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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; Leyser 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.*, 2010; Wahba *et al.*, 2011; Lin and Pasero, 2012) and as elevated HDR has been shown to occur in the *fas* mutants, it was plausible that HDR may be involved in the reduction of tandem repeats. Factors essential for HDR include Rad51 and its paralogs. As is the case for humans, *A. thaliana* has five RAD51 paralog proteins in addition to AtRAD51: AtRAD51B, AtRAD51C, AtRAD51D, XRCC2 and XRCC3 (for reviews, see Bleuyard *et al.*, 2006; Karpenshif and Bernstein, 2012; Osman *et al.*, 2011; Suwaki *et al.*, 2011) which all play important roles in recombination and DNA repair (Bleuyard and White, 2004; Abe *et al.*, 2005; Bleuyard *et al.*, 2005; Li *et al.*, 2005; Osakabe *et al.*, 2005; Durrant *et al.*, 2007; Wang and Baumann, 2008; Da Ines *et al.*, 2012, 2013; Serra *et al.*, 2013). While only RAD51C and XRCC3 are essential for meiotic recombination (Bleuyard and White, 2004; Abe *et al.*, 2005; Bleuyard *et al.*, 2005; Li *et al.*, 2005; Durrant *et al.*, 2007) RAD51B, RAD51D and XRCC2 are also expressed during Arabidopsis meiosis (Yang *et al.*, 2011) and recent results show meiotic hyper-recombination phenotypes in the absence of XRCC2 or RAD51B. Their roles are thus not limited to somatic recombination (Da Ines *et al.*, 2013).

Based on our previous results showing transgenerational loss of telomeres and rDNA repeats in *fas* mutants, we tested involvement of HDR in this process. We show that the loss of telomeres and 45S rDNA is mitotic and we thus introgressed the *rad51b* mutant allele into *fas* background to generate double *fas rad51b* mutants. Our working hypothesis presumed three possible scenarios of a potential HDR involvement: (i) sequence loss is reduced in *fas rad51b* when compared to *fas* – implicating HDR in the reduction of copy number; (ii) sequence loss is increased in *fas rad51b* – HDR does not contribute to the loss but 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

