a and AtBRCA2b in HR processes.
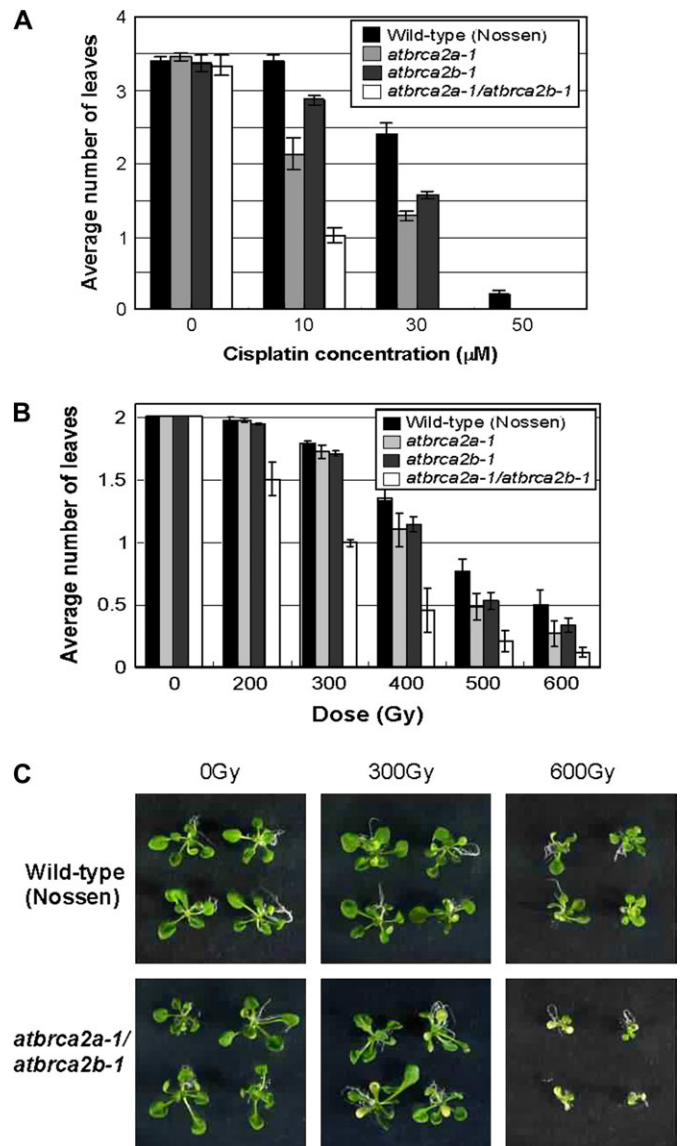
The sensitivity to cisplatin of both single mutants, *atbrca2a-1* and *atbrca2b-1*, and the *atbrca2a-1/atbrca2b-1* double mutant was tested first. Cisplatin sensitivity was scored based on the production of true leaves on MS agar medium containing cisplatin. This assay relies on the fact that the embryo of the mature *Arabidopsis* seed has already produced two fully formed cotyledons but has not yet initiated true leaves (Jiang *et al.*, 1997).

Both single mutants were hypersensitive to cisplatin compared to wild-type (Nossen) (Fig. 1A). Moreover, the *atbrca2a-1/atbrca2b-1* double mutant showed an additive increase in sensitivity to cisplatin treatment compared with that of the corresponding single mutants and wild-type plants (Fig. 1A). The effect was particularly apparent following treatment with 30 μM cisplatin, when the number of true leaves appearing in the double mutant plant was close to zero, single mutant plants produced an average of about 1.2–1.5 leaves, and wild-type plants about 2.4 leaves (Fig. 1A).

Next, to confirm cosegregation of *AtBRCA2a* or *AtBRCA2b* genotypes with sensitivity to cisplatin, the sensitivity to cisplatin of plants heterozygous for one or both of the *AtBRCA2a* and *AtBRCA2b* genes was tested. Accordingly, it was confirmed that sensitivity to cisplatin in *AtBRCA2a-1$^{+/-}$/AtBRCA2b-1$^{-/-}$* heterozygous plants (*a-1$^{+/-}$/b-1$^{-/-}$*) was the same as that in *atbrca2b* single mutant plants (*a-1$^{+/+}$/b-1$^{-/-}$*) (see Supplementary Fig. S2 at *JXB* online). Similarly, sensitivity to cisplatin in *AtBRCA2a-1$^{-/-}$/AtBRCA2a-1$^{+/-}$* heterozygous plants (*a-1$^{-/-}$/b-1$^{+/-}$*) was the same as that in *atbrca2a* single mutant plants (*a-1$^{-/-}$/b-1$^{+/+}$*) (see Supplementary Fig. S2 at *JXB* online). These results strongly suggest that *atbrca2a-1* and *atbrca2b-1* are recessive mutants, that the mutant phenotype is caused by the reduced activity of *AtBRCA2a* or *AtBRCA2b* products, and that both *AtBRCA2a* and *AtBRCA2b* are involved in DSB repair in somatic cells.

