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Replication protein A (AtRPA1a) is required for class I crossover formation but is dispensable for meiotic DNA break repair

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Replication protein A (RPA) is involved in many aspects of DNA metabolism including meiotic recombination. Many species possess a single RPA1 gene but *Arabidopsis* possesses five RPA1 paralogues. This feature has enabled us to gain further insight into the meiotic role of RPA1. Proteomic analysis implicated one of the AtRPA1 family (AtRPA1a) in meiosis. Immunofluorescence studies confirmed that AtRPA1a is associated with meiotic chromosomes from leptotene through to early pachytene. Analysis of an *Atrpa1a* mutant revealed that AtRPA1a is not essential at early stages in the recombination pathway. DNA double-strand breaks are repaired in *Atrpa1a*, but the mutant is defective in the formation of crossovers, exhibiting a 60% reduction in chiasma frequency. Consistent with this, localization of recombination proteins AtRAD51 and AtMSH4 appears normal, whereas the numbers of AtMLH1 and AtMLH3 foci at pachytene are significantly reduced. This suggests that the defect in *Atrpa1a* is manifested at the stage of second-end capture. Analysis of *Atrpa1a/Atmsh4* and *Atrpa1a/Atmlh3* double mutants indicates that loss of *AtRPA1a* predominantly affects the formation of class I, interference-dependent crossovers.
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Introduction

Meiotic recombination is initiated by the formation of DNA double-strand breaks (DSBs) catalyzed by the topoisomerase II-like protein SPO11 (Keeney *et al*, 1997). The DSBs undergo strand resection to form 3′ single-stranded DNA (ssDNA) tails. Initially, one of these invades the intact homologous duplexes of one of the non-sister chromatids of the other homologue. This displaces the corresponding sequence on the homologue to form a displacement loop (D-loop). DNA synthesis then occurs, extending the invading strand and the D-loop. This allows the capture of the 3′-end of the DNA from the other side of the DSB and following further DNA synthesis, ligation to form two four-way junctions termed double-Holliday junctions (dHjs) that connect the homologues. It was originally proposed that differential resolution of the dHjs resulted in the formation of crossover (CO) or non-crossover products (NCOs), but recent studies have led to a revised model which proposes that the CO/NCO decision occurs in early prophase I, such that most or all dHjs are resolved as COs, whereas NCOs arise through an early branch point in the homologous recombination (HR) pathway (Figure 1) (Allers and Lichten, 2001). In the budding yeast, *Saccharomyces cerevisiae* and in *Arabidopsis* this pathway accounts for the majority of meiotic COs. These, so-called class I COs, are dependent on the activities of a set of proteins referred to as the ZMM (Zip1, Zip2, Zip3, Zip4, Msh4, Msh5 and Mer3) complex (Boerner *et al*, 2004; Higgins *et al*, 2008b). Furthermore, class I COs are subject to interference, a mechanism which ensures that they do not occur in adjacent chromosomal regions (Jones, 1984). The remaining ZMM-independent (class II) COs do not exhibit interference and a proportion of these arise through a distinct pathway which is dependent on the activity of the Mus81/Mms4 protein complex (Figure 1) (de los Santos *et al*, 2003; Berchowitz *et al*, 2007; Higgins *et al*, 2008a).

Replication protein A (RPA) is an ssDNA-binding protein comprised of three subunits of ~70, ~32 and 14 kDa (Wold, 1997; Iftode *et al*, 1999). The RPA heterocomplex is implicated in a wide range of cellular activities associated with DNA metabolism, notably DNA replication, DNA repair and HR. In budding yeast the large RPA subunit is encoded by *RFA1*. Analysis of mutant alleles of *RFA1*, *rfa1-t11* and *rfa1-t48,* revealed an overall decrease in meiotic recombination frequency of up to 100-fold thereby confirming the crucial role played by RPA in meiosis (Soustelle *et al*, 2002). Immunolocalization studies in budding yeast have detected foci corresponding to RPA in nuclei at meiotic S-phase and in early prophase I where they co-localize with the recombination proteins Rad51 and Rad52 (Gasior *et al*, 1998). RPA is one of the several accessory proteins that participate in co-ordinating the assembly of the strand-exchange proteins Rad51 and Dmc1 on to 3′ ssDNA to form nucleoprotein filaments, which invade homologous duplex DNA. These studies have demonstrated that the strand-exchange activity of Rad51 is significantly enhanced by RPA. Paradoxically, prior saturation of the ssDNA with RPA before Rad51 addition interferes with loading of the recombinase and thus inhibits strand-exchange. This inhibition is overcome by the addition of Rad52 which stimulates Rad51 nucleation and strand-exchange and promotes post-invasion steps (reviewed in Sung *et al*, 2003).