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

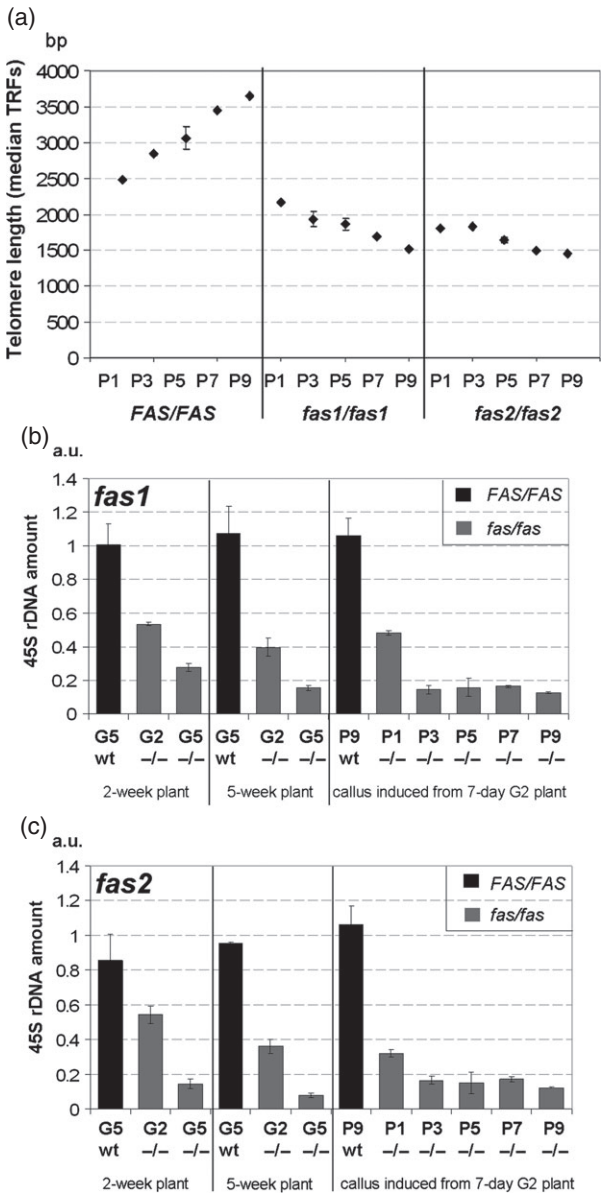

Figure 1. The loss of 45S rDNA and telomeres is mitotic.
Telomere lengths (a) and copy numbers of 45S rDNA (b, c) were measured in consecutive passages (P1–P9) of callus cultures derived from the second generation of *fas1* or *fas2* mutant plants as indicated. Data obtained in G2 and G5 generations 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.

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 *fas2rad51b* 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

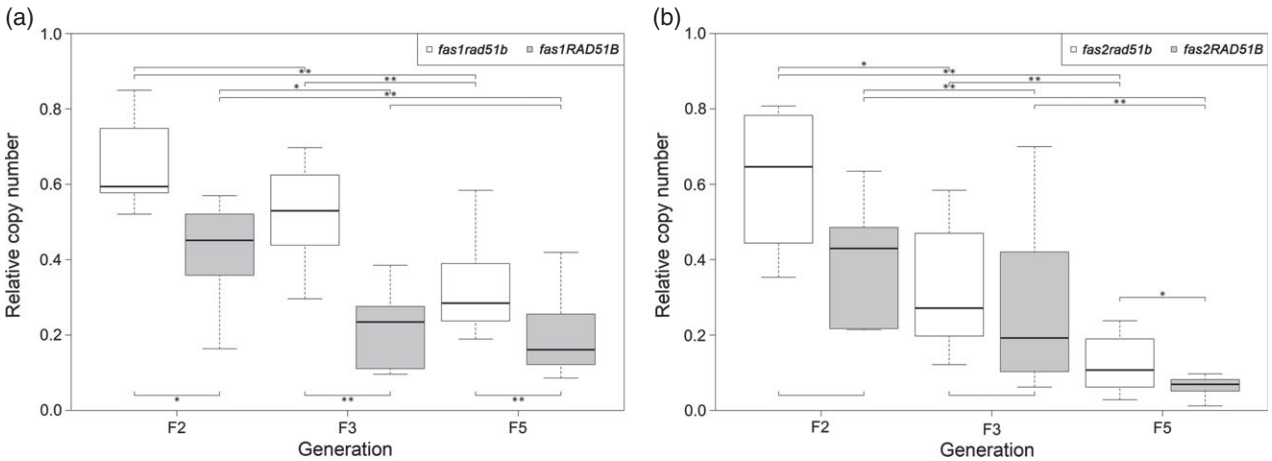

Figure 2. Loss of 45S rDNA repeats in *fas1* (a) and *fas2* (b) mutants with either functional or dysfunctional *RAD51B* gene.
45S rDNA copy numbers were measured by qPCR and normalized to the corresponding values obtained in WT plants. Statistical analysis and plots were performed by R software (http://www.r-project.org). The two-sided Mann–Whitney–Wilcoxon test ($\alpha = 0.05$), non-paired, was used. One asterisk denotes a *P*-value <0.05, two asterisks denote a *P*-value <0.01. Thick lanes inside the box plots indicate median values.