To confirm that the *Ds* insertion was responsible for the observed phenotypes of the *brca2a-1* and *brca2b-1* mutants, an RNAi construct designed to silence both genes simultaneously was cloned. This RNAi construct was placed under the control of a constitutive plant promoter (CaMV 35S promoter). Most plants transformed by the p35S::AtBRCA2–RNAi construct survived (95%), and exhibited a partially sterile phenotype under standard growth conditions. The sensitivity to cisplatin of *AtBRCA2*–RNAi plants was tested. *AtBRCA2*–RNAi plants show increased sensitivity to cisplatin compared to wild-type (Col.) (see Supplementary Fig. S2 at *JXB* online). This result strongly supports the hypothesis that the cisplatin sensitivity of these mutants is associated with the *Ds* insertion.

The sensitivity of *atbrca2a-1*, *atbrca2b-1*, and *atbrca2a-1/atbrca2b-1* mutants to γ-irradiation was examined next.

Fig. 1. (A) Sensitivity to cisplatin of single mutants *atbrca2a-1* and *atbrca2b-1*, and the double mutant *atbrca2a-1/atbrca2b-1*. Imbibed seeds were plated on MS agar medium containing 0–50 μM cisplatin. The numbers of true leaves in wild type (Nossen) and mutant plants were counted 14 d after plating. Data represent the mean ±SE of 50 plants in each group from three experiments. (B, C) Sensitivity of *AtBRCA2* mutants to γ-irradiation. (B) Imbibed seeds (4 d at 4 °C) were irradiated with increasing doses of ^{60}Co γ-rays. After γ-irradiation, the seeds were immediately plated on MS agar medium. The number of true leaves was counted 10 d after irradiation, and the average number of leaves was calculated according to Harlow *et al.* (1994). Data represent the mean ±SE of 34 plants in each group from three experiments. (C) Fourteen-day-old plantlets [upper panels: wild type (Nossen), lower panels: *atbrca2a-1/atbrca2b-1* double mutant plants] after γ-irradiation (0, 300, 600 Gy).

Seeds were irradiated with γ-rays as described in a previous study (Osakabe *et al.*, 2005). Prior to γ-irradiation, seeds were imbibed in water for 4 d at 4 °C. The seeds were then irradiated with ^{60}Co γ-rays at doses ranging from 100 to

700 Gy and immediately plated on MS agar medium. Four days after irradiation, germination rates were measured. Germination rates after each dose of γ-irradiation varied from 90–100% for wild type (Nossen), *atbrca2a-1*, *atbrca2b-1*, and *atbrca2a-1/atbrca2b-1* plants. Ten days after γ-irradiation, the plants were scored for the appearance of true leaves. Following doses of γ-irradiation of 400–600 Gy, both *atbrca2a-1* and *atbrca2b-1* were hypersensitive to γ-irradiation compared to wild-type (Nossen) regarding the formation of new leaves (Fig. 1B). Moreover, the *atbrca2a-1/atbrca2b-1* double mutant showed an additive increase in sensitivity to γ-irradiation compared to that of the single mutants (Fig.

1B, C). These data support the results of our sensitivity to cisplatin test.

