Homology-dependent repair is involved in 45S rDNA loss in plant CAF-1 mutants

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SUMMARY

Arabidopsis thaliana mutants in FAS1 and FAS2 subunits of chromatin assembly factor 1 (CAF1) show progressive loss of 45S rDNA copies and telomeres. We hypothesized that homology-dependent DNA damage repair (HDR) may contribute to the loss of these repeats in fas mutants. To test this, we generated double mutants by crossing fas mutants with knock-out mutants in RAD51B, one of the Rad51 paralogs of A. thaliana. Our results show that the absence of RAD51B decreases the rate of rDNA loss, confirming the implication of RAD51B-dependent recombination in rDNA loss in the CAF1 mutants. Interestingly, this effect is not observed for telomeric repeat loss, which thus differs from that acting in rDNA loss. Involvement of DNA damage repair in rDNA dynamics in fas mutants is further supported by accumulation of double-stranded breaks (measured as γ-H2AX foci) in 45S rDNA. Occurrence of the foci is not specific for S-phase, and is ATM-independent. While the foci in fas mutants occur both in the transcribed (intranucleolar) and non-transcribed (nucleoplasmic) fraction of rDNA, double fas rad51b mutants show a specific increase in the number of the intranucleolar foci. These results suggest that the repair of double-stranded breaks present in the transcribed rDNA region is RAD51B dependent and that this contributes to rDNA repeat loss in fas mutants, presumably via the single-stranded annealing recombination pathway. Our results also highlight the importance of proper chromatin assembly in the maintenance of genome stability.

Keywords: DNA repair, genome instability, 45S rDNA, chromatin assembly factor 1, Arabidopsis thaliana, FAS1, FAS2, RAD51B.

INTRODUCTION

Chromatin assembly factor 1 (CAF1) is a highly conserved heterotrimeric chaperone complex that facilitates nucleosome assembly by the formation and recruitment of (H3–H4)2 histone tetramers onto nascent DNA (Smith and Stillman, 1989, 1991; Winkler et al., 2012). The plant CAF1 is composed of three subunits: FASCIATA 1 (FAS1), FAS2 and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) (Kaya et al., 2001). Arabidopsis mutants in FAS1 and FAS2 subunits are viable, but show stem fasciation, abnormal leaf and flower morphology and disorganization of apical meristems (Reinholz, 1966; Leyer and Furner, 1992; Kaya et al., 2001). Among particular cell cycle-related defects reported in the fas mutants (reviewed in Ramirez-Parra and Gutierrez, 2007b) were 40-fold increased levels of homologous recombination (HR) (Takeda et al., 2004; Endo et al., 2006a, b; Kirik et al., 2006) and a slower progression of the S-phase (Schonrock et al., 2006; Ramirez-Parra and Gutierrez, 2007a). In addition, we observed that fas mutants showed progressive and specific loss of 45S rDNA and telomeres (Mozgova et al., 2010). These results suggested that stable maintenance of telomeres and 45S rDNA is particularly sensitive to disruption of replication-dependent chromatin assembly. We speculated that the specific sequence loss may be associated with the fact that both...
45S rDNA and telomeres are problematic templates for replication and thus may require additional steps and factors beyond those essential for general genomic DNA replication. In addition to their repetitive nature and potential to form specific local DNA and nucleoprotein structures (G4-structures, telomeric loops, replication fork barriers), interference between replication and transcription and formation of DNA-RNA hybrid structures (R-loops) are features of these loci that may contribute to the increased tendency for replication fork stalling (Hernandez et al., 1993; Lopez-Estrano et al., 1998, 1999; Boule and Zakian, 2006; Gilson and Geli, 2007; Buonomo et al., 2009; Sfeir et al., 2009; Bosco and de Lange, 2012; Stewart et al., 2012; Kasbek et al., 2013).

Although it is not clear whether 45S rDNA and telomere repeats are lost by independent pathways or through a common mechanism, our recent study has shown that telomere loss involves a telomerase-independent mechanism, which thus may be common to that of the loss of 45S rDNA (Jaske et al., 2013). Illegitimate HDR represents a major source of genomic instability throughout eukaryotic kingdoms (El Hage et al., 2006; Gilson and Geli, 2007; Buonomo et al., 2009; Sfeir et al., 2009; Bosco and de Lange, 2012; Stewart et al., 2012; Kasbek et al., 2013).