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

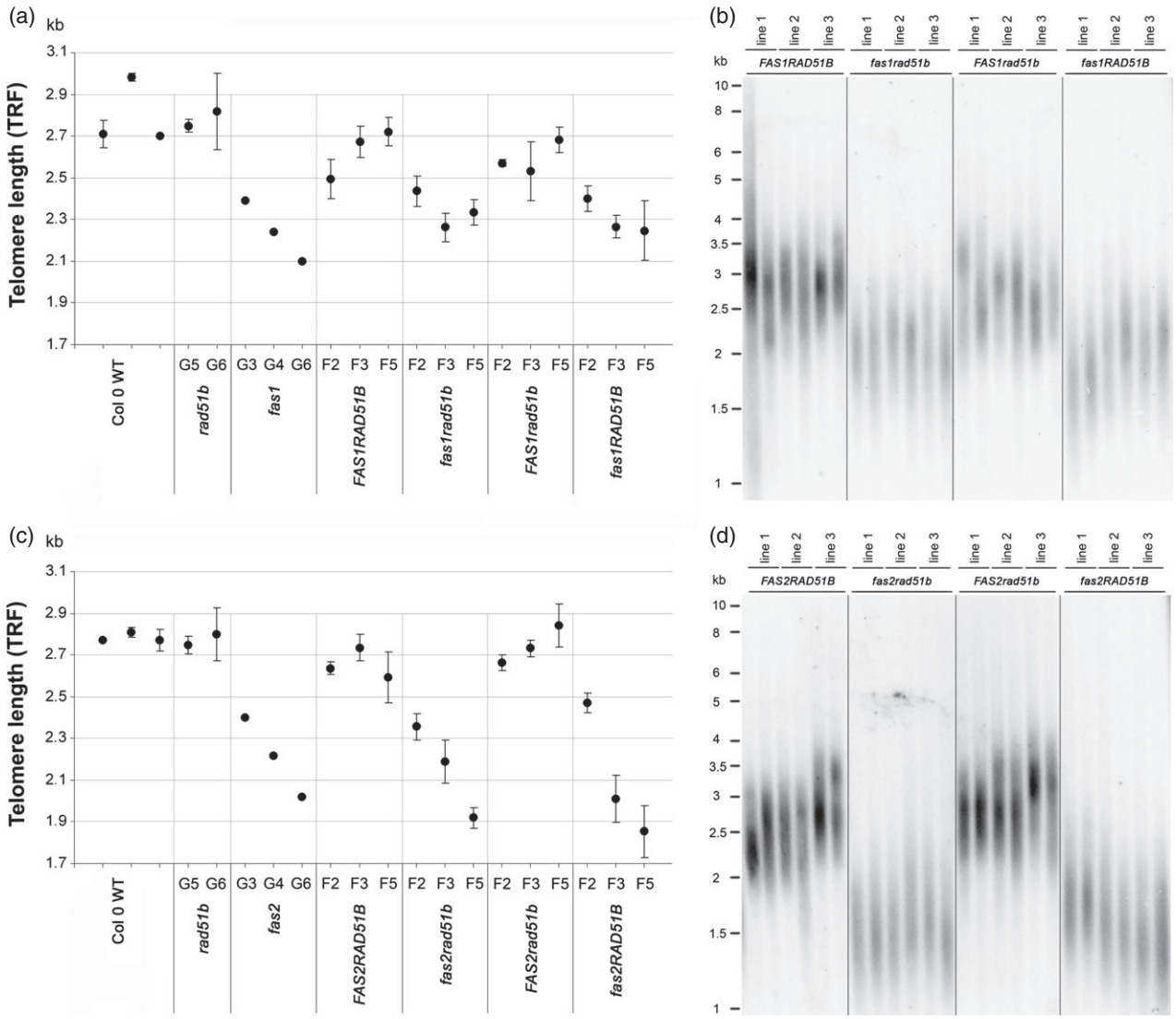

Figure 3. Loss of telomeric DNA repeats in *fas1* (a, b) and *fas2* (c, d) mutants with either functional or dysfunctional *RAD51B* gene.
Telomere lengths were measured by TRF analysis in F2, F3 and F5 generations of plants segregated from crossing between *fas1/2* and *rad51b* mutants, and mean telomere 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.

weaker in non-nucleolar foci when compared to nucleolar foci (62.5% versus 77% in *fas1*, and 54.2 versus 79.2% in *fas1rad51b* mutants, respectively – see also Table S1).

We note that no correspondence was observed between rDNA copy number and the numbers of γ-H2AX foci in plants with different total content of rDNA (Figure 5b). This concords with the notion that γ-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 *fas* mutants and WT plants (Mozgova *et al.*, 2010).

γ-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 γ-H2AX foci in S-phase nuclei. An EdU incorporation pulse was used to label S-phase nuclei and distributions of nucleolar and non-nucleolar γ-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 γ-H2AX foci is observed in EdU-positive nuclei, arguing against an origin due to problems of rDNA replication in these plants.