In mouse, as in budding yeast, *RPA1* is essential. Mice carrying a heterozygous point mutation in *RPA1* accumulate

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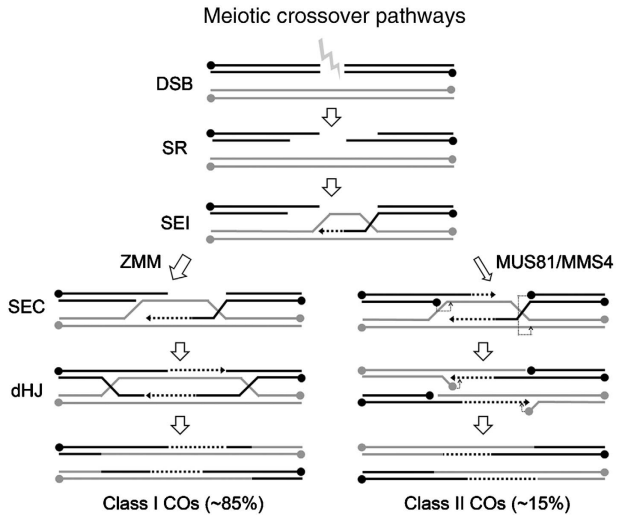

Figure 1 Meiotic crossover formation. Strand-resection (SR) of Spo11-induced DSBs enables Dmc1/Rad51-mediated single-end invasion (SEI) of the homologue followed by second-end capture (SEC). This intermediate is then processed to form either class I or class II COs. Class I, interference-dependent COs arise through a dHJ intermediate and require the ZMM group of proteins. They account for ~85% of total COs. Remaining, class II COs, are interference-independent and a proportion of them are Mus81/Mms4-dependent whereby the SEC intermediate is cleaved (thin dotted lines) to form a product with 5′ flaps and gaps. It is proposed that an isomerization step follows leading to formation of 3′ flaps, a second-round of Mus81 cleavage then follows prior to filling in of the gaps and ligation to form the CO product scheme based on Hollingsworth and Brill (2004). The formation of non-CO products is not shown.

lymphoid tumours and their litters exhibit early embryonic lethality (Wang *et al*, 2005). Cells from these mice have a reduced ability to repair DSBs induced by exposure to the replication inhibitor aphidicolin. Studies in mouse spermatocytes using both immunolocalization and immunogold labelling in conjunction with electron microscopy have also detected RPA foci during prophase I (Plug *et al*, 1997, 1998; Moens *et al*, 2007; Oliver-Bonet *et al*, 2007). RPA accumulates during late leptotene/early zygotene and persists through to pachytene, before dissociating from the chromatin at around the time CO-designated recombination intermediates (Marcon and Moens, 2003) acquire the MutL homologue, MLH1. Immunolocalization studies of RPA in human spermatocytes have also revealed that the complex is present throughout prophase I until mid-pachytene (Oliver-Bonet *et al*, 2007). These observations suggest that RPA may have a role at later stages of prophase I in addition to an earlier role but, because it has not proved possible to generate and analyse RPA-deficient spermatocytes, functional studies have not been conducted.

In contrast to budding yeast and mouse, it has been reported that *Arabidopsis* has five paralogues of *RPA1* (Table I, Supplementary Figure 1) (Shultz *et al*, 2007). The same authors also report two copies of the ~32 kDa RPA2 subunit as well as two copies of the 14 kDa RPA3 subunit (Shultz *et al*, 2007). Paralogues of RPA1 have been reported in a range of other plant species, notably rice, which also contains multiple copies (three) of the gene (Table I) (Ishibashi *et al*, 2006; Singh *et al*, 2007).