Morphology of atbrca2a-1, atbrca2b-1, and atbrca2a-1/atbrca2b-1 mutants

Both *atbrca2a-1* and *atbrca2b-1* single mutant and homozygous *atbrca2a-1/atbrca2b-1* double mutant plants showed substantially normal vegetative development when compared to wild-type plants (Nossen) (cf. Fig. 2A, C), although abnormal phyllotaxy was occasionally observed (Figs 2B, D, 3). In addition, these mutant plants were
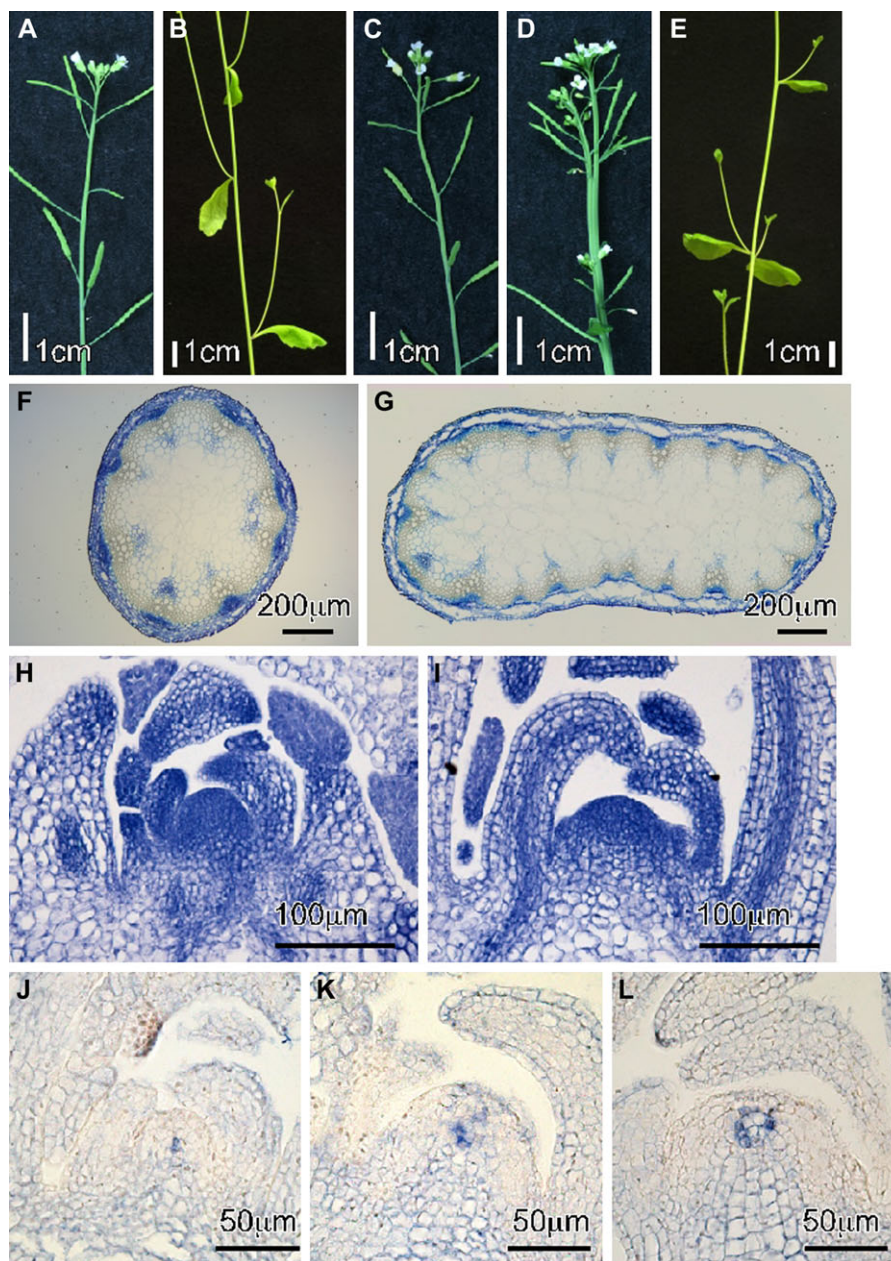

Fig. 2. Morphological phenotypes of *atbrca2* mutants. (A, B) Five-week-old wild-type plants (Nossen), (C, D, E) *atbrca2a-1/atbrca2b-1* double mutant plants. (F, G) Cross-sections of stems from 5-week-old plants: wild type (Nossen) (F) and *atbrca2a-1/atbrca2b-1* double mutant (G). (H, I) Longitudinal-sections of 2-week-old plants: wild type (Nossen) (H) and *atbrca2a-1/atbrca2b-1* double mutant plants (I). (J, K, L) *In situ* hybridization analysis of the *AtWUS* gene in wild-type (Nossen) (J) and *atbrca2a-1/atbrca2b-1* double mutant (K, L) plants.

fertile. By contrast, Siaud *et al.* (2004) reported that most plants transformed with an *AtBRCA2*–RNAi construct under the control of the *DMC1* promoter were affected in their fertility (Siaud *et al.*, 2004). Although at first glance this report appears inconsistent with our observations, if our mutants are partially deficient mutants, it is possible for the *atbrca2a-1*/*atbrca2b-1* double mutant to be partially fertile. However, all our *atbrca2* single and double mutants were as fertile as the wild type (data not shown). Next, to assess pollen grain viability, anthers were dissected from wild-type (Nossen) and mutant flower buds and stained with Alexander's solution (Alexander, 1969). As shown in Supplementary Table S1 and Fig. S3A at *JXB* online, more than 95% of wild-type anthers were full of red-coloured, viable pollen grains. On the other hand, although about 80% of double mutant anthers were similar to that of the wild type, about 20% of anthers in double mutant plants contained many green-coloured, non-viable pollen grains (see Supplementary Fig. S3B at *JXB* online). These results suggested that our *atbrca2a-1*/*atbrca2b-1* double mutant is partially defective in male gamete development, but that this defect does not affect overall fertility.