Our previous results showed progressive transgenerational loss of 45S rDNA and telomeres in fas mutants (Mozgova et al., 2010; Pontvianne et al., 2013). To address the question of whether this loss is mitotic or requires passage through meiosis, amounts of 45S rDNA and length of telomeres were measured in callus cultures derived from 1-week-old homozygous fas1 or fas2 second generation (G2) mutant plants (Figure 1). While telomere length increased in wild type derived callus cells [corresponding to our earlier findings (Riha et al., 1998)], we observed progressive telomere shortening in fas-derived calli. Quantification of 45S rDNA confirmed the decrease in copy number of the repeats (compared to parental G2 plants), clearly visible in the first passage (P1) and rapidly progressing until P3 of the callus cultures. After P3, the 45S rDNA levels reach a steady-state level corresponding approximately to that observed in G5 or later generations of the corresponding mutant plants. This supports our previous observation that 2-week-old plants of either fas1 or fas2 genotype showed higher numbers of 45S rDNA copies than the same plants analysed at 5 weeks (Mozgova et al., 2010). These results thus show that the loss of 45S rDNA and telomeres is mitotic and that passage through meiosis is not required in order to reach the low copy-number state observed in late generation fas mutants.

Instability of rDNA and telomeres

If HDR plays role in the instability of repeats in fas, absence of RAD51 paralogs acting during somatic HDR would be expected to impede the loss. The rad51b mutant was thus chosen to investigate the possible involvement of HDR in the rDNA repeat loss in fas mutant plant cells. fas1 or fas2 plants were crossed with rad51b mutants (Experimental procedures section). Three heterozygous plants were obtained from each of four independent crosses and wild-type (WT) (FAS1/2 RAD51B), fas (fas1/2 RAD51B), rad51b (FAS1/2 rad51b) and double mutant fas rad51b (fas1/2 rad51b) plants were selected from the F2 progeny (Figure S1).

Testing the effect of absence of RAD51B on rDNA and telomere instability shows that the absence of RAD51B partially suppresses the loss of 45S rDNA in fas1 or fas2 mutants (Figure 2a,b, respectively). This effect is not due to RAD51B alone as the rad51b knock-out alone does not rather minimizes/prevents further loss; and (iii) no change to repeat copy number, showing that the loss is independent on RAD51B-mediated HDR. The results presented here confirm that RAD51B-mediated HDR contributes to the rDNA loss but does not substantially affect telomere shortening in fas mutants.

RESULTS

Loss of 45S rDNA and telomeric repeats is mitotic

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result in any systematic/progressive changes in 45S rDNA copy number in either direction (Figure S2). However, RAD51B-dependent processing of DNA damage sites induced in fas mutants is deletion prone and, furthermore, in the absence of RAD51B there are other means to process the DNA damage which result in less loss of rDNA than the RAD51B-dependent pathway of HDR.

Surprisingly, these effects on rDNA were not observed in the telomeric DNA. The loss of telomere repeats is similar in both double mutant genotypes (fas2 rad51b, and fas1 rad51b) and single (fas1 or fas2) mutants with functional RAD51B (Figures 3 and S3). Also, rad51b knockout alone does not cause any detectable loss of telomeres when FAS is functional. Thus, RAD51B does not appear to act in the reduction of telomere length in fas mutants, implying that the mechanism of telomere loss in fas mutant plants differs from that responsible for the loss of 45S rDNA.

Lack of RAD51B does not increase sensitivity of fas mutants to genotoxic agents nor general levels of genome instability