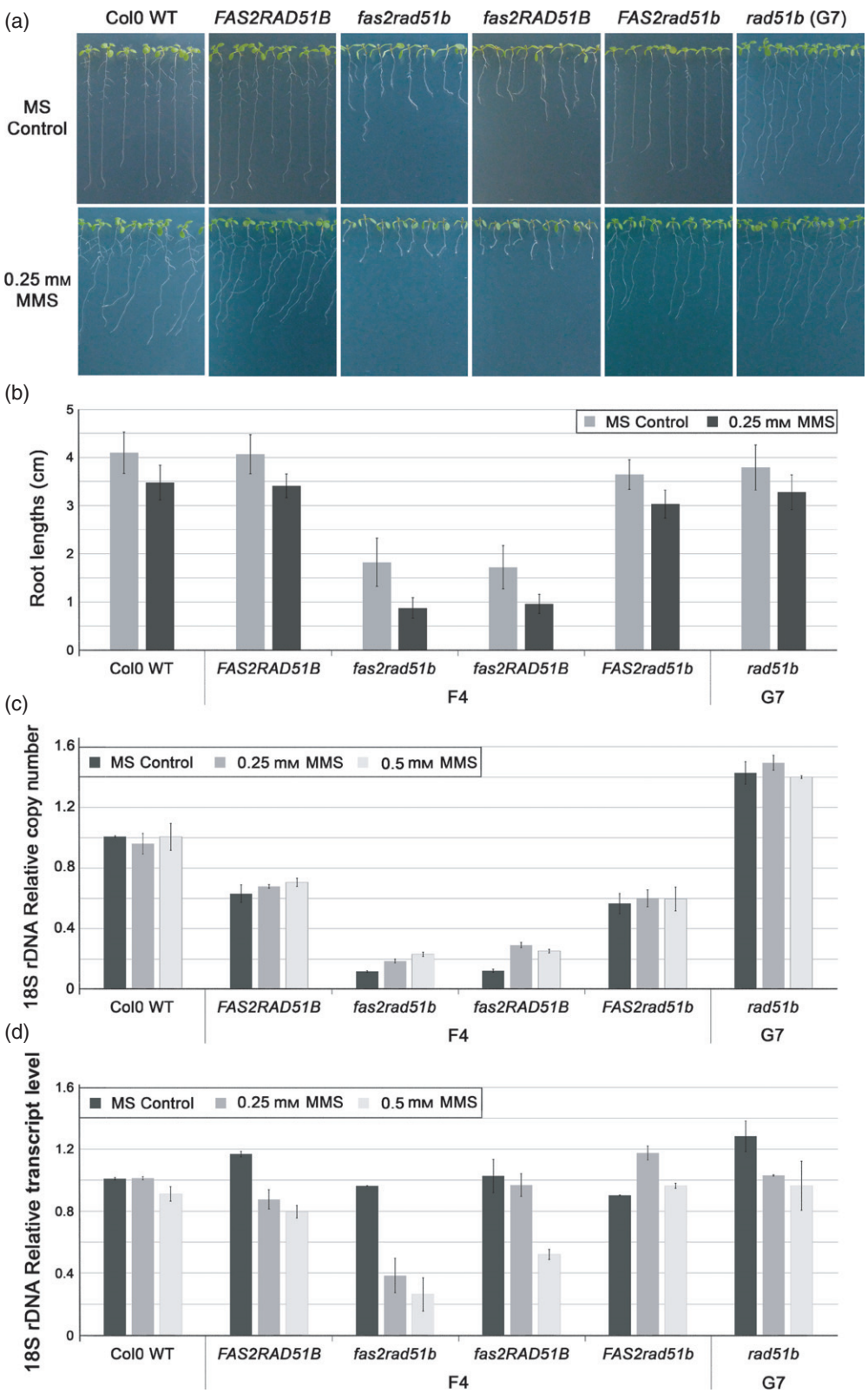

Figure 4. Sensitivity of seedlings to MMS.
Loss of RAD51B neither shows sensitivity to MMS, nor increases sensitivity of *fas* mutants to MMS [panels (a) and (b), respectively]. 18S rDNA copy numbers and transcript levels are shown in panels (c) and (d), respectively.