Table I Sequence homology of AtRPA1a with other RPA1-like proteins in *Arabidopsis* and other species

Species/gene identity	Amino acid identity (%)	Amino acid similarity (%)
A. thaliana At4g19130	49	68
A. thaliana At5g45400	45	62
A. thaliana At5g61000	32	53
A. thaliana At5g08020	32	52
S. cerevisiae	30	52
Schizosaccharomyces pombe	30	53
Mouse	33	53
Human	35	55
Oryza sativa (rice)	59	75
Pisum sativum (pea)	68	80
Vitis vinifera (grapevine)	73	84

Source: http://www.ncbi.nlm.nih.gov/.

In a survey of the meiotic proteome of *Brassica oleracea*, which is a close relative of *Arabidopsis* and provides an effective route to identifying *Arabidopsis* meiotic genes, we identified peptides corresponding to the predicted product of a member of the AtRPA1 family, At2g06510 (herein referred to as AtRPA1a) suggesting a possible role in meiosis (Sanchez-Moran *et al*, 2005). We report here the functional analysis of AtRPA1a during meiosis. We present evidence that AtRPA1a is expressed during prophase I of meiosis where it is required to ensure wild-type levels of meiotic COs. The phenotype of meiocytes lacking AtRPA1a indicates that the protein is not required for the repair of meiotic DSBs, but plays an essential role at later stages in the meiotic recombination pathway that is required for the formation of class I COs.

Results

Loss of AtRPA1a results in reduced fertility and meiotic defects

To investigate a potential role for AtRPA1a during meiosis a T-DNA knockout line (SALK_017580) was obtained from the Nottingham Arabidopsis Stock Centre (NASC). Molecular characterization of SALK_017580 confirmed a T-DNA insertion at the splice junction between the first intron and second exon 736 bp downstream from the ATG start codon resulting in a knockout of the *AtRPA1a* transcript (Supplementary Figure 2A–C). A homozygous line (*Atrpa1a*) was identified that showed normal vegetative growth, but with reduced fertility (Supplementary Figure 3). Mean silique length was reduced from 15.84 mm ($n = 50$) in wild-type to 10.84 mm ($n = 50$) in *Atrpa1a*. Mean seed-set per silique in the mutant was 17.72 ($n = 50$) compared with 56.08 ($n = 50$) in wild-type representing a decrease of 68.4%. Pollen viability determined by Alexander's staining revealed a large proportion of non-viable pollen (data not shown) (Alexander, 1969).

To determine if the reduced fertility phenotype of *Atrpa1a* was due to a defect in meiosis, chromosome spreads prepared from pollen mother cells (PMCs) were examined using fluorescence microscopy (Figure 2). Early prophase I from G2 through to diplotene was indistinguishable from wild-type, with the chromosomes apparently pairing normally and attaining full synapsis at pachytene (Figure 2A–C, G–I). However, as the chromosomes desynapsed and began to condense during late diplotene/diakinesis, it became apparent that not all the homologous chromosomes pairs were