To investigate a possible contribution of rad51b to the reported increased sensitivity of fas mutants to genotoxic agents (Takeda et al., 2004; Ramirez-Parra and Gutierrez, 2007a), the different fas1/2 rad51b genotype plants were tested for sensitivity to DNA damage (methyl methanesulfonate, MMS) or replication fork retardation/stalling induced by dNTP pool depletion (hydroxyurea, HU). While fas mutants are sensitive to MMS (0.25 mM) (fas2 > fas1), rad51b mutants show no detectable sensitivity at this MMS concentration, and the absence of RAD51B does not increase the MMS sensitivity of fas mutants (in fas rad51b double mutants) (Figure S4). To get a more precise data on the possible MMS effect, the experiment was repeated twice in parallel, each with 40 seedlings. In addition to root length measurements (Figure 4a,b), 18S rDNA copy numbers and levels of the corresponding transcripts were also investigated (Figure 4c,d, respectively). In both fas2 and fas2 rad51b mutants, MMS treatment resulted in a lesser loss of rDNA copies with respect to Murashige and Skoog medium (MS) controls in these mutants, in accord with the slower growth of seedlings in the presence of MMS. Interestingly, notable differences in 18S rDNA transcript levels between fas2 and fas2 rad51b were observed in response to MMS: while both mutants showed a decrease in 18S rDNA transcript levels with respect to the corresponding controls grown on MS (which are similar to the levels in WT plants), absence of RAD51B in fas2 mutants results in noticeable decrease in transcript levels already at 0.25 mM MMS (to approximately 40% of the MS control) and a further decrease at 0.5 mM MMS (to 27% of the MS control). This contrasts with fas2 mutants with functional RAD51B, in which the effect of 0.25 mM MMS is negligible and 0.5 mM MMS decreases transcript levels to approximately 50% of the corresponding MS control.

fas mutants show no detectable sensitivity to 1 mM HU at the tested time (10 days after germination [DAG]) as previously described (Ramirez-Parra and Gutierrez, 2007a), and no increase in sensitivity was seen in the absence of RAD51B (fas2 rad51b) after HU treatment. Although higher

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Figure 1. The loss of 45S rDNA and telomeres is mitotic. Telomere lengths (a) and copy numbers of 45S rDNA (b, c) were measured in consecutive passages (P1-P9) of callus cultures derived from the second generation of fas1 or fas2 mutant plants as indicated. Data obtained in G2 and G5 generations of mutant plants are shown for comparison. Relative content of 45S rDNA is expressed in arbitrary units (a.u.) with respect to the level in WT plants with no mutational history.
HU concentrations (2.5 mM) do affect growth, they show similar effects on fas mutants and WT seedlings (Figure S5).

The results indicate that RAD51B is not essential in DNA damage response stimulated by MMS in fas1/2. However, MMS induces a drop in 18S rDNA transcript levels in fas mutants, and RAD51B function mitigates this effect. HU-induced fork stalling in fas does not affect root length when compared with WT, and the same holds true in the absence of RAD51B in the fas background.

The occurrence of mitotic anaphase bridges was assessed in the fas and rad51b plants to check for effects on the general level of genome instability in these plants (Table 1). Both rad51b and fas1 mutant plants have increased numbers of bridges (0.3% of anaphase nuclei with a visible bridge) compared to WT plants (0.07%), however no further increase was observed in double fas1 rad51b mutants. With 1.1% nuclei with a visible bridge, fas2 plants have a higher level of bridges than fas1 (or rad51b) mutants, but again, the absence of RAD51B does not further increase this in the double fas2 rad51b mutant. We note that the observed effects are mild and caution is needed in drawing conclusions, but the absence of additive effects in the fas2 rad51b mutant implies that these two proteins are affecting the same process.

RAD51B participates in intranucleolar DNA damage repair

An active role of RAD51B (and thus of RAD51-dependent HDR) in the loss of rDNA copies in fas mutants, implies activation of DNA damage signalling in these sequences and this can be quantified by counting numbers of γ-H2AX foci. γ-H2AX foci were observed in both fas1 and fas2 mutant plants, while no foci were detected in WT control plants as expected (Figure 5a). That this is a reflection of a DNA damage and recombination processes is supported by the further increase in numbers of γ-H2AX foci observed in the double fas1 rad51b and fas2 rad51b plants compared to the single fas mutants. As expected (Da Ines et al., 2013), rad51b mutant controls showed only rare γ-H2AX foci (mean of 0.06 foci/nucleus), similar to the WT plants. Given our previous results (Pontvianne et al., 2013) showing that both originally active and originally inactive rDNA copies are lost in the fas mutants, we extended this analysis by classifying the γ-H2AX foci as intra- or extra-nucleolar. This classification showed that 55% of the foci are intranucleolar in both fas1 and fas2 plants, suggesting that both active (intranucleolar) and inactive (extra-nucleolar) 45S rDNA copies are affected to a similar extent in fas mutants.