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 γ-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

Table 1 Anaphase bridges

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

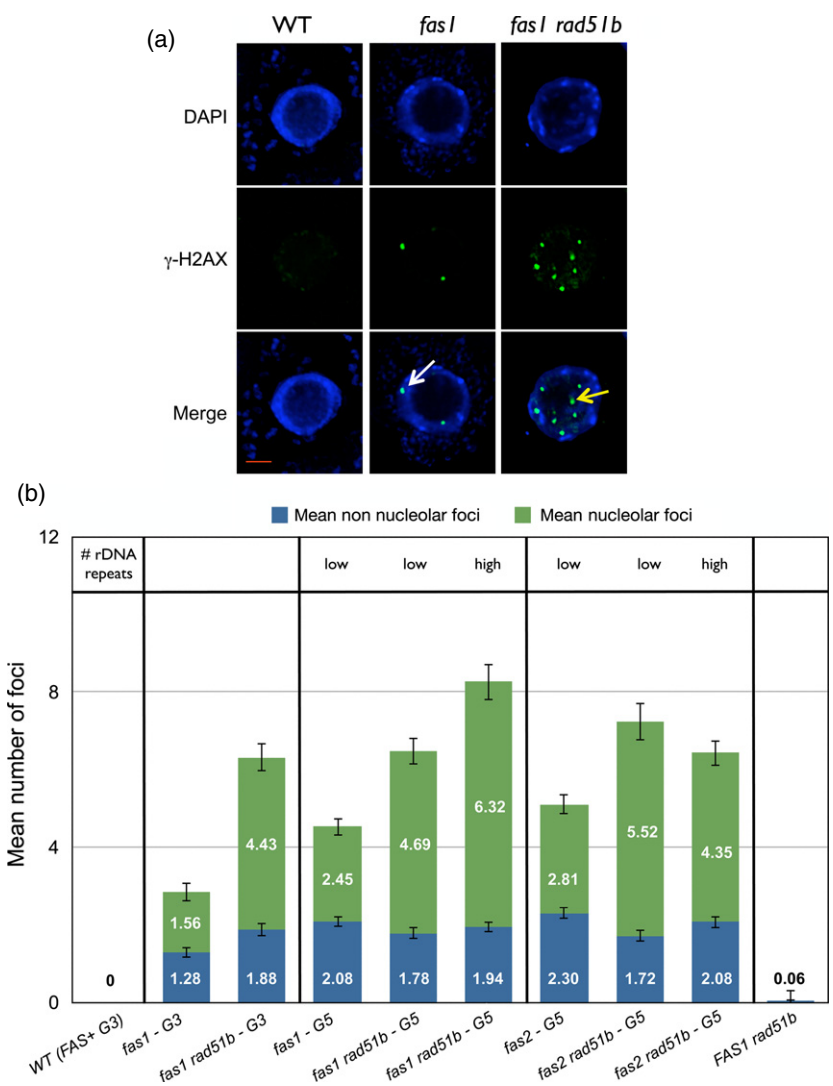

Figure 5. γ-H2AX foci in *fas* and *fas rad51b* mutant plants.
(a) Immunofluorescence of root tip interphase nuclei indicates γ-H2AX foci formation in *fas1* and *fas1 rad51b* mutants. DNA is stained with DAPI (blue), γ-H2AX foci are colored in green and merged images overlay γ-H2AX foci onto chromosomes. Scale bar, 2 μm. An example of a γ-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 γ-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.