Homozygous *atbrca2a-1*/*atbrca2b-1* plants grew a little slower than the wild type (Nossen) (data not shown), and showed stem fasciation (flattened and thick stems and fused organs; Fig. 2D, G) and/or abnormal phyllotaxy (resulting in an irregular branching pattern; Fig. 2E) in approximately 5–40% of the double mutant plants. Figure 2G shows a cross-section of the fasciated stem of the double mutant. The fasciation phenotype represents disintegration in the pattern of organogenesis and is associated with an enlargement of the shoot apical meristem (SAM) (Leyser and Furner, 1992). In double mutant plants, the SAM occasionally developed into an enlarged mass compared to wild-type (Fig. 2H, I). Fertility of the double mutant plant was similar to that of the wild type and corresponding single mutant plants.

γ-Ray-induced fasciation phenotype in atbrca2 mutants

The long-term effects of γ-irradiation on development in *atbrca2* mutants transplanted in soil were investigated further. After high doses of γ-irradiation, although seedling growth of the wild type (Nossen), and *atbrca2* single and double mutants was retarded (Fig, 1B, C), all these seedlings were able to survive and growth of these plants recovered (approximately 40–70% of control). One month after irradiation, shoot meristems underwent a transition from vegetative to inflorescence development, and the plants formed stems and floral meristems. It was found that γ-irradiation (600 Gy) accelerated the stem fasciation phenotype (including abnormal phyllotaxy) in *atbrca2a-1* and *atbrca2b-1* single mutants and in the double mutant plants (Nossen) (Fig. 3). Most interestingly, these phenotypes were also induced by γ-irradiation in wild-type plants (Nossen) (Fig. 3). Without γ-irradiation, the double mutant exhibited stem fasciation in 6.6% and 40% of plants transplanted to soil in the first and second experiments, re-
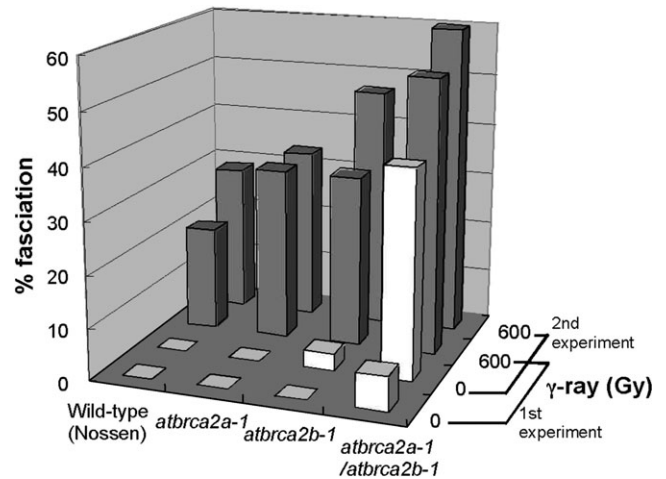
Fig. 3. γ-irradiation induces fasciation in wild-type (Nossen), *atbrca2a-1* and *atbrca2b-1* single mutants, and the *atbrca2a-1*/ *atbrca2b-1* double mutant.

spectively. Thus, the proportion of plants exhibiting stem fasciation differed markedly between the first and second experiments. Although every attempt was made to grow all the *Arabidopsis* plants in both experiments under identical conditions, even with sophisticated environmental control systems, subtle variations in conditions may occur. It is possible that such slight variations could have an effect on the ratio of plants that show stem fasciation. When imbibed seeds of the double mutant plants were irradiated with a 600 Gy dose of γ-rays, the frequency of stem fasciation increased by more than 20%. The frequency of stem fasciation was further increased by irradiation with a γ-ray dose of 700 Gy (data not shown). Thus, the proportion of plants showing stem fasciation increased with γ-irradiation in a dose-dependent manner. Each single mutant also showed stem fasciation at a low frequency without γ-irradiation, while γ-irradiation induced stem fasciation. Importantly, in wild-type plants, the frequency of stem fasciation was very low (<0.1%) under the growth conditions used, but these phenotypes were induced by γ-irradiation. In this study, the Nossen ecotype was used as a control plant since the mutant background is the Nossen ecotype. Imbibed seeds of several other ecotypes were also irradiated, and it was found that Nossen was one of ecotypes with relatively higher sensitivity to γ-irradiation. Ecotype Columbia was less sensitive to γ-irradiation than the other ecotypes (data not shown). These data suggest that the fasciation phenotypes in the *atbrca2* single and double mutants could be linked to sensitivity to γ-irradiation.