Figure 5(b) shows that absence of RAD51B leads to significant increases of numbers of γ-H2AX foci in both fas1 and fas2 mutant plants. This increase presumably reflects a slower repair of DNA damage events in fas mutants upon the loss of the deletion-prone RAD51B-dependent pathway. Strikingly, this increase is due to higher numbers of intranucleolar foci in all cases, with no observed increases in numbers of extra-nucleolar foci. Again, this strongly implicates effects on rDNA loci, and specifically on active rDNA copies (including originally inactive rDNA copies which became activated to compensate for the lost active copies). Blocking HDR in fas mutants thus specifically increases numbers of intranucleolar γ-H2AX foci. To confirm and extend this observation, we carried out combined γ-H2AX immunodetection and 45S rDNA fluorescence in situ hybridization (FISH) analyses on these plants. The results of these analyses (Figure 6) show clearly that a majority of foci co-localize with rDNA in both fas1 (69%) and fas1-rad51b mutants (67%). Co-localization with rDNA appears
weaker in non-nucleolar foci when compared to nucleolar foci (62.5% versus 77% in \textit{fas1}, and 54.2 versus 79.2% in \textit{fas1rad51b} mutants, respectively – see also Table S1).

We note that no correspondence was observed between rDNA copy number and the numbers of \textit{\textgamma}-H2AX foci in plants with different total content of rDNA (Figure 5b). This concords with the notion that \textit{\textgamma}-H2AX foci arise mostly in transcriptionally active rDNA copies, and this portion is relatively stable (contrary to the total copy number) in the analysed lines to ensure comparable level of 45S rDNA transcription in the \textit{fas} mutants and WT plants (Mozgova et al., 2010).

\textit{\textgamma}-H2AX foci are ATM-independent and occur independently of ongoing replication

The possibility that DNA breaks in the nucleolus occur in connection with rDNA replication was examined by determining the number of nucleolar \textit{\textgamma}-H2AX foci in S-phase nuclei. An EdU incorporation pulse was used to label S-phase nuclei and distributions of nucleolar and non-nucleolar \textit{\textgamma}-H2AX foci in EdU-positive nuclei were compared to the distribution in the population as a whole. As seen in Figure 7, no enrichment of nucleolar \textit{\textgamma}-H2AX foci is observed in EdU-positive nuclei, arguing against an origin due to problems of rDNA replication in these plants.

**Figure 3.** Loss of telomeric DNA repeats in \textit{fas1} (a, b) and \textit{fas2} (c, d) mutants with either functional or dysfunctional RAD51B gene.

Telomere lengths were measured by TRF analysis in F2, F3 and F5 generations of plants segregated from crossing between \textit{fas1/2} and \textit{rad51b} mutants, and mean telomere lengths with corresponding error bars were plotted to the diagrams depicted in panels (a) and (c). Corresponding generations of parental mutant plants were analysed in parallel together with Col0 WT plants without a mutation history. Source TRF patterns of the last generation are shown in panels (b) and (d), data from the earlier generations are shown in Figure S3.
Loss of RAD51B neither shows sensitivity to MMS, nor increases sensitivity of fas mutants to MMS (panels (a) and (b), respectively). 18S rDNA copy numbers and transcript levels are shown in panels (c) and (d), respectively.

Figure 4. Sensitivity of seedlings to MMS.
Loss of RAD51B neither shows sensitivity to MMS, nor increases sensitivity of fas mutants to MMS (panels (a) and (b), respectively). 18S rDNA copy numbers and transcript levels are shown in panels (c) and (d), respectively.
H2AX phosphorylation has been clearly shown to depend upon ATM (Ataxia Telangiectasia Mutated) and/or ATR (ATM and Rad3-related) in Arabidopsis (Friesner et al., 2005; Amiard et al., 2010, 2011). To identify which of these two kinases is responsible for phosphorylation of H2AX in fas mutants, we employed the ATM inhibitor, IATM, which we have previously shown to inhibit the ATM kinase in Arabidopsis (Amiard et al., 2010). The results presented in Figure 8 show clearly that inhibition of the ATM kinase does not affect numbers of \(\gamma\)-H2AX foci in WT, fas1 and fas1rad51b mutant plants.