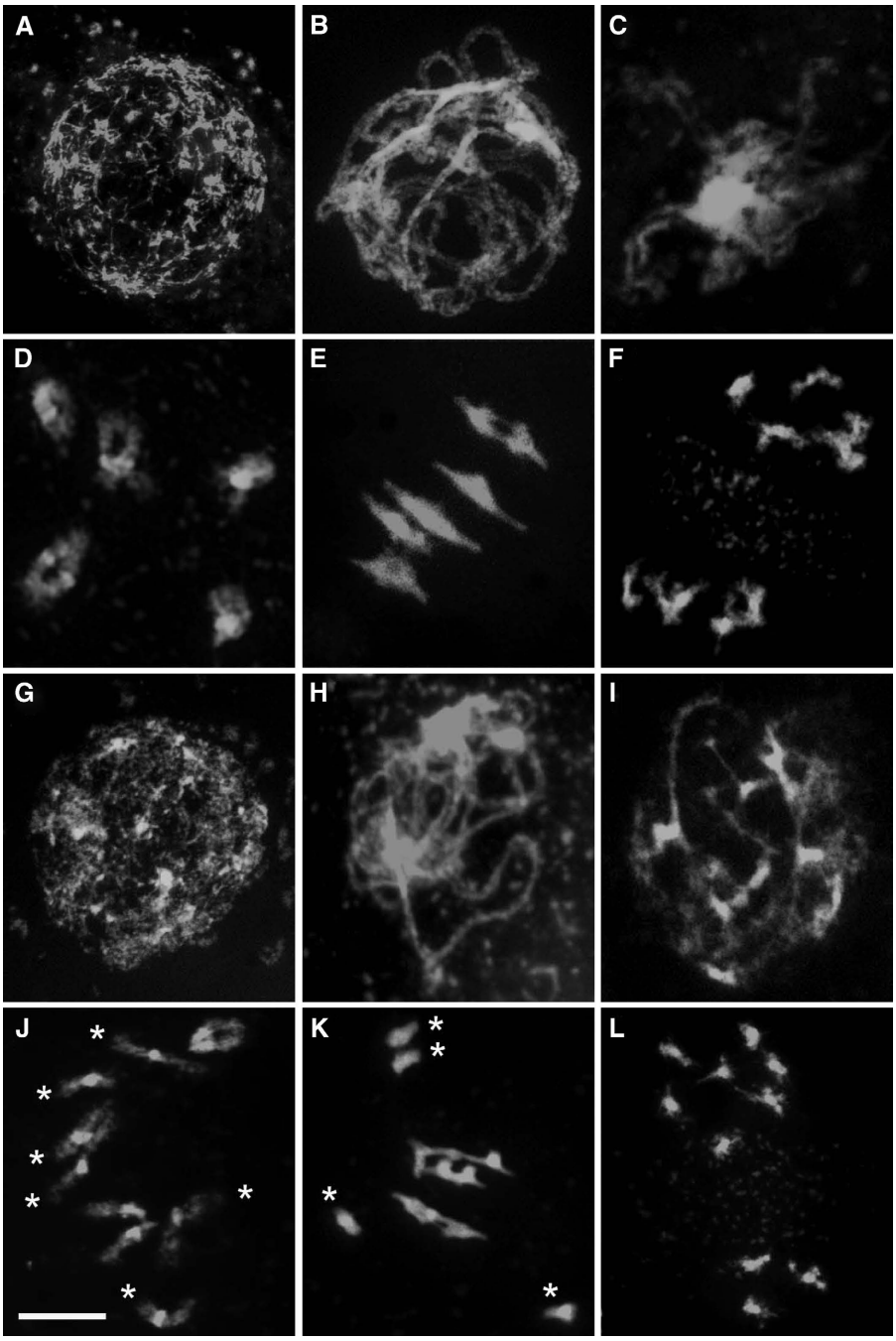

Figure 2 Meiotic stages from wild-type (**A–F**) and *Atrpa1a* (**G–L**) pollen mother cells. (A, G) leptotene. (B, H) pachytene. (C, I) diplotene. (D, J) diakinesis. (E, K) metaphase I. (F, L) metaphase II. Stages to diplotene (A, B, C, G, H, I) are very similar in appearance in wild-type and *Atrpa1a*. At diakinesis and metaphase I univalents (asterisks) are apparent in *Atrpa1a* (J, K) which can lead to unbalanced chromosome segregation at the second division (L). Bar, 10 μm.

linked by chiasmata, leading to the presence of univalents at metaphase I (Figure 2D, E, J and K). As a result, missegregation occurred at the first meiotic division leading to unbalanced dyads and subsequent aneuploid tetrads following the second meiotic division (Figure 2F and L). These observations demonstrate that *AtRPA1a* is essential for normal progression through meiosis. Nevertheless, the absence of any evidence of chromosome fragmentation would suggest that meiotic DSB repair *per se* is not dependent on the activity of AtRPA1a.

Subsequent cytological analysis of an independently isolated *Atrpa1a* allele (GABI_406D08) revealed the same meiotic phenotype (Supplementary Figures 2A and 4).