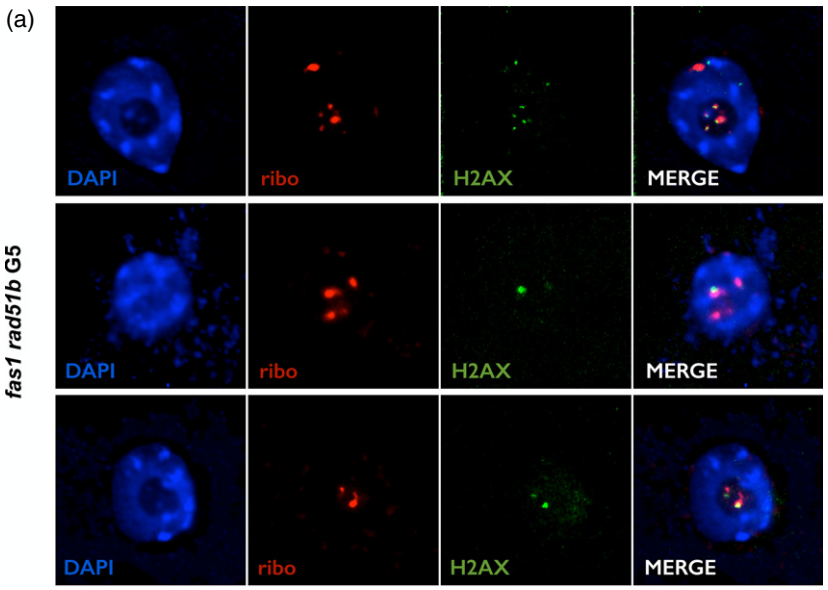

Figure 6. γ-H2AX foci colocalise with 45S rDNA FISH in the f*as1 rad51b* mutant.
(a) Immunostaining and 45S rDNA FISH labelling of root tip nuclei of *fas1* and *fas1 rad51b* mutants. Nuclei were stained with DAPI (blue), γ-H2AX foci are colored in green and FISH signals are colored in red. Images are 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.

(b)

	Colloc.	Not colloc.	Total	% colloc.
***fas1* - G5**	20	9	29	69 %
***fas1 rad51b* G5**	32	16	48	67 %

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 size 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

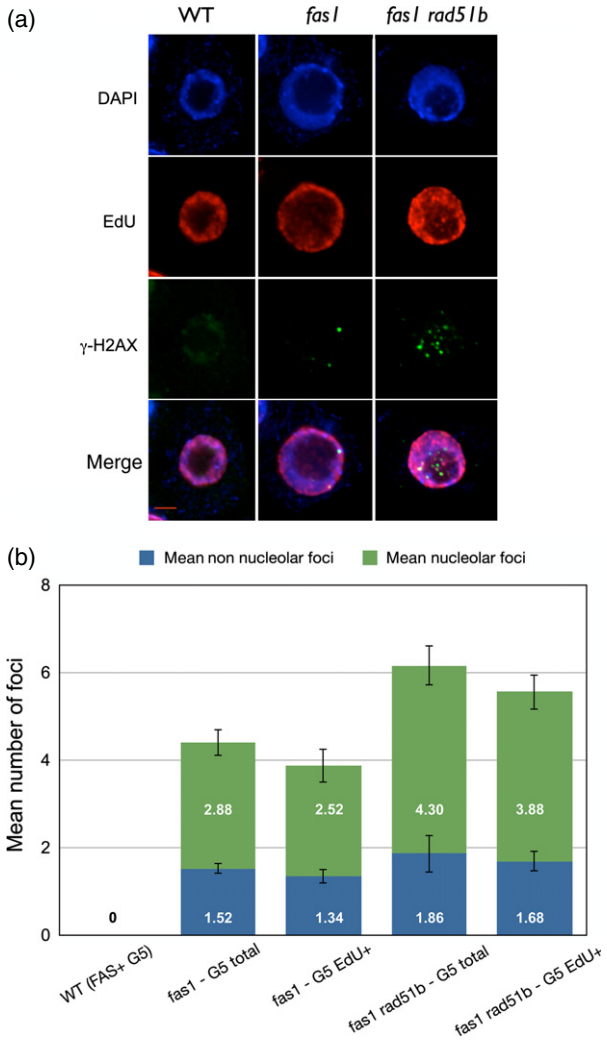

Figure 7. γ-H2AX foci formation does not depend on replication.
(a) Immunofluorescence of root tip nuclei labelled with EdU showing γ-H2AX foci formation in WT, *fas1* and *fas1 rad51b* replicated nuclei. DNA is stained with DAPI (blue), EdU incorporation in red and γ-H2AX foci in green. Scale bar, 2 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.