**DISCUSSION**

The reduction of rDNA loss in fas mutants in the absence of RAD51B indicates that RAD51B-dependent recombination is involved in the HDR of the rDNA damage induced in the fas mutants. It was shown recently in *A. thaliana*, that the RAD51 paralogs RAD51B, RAD51D and XRCC2

| Genotype           | Total number of anaphases | Number of anaphase bridges | Total % |
|--------------------|---------------------------|----------------------------|---------|
| FAS1-4RAD51B       | 1446                      | 1                          | 0.07    |
| fas1-4RAD51B       | 992                       | 3                          | 0.3     |
| FAS1-4rad51B       | 1306                      | 4                          | 0.3     |
| fas1-4rad51B       | 2479                      | 6                          | 0.24    |
| fas2-4RAD51B       | 1174                      | 13                         | 1.1     |
| fas2-4rad51B       | 1264                      | 13                         | 1       |

**Figure 5.** \(\gamma\)-H2AX foci in fas and fas rad51b mutant plants. 
(a) Immunofluorescence of root tip interphase nuclei indicates \(\gamma\)-H2AX foci formation in fas1 and fas1 rad51b mutants. DNA is stained with DAPI (blue), \(\gamma\)-H2AX foci are colored in green and merged images overlay \(\gamma\)-H2AX foci onto chromosomes. Scale bar, 2 \(\mu\)m. An example of a \(\gamma\)-H2AX focus located outside the nucleolus is indicated by a white arrow in the fas1 mutant and an example of a focus located inside the nucleolus is indicated by a yellow arrow in the fas1 rad51b mutant. 
(b) Graphical representation of the number of intra- and extra-nucleolar \(\gamma\)-H2AX foci counted in WT, rad51b, fas1, fas1 rad51b, fas2 and fas2 rad51b plants of third and fifth mutant generations in plants with low or high number of rDNA repeats (indicated above the graph). Mean values are from counting foci of 100 interphase nuclei. Error bars indicate standard error.
participate not only in RAD51-dependent HR, but surprisingly also in single-strand annealing (SSA) recombination (Serra et al., 2013), possibly through participation in the annealing of the exposed repeat sequences flanking the DSB. The SSA mechanism of HDR promotes recombination between direct tandemly repeated DNA sequences flanking a double-stranded break and has been shown previously to mediate chromosome fusions following telomere loss (Wang and Baumann, 2008). In contrast to RAD51-dependent HR, SSA does not involve DNA-strand invasion and has been shown to be independent of RAD51. Consistent with the SSA model and experimental data, this kind of homology-dependent DSB repair pathway leads to deletion of the DNA sequence lying between the flanking repeats and thus at least one of the repeated homologous sequences (for reviews see Heyer et al., 2010; Krogh and Symington, 2004). In the case of direct tandem repeats, such as telomeric (TTTAGGG)n or rDNA units, the size of deletion per SSA event is thus proportional to the length of the repeating unit (7 bp and 10.7 kb, respectively). This would explain why the contribution of RAD51B to the loss of telomeric repeats is hardly detectable, while it is significant in the case of rDNA. Importantly, the involvement of the SSA pathway in the observed instability of rDNA in fas mutants would also explain the strong bias for deletion of sequences rather than a balance between duplications and deletions, an expectable outcome of RAD51-dependent HR. Another (non-exclusive) possible explanation for this apparent difference in the implication of RAD51B in the loss of 45S rDNA and telomeres may be a general prevention of HDR at telomeres (Palm and de Lange, 2008; Zimmermann et al., 2013). This would accord with our previous results (Jaske et al., 2013) showing that telomeres in fas mutants are functional despite their shortening, and that occurrence of telomeric fusions is relatively low in these plants when compared with early generations of tert mutants with similar telomere lengths.

We note that our data do not show a specific association between ongoing replication and γ-H2AX foci in fas mutants, suggesting rather that these events are linked to the high transcriptional activity of the repeated sequence arrays. Transcriptional activation could be promoted by the loss of CAF-1-dependent replicative deposition of H3.1 whose selective K27 methylation ensures mitotic inheritance of heterochromatin, while the H3.3 variant which is deposited independently of replication and CAF-1 into transcriptionally active regions cannot undergo this modification (Jacob et al., 2014).