To elucidate the relationship between the fasciation phenotype and the sensitivity to γ-irradiation further in the *atbrca2a-1*/*atbrca2b-1* double mutant, *in situ* hybridization analysis of the *WUSCHEL* (*WUS*) gene was performed with wild-type (Nossen) and *atbrca2a-1*/*atbrca2b-1* plants. WUS is one of the regulatory factors required for maintenance of stem cell identity, and the *WUS* gene is expressed in a small group of cells in the centre of the SAM (Mayer *et al.*, 1998). It was found that the region in which the *WUS*

gene is expressed expanded laterally but not uniformly in double mutant plants (Fig. 2K, L). Occasionally, the *WUS* expression region was also shifted to the peripheral zone in the double mutant. These observations suggested that the defect in DSB repair in the *atbrca2a/atbrca2b* double mutant might lead to SAM disorganization via ectopic expression of the *WUS* gene.

Cell cycle regulation in atbrca2 *mutants*

Previous studies have indicated that cell cycle control is essential for normal development of the apical meristem in plants (Endo *et al.*, 2006; Andersen *et al.*, 2008). To study the relationship between disorganization of meristem structure and cell cycle progression, especially tissue-specific effects, expression of the *AtCYCB1;1::GUS* repoter gene was examined. In this fusion construct, transcription of the GUS gene is driven by a *AtCYCB1;1* promoter, and GUS protein degradation is controlled by the Cyclin B mitotic destruction sequence (Colon-Carmona *et al.*, 1999). This GUS fusion protein accumulates on entry into G_2, and is degraded just before anaphase during mitosis. Thus, products of this GUS fusion gene accumulate from late G_2 until M phase (Colon-Carmona *et al.*, 1999; Culligan *et al.*, 2004, 2006). *Arabidopsis* Col plants transformed with *AtCYCB1;1::GUS* (Colon-Carmona *et al.*, 1999) were crossed with the *atbrca2a-1/atbrca2b-1* double mutant. As shown in Figs 4 and 5, cells expressing GUS activity appeared sporadically only in the root tip of wild-type plants without any treatment. By contrast, significantly large numbers of cells in the shoot apex, young leaves, and root tips of the *atbrca2a-1/atbrca2b-1* double mutant showed strong GUS activity (Figs 4, 5). These results suggest that disorganization of the meristem structure is correlated with the defect in cell cycle progression in the *atbrca2a-1/atbrca2b-1* double mutant. In addition, *AtBRCA2–RNAi* construct was transformed to *Arabidopsis* Col plants with *AtCYCB1;1::GUS*. *AtBRCA2–RNAi* plants show strong GUS activity in the shoot apex, young leaves, and root tips (see Supplementary Fig. S4 at *JXB* online). This result supports the hypothesis that the strong expression of AtCYCB1;1 in meristematic tissues of the double mutant is associated with the *Ds* insertion.

Next, the effect of the *atbrca2* mutation on cell cycle regulation in response to aphidicolin was studied. Aphidicolin is an inhibitor of the replicative DNA polymerases δ and ε. As shown in Fig. 4, meristematic cells of shoots and roots in aphidicolin-treated wild-type and *atbrca2a-1/atbrca2b-1* double mutant plants displayed a substantial increase in the number of *AtCYCB1;1::GUS* expressing cells (Fig. 4). This property is especially pronounced in the *atbrca2a-1/atbrca2b-1* double mutant (Fig. 4). These results indicate that cell cycle progression of dividing cells in wild-type plants was delayed due to the replicational stress caused by aphidicolin, and that the brca2 mutation exacerbates this effect.