Complementation of Atrpa1a restores fertility

To confirm that the meiotic phenotype was due to the insertion in *AtRPA1a* a full-length cDNA of the gene was placed under the control of the AtDMC1 promoter in pPF408 and was transformed into *Atrpa1a*. Three plants from each of two independently generated *Atrpa1a* homozygotes were

transformed in duplicate. Here, 58 transformants were obtained which on visual inspection were found to have full or partial restoration of fertility. Three lines that showed restoration to full fertility were analysed further. The presence of the transgene and the endogenous knockout gene was confirmed by PCR and cytological examination revealed that normal meiosis had been restored (Supplementary Figure 5). *Atrpa1a* controls transformed with empty pPF408 did not exhibit any restoration of fertility and meiosis remained aberrant.

AtRPA1a localizes to meiotic chromosomes during prophase I

The distribution of AtRPA1a during meiosis was investigated using an antibody (Ab) against a recombinant polypeptide comprising amino acid residues 2–171 of the protein expressed in *Escherichia coli* (Figure 3). Immunolocalization studies on chromosome spread preparations of wild-type PMCs using the anti-AtRPA1a Ab in conjunction with Abs recognizing the axis-associated protein, ASY1 (Caryl *et al*, 2000; Armstrong *et al*, 2002) or the synaptonemal complex (SC) transverse element protein, ZYP1 (Higgins *et al*, 2005) to aid meiotic staging revealed that AtRPA1a was first detectable as chromatin-associated foci at early leptotene (Figure 3A and E). The number of foci increased to a maximum (175.8 ± 12, $n = 5$) at late leptotene/early zygotene before gradually reducing during pachytene (Figure 3B, C, F and G). By late pachytene the protein was no longer detectable (Figure 3D and H). When the anti-AtRPA1a Ab was applied to spread preparations of *Atrpa1a* prophase I chromosomes no signal was detectable, thereby confirming the specificity of the Ab (Figure 3I–P) and the RT–PCR analysis which had revealed that the *AtRPA1a* transcript was absent in the mutant (Supplementary Figure 2C). Identical results were obtained when the studies were conducted with an anti-AtRPA1a Ab based on a peptide comprising residues 116–135 of the protein (Supplementary Figure 6).

Later stages of meiotic recombination are affected in Atrpa1a

To investigate the meiotic defect in *Atrpa1a* in more detail, immunolocalization studies were carried out on spread preparations of PMCs from the mutant at various stages throughout prophase I. Localization of ASY1 and ZYP1 in *Atrpa1a* was indistinguishable from the wild-type control (Figure 4A–L). In both *Atrpa1a* and wild-type an abundant ASY1 signal was detectable at late leptotene/early zygotene (Figure 4A and D) which by zygotene through pachytene was clearly discernable as a linear axis-associated signal (Figure 4B, C, E and F). ZYP1 was detectable as foci or very short linear signals at late leptotene/early zygotene before progressively polymerizing to form a continuous linear signal through zygotene into pachytene indicative of full synapsis (Figure 4G–L). This indicates that there are no major defects in axis formation and assembly of the SC in *Atrpa1a*.

The observation that chromosome synapsis was normal suggested that early stages in the recombination pathway were not substantially perturbed. Immunolocalization with an Ab against the strand-exchange protein AtRAD51 was consistent with this conclusion. This revealed no obvious difference in the number of AtRAD51 foci in *Atrpa1a* and wild-type chromosome spread preparations at early prophase I (Figure 5A and E). To assess the effect of loss of AtRPA1a at later stages of the recombination pathway, immunolocalization studies were conducted using Abs against the mismatch repair proteins AtMSH4 and AtMLH1 (Higgins *et al*, 2004, 2008b; Jackson *et al*, 2006).