The results of analyses of γ-H2AX foci in fas and fas rad51b plants show that they occur both inside and outside

Figure 6. γ-H2AX foci colocalise with 45S rDNA FISH in the fas1 rad51b mutant. (a) Immunostaining and 45S rDNA FISH labelling of root tip nuclei of fas1 and fas1 rad51b mutants. Nuclei were stained with DAPI (blue), γ-H2AX foci are colored in green and FISH signals are colored in red. Images are a single focal plane from a deconvolved three-dimensional image. Bar, 2 μm. (b) Numerical results recapitulating the number and the percentage of co-localization of γ-H2AX foci with the rDNA probe.
of the nucleolus in fas mutants, but are preferentially enriched inside the nucleolus upon RAD51B knock-out in fas plants. This suggests that RAD51B-mediated repair takes place in the nucleolus of fas mutants, again linking to the high transcriptional activity of these sequences. In accordance with this, RAD51B function partially mitigates the observed drop in rDNA transcripts induced in fas mutants by MMS treatment (Figure 4d).

We conclude that a RAD51B-dependent DNA repair process, very possibly SSA, is involved in the observed loss of rDNA sequences in fas mutants, and that due to the very short repeat-length, its contribution to the loss of telomeres is not detectable. This ATM-independent process preferentially acts on transcriptionally active rDNA inside the nucleolus and is associated to the transcriptional activity of these sequences rather than to difficulties in their replication.

Controversial results exist on the presence of nucleosomes on the PolI-transcribed fraction of rDNA (Hamperl et al., 2013). While classical electron microscopic studies suggest a complete removal of nucleosomes at actively transcribed fraction of rDNA genes (Miller and Beatty, 1969), other reports show a remodeled, dynamic, but still nucleosomal arrangement of the transcribed rDNA fraction (Jones et al., 2007), with a lower nucleosome occupancy in the coding region relative to the intergenic spacer (IGS) (Zentner et al., 2011). Our findings of rDNA nucleosomes in MNase-digested nuclei of G4 fas mutants [where the remaining rDNA copies are transcriptionally active (Mozgova et al., 2010)] support rather the latter view, i.e. partial depletion of nucleosomes. While the inactive rDNA fraction remains in the closed chromatin state throughout the cell cycle, the active rDNA fraction switches between the open (outside of S-phase) and closed conformation (in S-phase) (Wittner et al., 2011) to balance between the necessity to supply cell with structural components of the ribosome, and ensuring genome integrity, respectively. Therefore, the requirement of actively transcribed (nucleolar) rDNA fraction for chromatin assembly factors is higher. The active transcription, when combined with the decreased

![Figure 7. γ-H2AX foci formation does not depend on replication.](a) Immunofluorescence of root tip nuclei labelled with EdU showing γ-H2AX foci formation in WT, fas1 and fas1 rad51b replicated nuclei. DNA is stained with DAPI (blue), EdU incorporation in red and γ-H2AX foci in green. Scale bar, 2 μm. (b) Graphical representation of results for the appearance of γ-H2AX foci in total interphase nuclei or in replicating (EdU+) nuclei of fas1 or fas1 rad51b mutant. Mean numbers of foci counted on 100 interphase or S-phase nuclei. Error bars indicate standard error.

![Figure 8. γ-H2AX foci in fas1 and fas1rad51b mutants are ATR dependent.](Graphical representation of the number of γ-H2AX counted inside or outside the nucleolus in WT, rad51b, fas1, fas1 rad51b, fas2 and fas2 rad51b, with or without IATM. Mean numbers of foci counted on 100 interphase nuclei. Error bars indicate standard error.)
EXPERIMENTAL PROCEDURES

Plant material and genotyping of the mutants

All Arabidopsis plants were on a Columbia 0 background (Col 0). The fas1-4 (NASC: N239822, SAIL_662_D10; Exner et al., 2006), fas2-4 (NASC: N532328, SALK_032328 (Exner et al., 2006), and rad51b (NASC: N524755, SALK_024755 (Alonso et al., 2003; Bleu- yard et al., 2005) were used. Double mutants were created by crossing the first generation of fas1 or fas2 plants with third generation of rad51b plants (see Figure S1) in three independent lines. The several heterozygous plants were obtained from each cross (F1 generation) and their progenies were screened for segregated double WT plants (fas1/2RAD51B, fas1/2 (fas1/2RAD51B, rad51b (FAS1/2RAD51b) and double mutant plants (fas1/2rad51b) and then propagated into the F5 consecutive generation. All used seeds were sterilized by washing in 70% ethanol for 10 min and in 99% ethanol for 5 min, stratified for 2 days on 4°C, pre-grown on germination half-strength Murashige and Skoog medium (Duchefa, http://www.duchefa-biochemie.com/) for 2 weeks then moved into the soil and grown under the long day conditions (16 h light at 21°C, 8 h dark at 19°C, with 50–60% relative humidity).