In addition, *AtCYCB1;1* is strongly induced by DNA damaging agents (Culligan *et al.*, 2006; Endo *et al.*, 2006). The induction of *AtCYCB1;1* in response to cisplatin in the *atbrca2a-1/atbrca2b-1* double mutant compared to that of
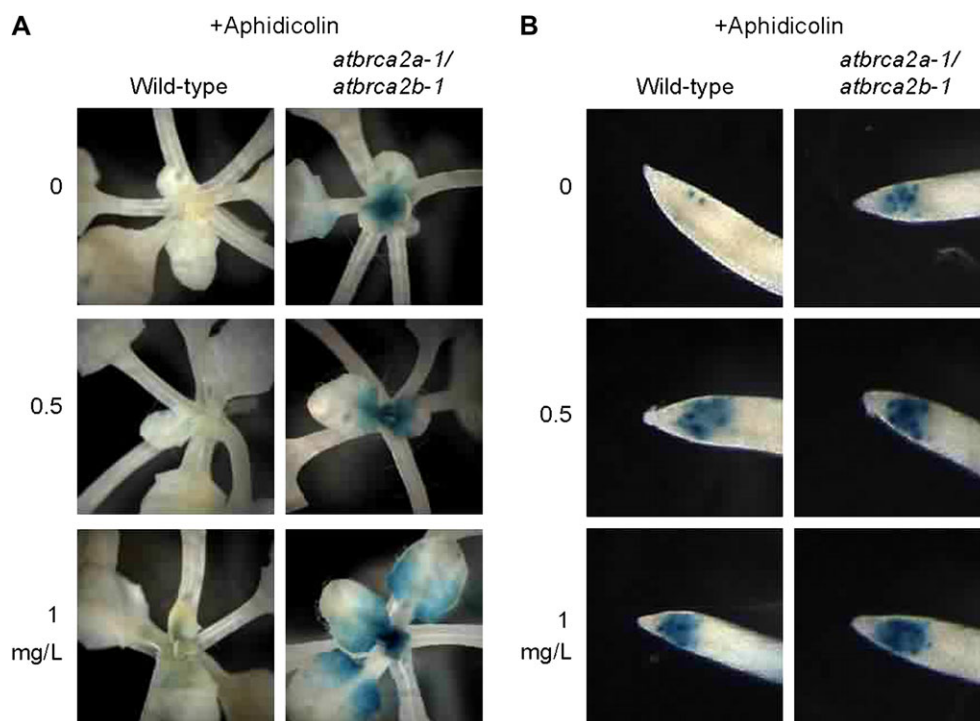
Fig. 4. Histochemical assay of the AtCYCB1;1::GUS reporter gene (Colon-Carmona *et al.*, 1999) in 7-d-old wild-type and *atbrca2a-1/atbrca2b-1* mutant seedlings with or without aphidicolin (A, B). GUS activity was detected after 3 d of treatment with aphidicolin. (A) GUS staining of SAMs and leaves. (B) GUS staining of root tips.
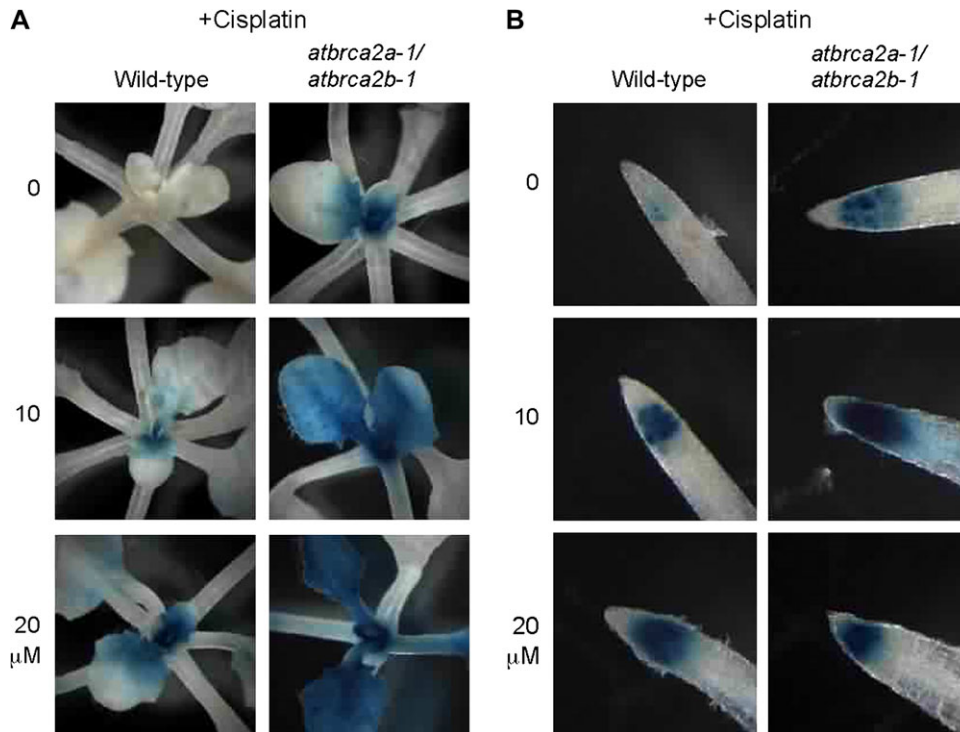
Fig. 5. Histochemical assay of the AtCYCB1;1::GUS reporter gene (Colon-Carmona *et al.*, 1999) in 7-d-old wild-type and *atbrca2a-1/ atbrca2b-1* mutant seedlings with or without cisplatin (A, B). GUS activity was detected after 3 d of treatment with cisplatin. (A) GUS staining of SAMs and leaves. (B) GUS staining of root tips.