The MutS homologue Msh4 functions as a heterodimer with Msh5 and is essential for the formation of class I COs (Ross-Macdonald and Roeder, 1994; Boerner *et al*, 2004; Higgins *et al*, 2004). In wild-type *Arabidopsis*, numerous AtMSH4 foci are detected in chromosome spread preparations at leptotene and persist until early pachytene, but with a gradual reduction in number (Higgins *et al*, 2004). Comparison of *Atrpa1a* and wildtype did not reveal any obvious difference in the number of AtMSH4 foci per nucleus at early prophase I (mean *Atrpa1* = 86 versus wildtype = 83, $n = 5$), suggesting that the protein associates as normal with the recombination intermediates (Figure 5B and F). Moreover, there was no discernable difference in the progressive reduction in the number of AtMSH4 foci thereafter. Dual-immunolocalization with ZYP1 indicated that by pachytene few AtMSH4 axis-associated foci remained in both *Atrpa1a* and wild-type nuclei (Figure 5C and G). Hence, it appears that the dynamics of AtMSH4 turnover in the mutant are indistinguishable from wild-type.

The MutL homologue, Mlh1, forms a heterodimer with Mlh3 which co-localizes to CO sites where it is required to ensure resolution of dHjs as COs rather than non-COs (Hunter and Borts, 1997; Wang *et al*, 1999; Marcon and Moens, 2003; Jackson *et al*, 2006). Immunolocalization of AtMLH1 in *Atrpa1a* chromosome spreads at pachytene revealed the presence of foci corresponding to the protein. However, there was a significant reduction in their number compared with wildtype, which typically had 8–10 AtMLH1 foci per nucleus. Although *Atrpa1a* nuclei with up to five AtMLH1 foci were observed, the majority contained 0–3 foci (Figure 5D and H). When an Ab against AtMLH3 was used in place of the anti-AtMLH1 Ab a corresponding reduction in AtMLH3 foci was observed (Supplementary Figure 7).

Together, these data suggest that recombination is initiated as normal in the absence of AtRPA1a, but that at later stages of prophase I a proportion of the recombination intermediates lose their CO designation and are repaired as non-COs.

Residual chiasmata are randomly distributed in Atrpa1a

Previously, we have shown that mutants lacking AtMSH4 and AtMLH3 are, to different degrees, defective in the formation of chiasmata and that the numerical distribution of residual chiasmata per nucleus is random (Higgins *et al*, 2004; Jackson *et al*, 2006). Our initial analysis of *Atrpa1a* revealed a reduction in chiasma formation. Moreover, the immunolocalization studies indicated that the number of MLH1/MLH3 foci is significantly reduced in *Atrpa1a*. Hence, to gain further insight into the effect of loss of AtRPA1a in relation to these observations, we conducted a quantitative cytological analysis to determine the frequency and distribution of the residual chiasmata in *Atrpa1a* at metaphase I (Figure 6). Nuclei containing between 0 and 8 chiasmata were observed giving an overall mean chiasma frequency of 3.98 ($n = 50$) per cell, whereas in wild-type nuclei the number of chiasmata range between 8 and 12 per cell and the overall mean chiasma frequency is 9.86 (Higgins *et al*, 2004). Moreover, the numerical distribution of chiasmata in *Atrpa1a* does not significantly deviate from a Poisson distribution ($\chi^2_{(4)} = 8.947$