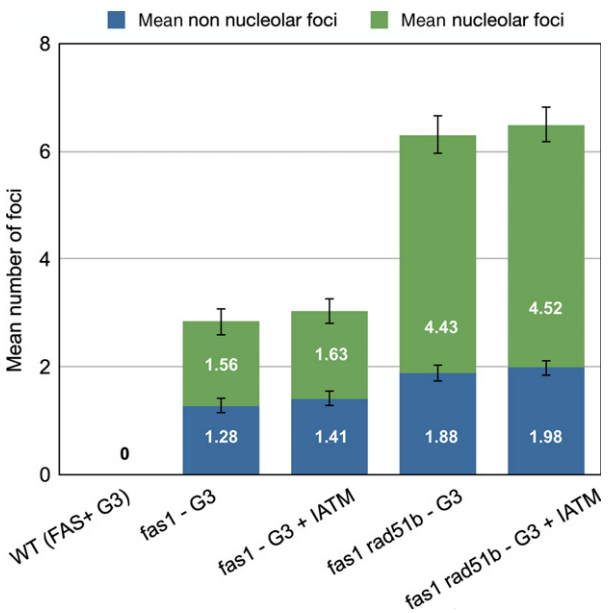

Figure 8. γ-H2AX foci in *fas1* and *fas1rad51b* mutants are ATR dependent.
Graphical representation of the number of γ-H2AX counted inside or outside the nucleolus in WT, *rad51b*, *fas1*, *fas1 rad51b*, *fas2* and *fas2 rad51b*, with or without IATM. Mean numbers of foci counted on 100 interphase nuclei.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

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 copies 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 *Pol*I-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

protection of DNA in nucleosomes, may result in increased formation of RNA-DNA hybrids which promote aberrant DNA recombination (Salvi *et al.*, 2014). This would explain the observed prevailing effect of the absence FAS proteins on transcriptionally active rDNA fraction, and implicates CAF1/FAS as a factor important for nucleosome dynamics, primarily in actively transcribed rDNA.

EXPERIMENTAL PROCEDURES

Plant material and genotyping of the mutants

All Arabidopsis plants were on a Columbia 0 background (Col 0). The *fas1-4* (NASC: N828822, SAIL_662_D10; (Exner *et al.*, 2006), *fas2-4* (NASC: N533228, SALK_033228 (Exner *et al.*, 2006), and *rad51b* (NASC: N524755, SALK_024755 (Alonso *et al.*, 2003; Bleuyard *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 $NaNO_3$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 3.5 μM $ZnSO_4$, 0.4 μM $MnSO_4$, 0.1 μM $CuSO_4$, 16 μM H_3BO_3, 0.1 μM KI, 0.1 μM $AlCl_3$, 0.1 μM $NiCl_2$, 0.1 mM Na_2EDTA, 0.1 mM $FeSO_4$, 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 *fas1* and *fas2* was done as described previously in Mozgova *et al.* (2010). *RAD51B* WT locus was assayed using rad51b F: 5′-CCTTGTGTGGTGGGATACCTT-3′ and rad51b R: 5′-GGAAACGAAGCTGATAACGCA-3′ primers. Mutant *rad51b* locus was detected using rad51b F and o5: 5′-CAACACTCAACCCTATCTCGG-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(*Xba*)F: 5′-CTAGAGCTAATACGTG CAACAAAC-3′ 18S(*Hpa*)R 5′-TTGCAATGATCTATCCCCATC-3′] normalized to *UBIQUITIN 10* (primer combination UBQ10F: 5′-AA CGGGAAAGACGATTAC-3′ and UBQ10R: 5′-ACAAGATGAAG 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–Witney–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 *Mse*I (NEB), separated by agarose gel electrophoresis followed by Southern hybridization with [^{32}P]-labelled telomeric probe TR4C $(CCCTAAA)_4$. 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 $\sum(OD_i \times L_i)/\sum(OD_i)$, where OD_i is the signal intensity above background within interval i, and L_i is the molecular weight at the mid-point 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 antiserum was raised and purified against a phospho-specific Arabidopsis H2AX 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 F17A22 BAC from ribosomic regions of Arabidopsis chromosome 2, labelled with biotin (Amersham, http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-uk/brands/amersham/) 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 wather, 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 transfered 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/cz/en/home/brands/ambion.html) for 40 min to eliminate contaminant DNA and quality was checked on 0.8% agarose gel. The synthesis of cDNA was performed on 1 μg of RNA with the random hexamers (Finnzymes, http://www.thermoscientificbio.com/finnzymes/) using an M-MuLV reverse transcriptase kit (Finnzymes).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

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.

Table S1. Distribution of γ-H2AX-foci.

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