the wild type was also studied. As in the case of aphidicolin treatment, double mutant plants particularly displayed an increased level of *AtCYCB1;1::GUS*-expressing cells in SAMs (Fig. 5). These results suggested that *AtCYCB1;1* is strongly induced by cisplatin in wild-type plants and that the *AtBRCA2* mutation enhances this induction. As described above, cisplatin is a cross-linking reagent that forms DNA intra- and inter-strand cross-links (Zamble and Lippard, 1995). Inter-strand cross-links are repaired exclusively by HR, and not by NHEJ (Essers *et al.*, 2000; De Silva *et al.*, 2002; Sasaki *et al.*, 2004). Particularly in the S phase of the cell cycle, inter-strand cross-links are repaired by HR using sister chromatids as homologous templates. The brca2 mutation may limit this step in plant meristems.

Discussion

Previously, Siaud *et al.* (2004) reported the role of AtBRCA2a and AtBRCA2b in meiotic recombination in *Arabidopsis.* However, the function of AtBRCA2 in HR repair, especially in somatic cells, was not determined. In this study, it has been shown that AtBRCA2a and AtBRCA2b are important for DSB repair in somatic cells. *atbrca2a-1* and *atbrca2b-1* single mutants exhibited hypersensitivity to genotoxic stresses compared to wild-type plants, and the *atbrca2a-1/atbrca2b-1* double mutant showed an additive increase in sensitivity to genotoxic stresses compared to each single mutant (Fig 1). These results suggested that AtBRCA2a and AtBRCA2b play

essential roles in DNA repair in somatic cells. Siaud *et al.* (2004) reported that most plants transformed with an *AtBRCA2*–RNAi construct under the control of a constitutive plant promoter (p35S) became bleached and died, thus indicating that *AtBRCA2* genes are necessary for plant survival. On the other hand, our *atbrca2a-1/atbrca2b-1* double mutation is not lethal. In addition, AtBRCA2–RNAi plants were obtained. Taken together, these observations suggest that the production and/or activity of AtBRCA2a and AtBRCA2b are partially deficient in our mutants and that the silencing of both *BRCA2* genes also defective in our *AtBRCA2*–RNAi plants.

In addition, it was found that *atbrca2* mutant plants displayed fasciation and abnormal phyllotaxy phenotypes. Bundock and Hooykaas (2002) identified mutants of the *Arabidopsis MRE11* gene. One of these mutants, *mre11-1*, is hypersensitive to the alkylating reagent methyl methane sulphonate (MMS) and shows fasciation (Bundock and Hooykaas, 2002). On the other hand, mutants impaired in the NHEJ repair pathway [*ku70* (Riha *et al.*, 2002), *ku80* (Friesner and Britt, 2003; Gallego *et al.*, 2003; West *et al.*, 2002), *ligIV* (Friesner and Britt, 2003; van Attikum *et al.*, 2003)] do not cause fasciation phenotypes. Indeed, it was confirmed that abnormal phyllotaxy and stem fasciation were not increased in the *ku80* mutant (WS ecotype background; West *et al.*, 2002) compared to the wild type, with or without γ-irradiation. Thus, in contrast to the HR repair pathway, inefficient DSB repair via NHEJ does not seem to induce disorganization of apical meristem cells. Therefore, the fasciation phenotype induced by γ-irradiation

may be associated with the type of repair system, and may also depend on the cell cycle because NHEJ repair occurs during G_1 to early S-phase, and HR during late S to G_2 phase (Essers *et al.*, 2000; Couedel *et al.*, 2004; Bleuyard *et al.*, 2006).

Moreover, it was found that the ratio of plants displaying fasciation and abnormal phyllotaxy phenotypes increased upon γ-irradiation in the *atbrca2* mutant. Interestingly, these phenotypes were also induced by γ-irradiation in wild-type plants (Nossen). These results suggest that the fasciation phenotypes in the *atbrac2* single and double mutants could be linked to sensitivity to γ-irradiation. The *clv1* and *clv3* mutants of *Arabidopsis* are well known as showing stem fasciation. The *CLAVATA3* (*CLV3*) gene encodes a putative ligand for a transmembrane receptor kinase, CLAVATA1 (CLV1). Reddy *et al.* (2005) reported that a *CLV3* RNAi construct induced by dexamethasone also shows stem fasciation (Reddy and Meyerowitz, 2005). Such reports indicate that developmental programmes in plant SAMs can be changed by exogenous factors during development. This notion is in agreement with our results, which clearly demonstrate that developmental programmes in *Arabidopsis* SAMs are changed by γ-irradiation as an exogenous factor.