For callus induction, 7 DAG seedlings were placed on callus induction plate containing V4 medium (10 mM KCl, 7 mM NaNO3, 1 mM MgSO4, 1 mM NaH2PO4, 0.5 mM CaCl2, 3.5 μM ZnSO4, 0.4 μM MnSO4, 0.1 μM CuSO4, 16 μM H2BO3, 0.1 μM KI, 0.1 μM AlCl3, 0.1 μM NiCl2, 0.1 mM Na2EDTA, 0.1 mM FeSO4, 0.1 g L−1 inositol, 1 g L−1 yeast extract, 30 g L−1 sucrose, 0.1 mg L−1 NAA, 0.1 mg L−1 2,4-D, 0.01 mg L−1 thiamine, 0.01 mg L−1 pyridoxine, 0.05 mg L−1 nicotinic acid, 0.3 mg L−1 glycine, 0.8% (w/v) plant agar). The induced calli were cultivated at 24°C in the dark and were passaged every 4 weeks onto a new V4 medium plate; the remaining material was collected and DNA was isolated as described (Dellaporta et al., 1983).

DNA extraction and plant genotyping

Plant DNA was extracted from 16-day-old seedlings or 5-week-old leaves in accordance with Dellaporta et al. (1983) and quality of DNA was checked by gel electrophoresis, 0.8% (w/v) in agarose gel stained with ethidium bromide. Genotyping of rad51b locus was assayed using rad51b F: 5′-CCCTGGTGTTGGTTGGGA- TACCTT-3′ and rad51b R: 5′-GGAAACGAGCTGATAACGCA-3′ primers. Mutant rad51b locus was detected using rad51b F and F05: 5′-CAACACTCAACCTTCTCGG-3′ primers. For each polymerase chain reaction (PCR) 0.25 μM primers, 0.5 U MyTaq DNA polymerase (Bioline, http://www.bioline.com/), and 6–8 ng of genomic DNA were used. The conditions of PCR reaction were incubation at 96°C for 2 min, followed by 30 cycles of 95°C for 10 sec, 55°C for 10 sec, and 72°C for 10 sec, with a final incubation at 72°C for 5 min. PCR products were separated by electrophoresis in 1% (w/v) agarose gel stained with ethidium bromide and product lengths were compared with a Gene Ruler 1 kb DNA Ladder (Fermentas, http://www.thermoscientificbio.com/fermentas/).

Quantitative PCR (qPCR) analysis of 45S rDNA copy number

qPCR was performed in triplicates for all samples to analyse 18S rDNA [primer combination 18S(XbaF): 5′-CTAGAGCTAATACGTTACAAACAC-3′ and 18S (HpaR): 5′-TTGCAATGATCCTACCCCCACCT-3′] normalized to UBIQUITIN 10 (primer combination UBQ10F: 5′-AA CGGGAAAGACGATTAC-3′ and UBQ10R: 5′-ACAAGATGAA GGTGGAC-3′) under the following conditions: initial denaturation 95°C/7 min, 35 cycles of 95°C/30 sec, 56°C/30 sec, 72°C/30 sec with final incubation at 75°C/5 min followed by the standard melting analysis. The analysis was performed by StepOnePlus Real-Time PCR system (Applied Biosystems, http://www.appliedbiosystems.com/absite/us/en/home.html) using FastStart SYBR Green Master (Roche, http://www.roche.com/).

Statistical analysis

Statistical analysis and plots were done in R software (Free Software Foundation, http://www.r-project.org) using the non-paired two-sided Mann-Whitney-Wilcoxon test ($\alpha = 0.05$).

Evaluation of telomere lengths using analysis of terminal restriction fragments

Five hundred nanogram of genomic DNA were analysed according to Ruckova et al. (2008), Samples were digested by Msel (NEB), separated by agarose gel electrophoresis followed by Southern hybridization with [32P]-labelled telomeric probe TRC (CCGCTAAA). Signals on membranes were visualized using the FLA7000 imager (Fujifilm, http://www.fujifilm.com/) and a gray-scale intensity profile was generated by Multi Gauge software (Fujifilm). The unweighted mean of terminal restriction fragments (TRF) length was calculated as $\Sigma (OD_i \times L_i)/(\Sigma OD_i)$, where OD is the signal intensity above background within interval $i$, and $L_i$ is the molecular weight at the midpoint of interval $i$ (Fojtova et al., 2011).