In our results, a large number of cells in the shoot apex, young leaves, and root tips of the *atbrca2a-1/atbrca2b-1* double mutant showed strong GUS activity upon expression of *AtCYCB1;1::GUS*, whereas cells expressing GUS activity appeared sporadically only in the root tip of wild-type plants (Figs 4, 5). These results indicate that cell cycle progression is important for the regulation of the pattern of cell division and differentiation in plant development. Other studies support our results. For example, *Arabidopsis fas1* and *fas2* mutants show stem fasciation (Kaya *et al.*, 2001)—FAS1 (CAF-1 p150) and FAS2 (CAF-1 p60) are subunits of the chromatin assembling factor complex (Smith and Stillman, 1989). The absence of CAF-1 could cause the delay or down-regulation of chromatin assembly. Delayed chromatin assembly might lead to a prolongation of the cell cycle at the S or G_2/M phase. Recently, it was found that *fas1* and *fas2* mutants exhibited an increased level of DSBs and G_2 phase retardation (Endo *et al.*, 2006). Our report suggested that DSBs were induced during the late S to G_2 phase of the cell cycle in these *fas* mutants. In *teb* mutants, whose causative gene encodes a homologue of *Drosophila* MUS308 and mammalian DNA polymeraseθ, morphological defects such as fasciation, serrated leaves, and short roots are observed (Inagaki *et al.*, 2006). These mutants also show a defect in G_2/M cell cycle progression. The mutation and suppression of condensin (*AtCAP-E1* and *AtCAP-E2*), which is involved in chromatin condensation, also causes stem fasciation. Although the functions of AtCAP-E1 and AtCAP-E2 in DSB repair and cell cycle progression have not been determined (Siddiqui *et al.*, 2003), studies in yeast indicate that condensin is required for the arrest caused by DNA replication inhibition, the activation of checkpoints, and DNA repair (Aono *et al.*, 2002). Recently, Andersen *et al.* (2008) demonstrated that CDKB2;1 and CDKB2;2 are

necessary both for cell cycle progression and for meristem organization. Plants transformed with microRNAs of both CDKB2;1 and CDKB2;2 genes show dwarfism, abnormal structure of the shoot meristem and phyllotaxis defects (Andersen *et al.*, 2008). These results indicate directly that progression of the cell cycle is linked closely to the regulation of meristem organization in plants. Reidt *et al.* (2006) demonstrated that BARD1 plays an important role in the regulation of DNA repair in somatic cells in *Arabidopsis* (Reidt *et al.*, 2006). Interestingly, Han *et al.* (2008) suggested that BARD1 regulates SAM organization and maintenance by limiting expression of the *WUS* gene in plants (Han *et al.*, 2008). Although the latter authors did not discuss any relationship between SAM organization and the cell cycle, it is likely that cell cycle regulation is involved in the regulation of SAM organization by BARD1.

In conclusion, our results demonstrate that AtBRCA2a and AtBRCA2b are important for DSB repair in somatic cells. In addition, extrinsic DSBs and inefficient repair of DSBs induce an abnormal morphological phenotype in *Arabidopsis*, including stem fasciations. Moreover, disorganization of the meristem structure is connected to the defect in cell cycle progression in *Arabidopsis* plants.

Supplementary data

The following supplementary data relating to this study are available at *JXB* online.

Supplementary Fig. S1. *Ds*-transposon insertion sites of *atbrca2* mutants.

Supplementary Fig. S2. Sensitivity to cisplatin of heterozygous plants of both *AtBRCA2a* and *AtBRCA2b* genes, and *AtBRCA2*–RNAi plants.

Supplementary Fig. S3. Anthers of wild-type (A) and *atbrca2a-1/atbrca2b-1* double mutant (B) stained with Alexander's solution (Alexander, 1969).

Supplementary Fig. S4. Histochemical assay of the AtCYCB1;1::GUS reporter gene (Colon-Carmona *et al.*, 1999) in 7-d-old wild-type, *atbrca2a-1/atbrca2b-1* mutant and *AtBRCA2*–RNAi seedlings.

Supplementary Table S1. Pollen grain viability in *atbrca2* mutants.

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