Plant treatments

To inhibit ATM kinase, Arabidopsis plants were sown on Petri plates containing ku55933 (ATM inhibitor or IATM – Calbiochem, http://www.calbiochem.com/) at 10 μM (Amiard et al., 2010).

For EdU incorporation, Arabidopsis seedlings were germinated as usual and after 5 days were transferred to liquid medium containing 10 μM of EdU for 1 h. Seedlings were then fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) and washed three times in PBS. After permeabilization in Triton 0.5%, EdU detection was performed as indicated by the manufacturer (Invitrogen-click-it EdU Alexa fluor 594 Imaging kit, http://www.lifetechnologies.com/cz/en/home/brands/invitrogen.html) and previously described (Amiard et al., 2010).

Slide preparation, immunostaining and FISH

The γ-H2AX antisem was raised and purified against a phospho-specific Arabidopsis HAX2 peptide and immunostaining was carried out as previously described (Charbonnel et al., 2010).

Fluorescence in situ hybridization was carried out according as previously described (Vannier et al., 2009), using FITC222 BAC from ribosomal regions of Arabidopsis chromosome 2, labelled with biotin (Athersham, http://www.gelifesciences.com/webapp/ wcs/stores/servlet/catalog/en/CELlifeSciences-uk/brands/athersham/) by nick translation (Roche). For the detection of biotin-labelled probe, avidin conjugated with Texas Red (1:500; Vector Laboratories)
followed by goat anti-avidin conjugated with biotin (1:100, Vector Laboratories, https://www.vectorlabs.com/) and avidin-Texas Red (1:500) were used. FISH after immunostaining require a post-fixation step of 30 min in 4% formaldehyde. Slides were observed by fluorescence microscopy and images were further processed and enhanced using Adobe Photoshop (Adobe, http://www.adobe.com/) software.

Analysis of anaphase bridges formation

Arabidopsis immature floral buds were collected, fixed, excised and processed according to Mokros et al. (2006). We used mix of three enzymes (0.3% each), in 10 mM citrate buffer – cellulase (Onozuka R10, Serva), pectolyase (Duchefa) and cytohelicase (Sigma, https://www.sigmaaldrich.com/). Slides were then stained with DAPI (1 μg ml⁻¹) in Vectashield (Vector Laboratories) and anaphases were count on each slide using Zeiss Axioimager Z1 with specific filter corresponding to DAPI excitation and emission spectra (AHF Analysentechnik, http://www.ahf.de/).

Sensitivity test

Seeds were surface sterilized in bleach for 7 min, washed four times in sterile water, put on ½MS (Murashige and Skoog) medium plates supplemented with 1% sucrose, incubated for 2 days at 4°C and grown under long day (LD) conditions. After 4 days the seedlings were transferred onto ½MS medium +1% sucrose control plates or ½MS medium +1% sucrose medium supplemented with 0.25 mM MMS, 0.5 mM MMS or 1 and 2.5 mM HU. Seedlings were grown for additional 6 days and documented. The plants growing on control and 0.25 or 0.5 mM MMS treated plates were then transferred to another freshly prepared plates, kept at LD conditions for another 6 days and finally harvested for RNA and genomic DNA isolation.

RNA extraction and reverse transcription

Total RNA was extracted from 16-day-old seedlings using the RNeasy plant mini kit (Qiagen, http://www.qiagen.com). RNA was treated with Turbo DNase (Ambion, http://www.lifetechnologies.com/finnzymes/) using an M-MuLV reverse transcriptase kit (Finnzymes). Table S1. Distribution of γ-H2AX-foci.

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Figure S1. Schematic diagram of plant crossing and propagation.

Figure S2. The copy number of 45S rDNA in parental rad51b mutants and segregated rad51b mutants with functional FAS1 and FAS2 genes.

Figure S3. Loss of telomeric DNA repeats in fas1 (A) and fas2 (B) mutants with either functional or dysfunctional RAD51B gene.

Figure S4. Sensitivity of seedlings to MMS. Loss of RAD51B neither shows sensitivity to MMS, nor increases sensitivity of fas mutants to MMS.

Figure S5. Sensitivity of seedlings to HU. Relative sensitivity to HU is similar in WT and fas mutant plants and loss of rad51b does not contribute to the effect of corresponding HU doses.
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