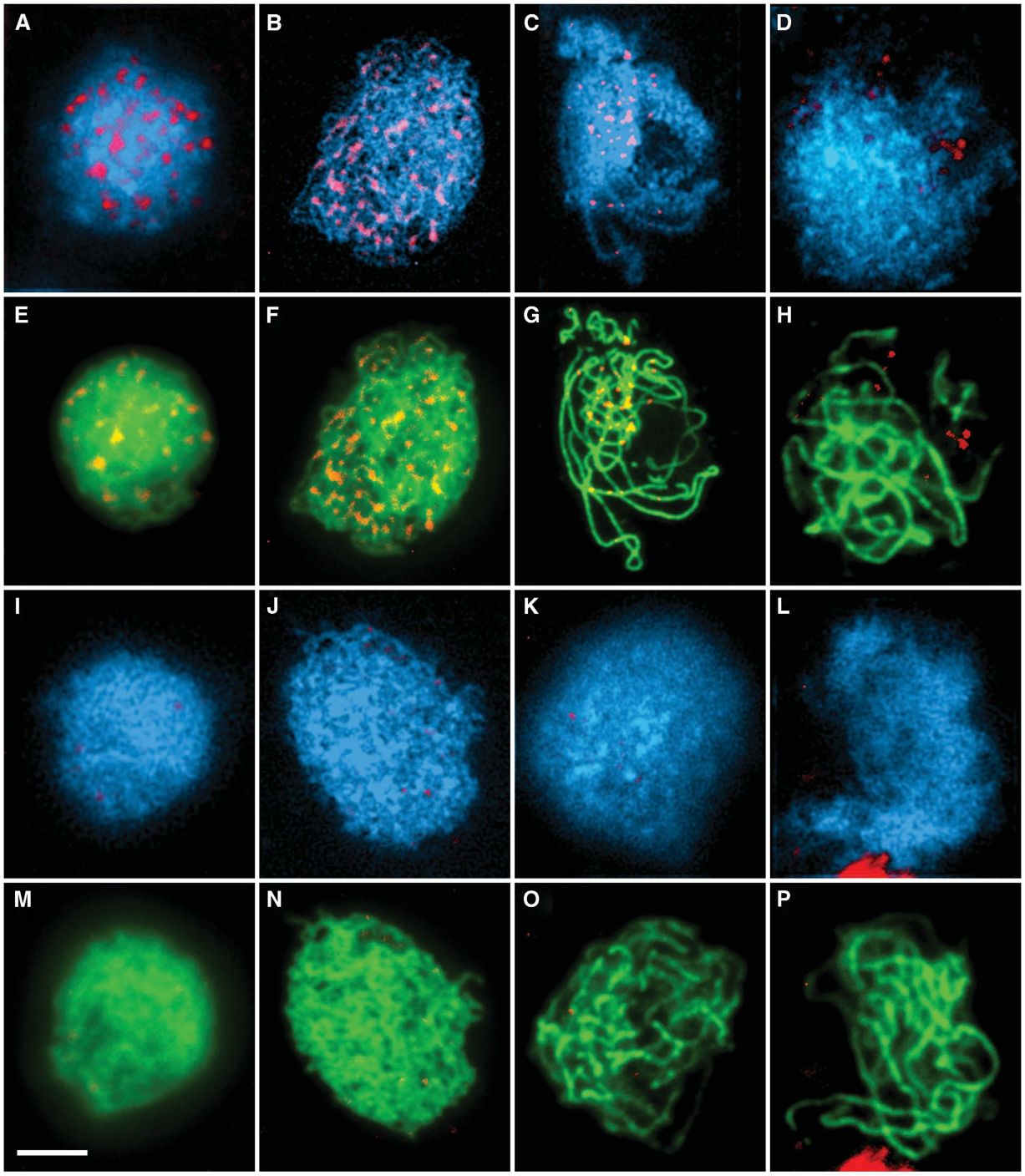

Figure 3 Immunolocalization of AtRPA1a (red) to wildtype (**A–H**) and *Atrpa1a* (**I–P**) prophase I nuclei. In wild-type nuclei AtRPA1a foci first appear during late leptotene/early zygotene (A), reach a maximum number during zygotene (B), reduce in number throughout pachytene (C) before disappearing by late pachytene (D). Corresponding images showing colocalization with AtASY1 (E, F) and AtZYP1 (G, H) (green) show that AtRPA1a foci localize along chromosome axes. Corresponding stages in mutant nuclei show localization of AtASY1 (M, N) and AtZYP1 (O, P) but no AtRPA1 localization (I–P). Nuclei in A–D and I–L are counterstained with DAPI. Bar, 10 μm.

$P > 0.05$), indicating that the residual distribution of chiasmata among cells is random. In contrast the distribution of chiasmata in wild-type cells deviates significantly from a Poisson distribution (Higgins *et al*, 2004).

AtRPA1a is required for normal levels of class I crossovers

In principle, the reduction in chiasma formation observed in *Atrpa1a* could be due to a reduction in type I COs or a general reduction in both classes of CO. Previously, we have shown that formation of class I COs is dependent on the activity of AtMSH4 (Higgins *et al*, 2004). Loss of the protein results in a dramatic reduction of COs/chiasmata to around 15% of wild-type levels and the residual class II COs exhibit a random numerical distribution consistent with a loss of CO interference. Thus, to determine if AtRPA1a is implicated in one or both CO pathways we constructed an *Atrpa1a/Atmsh4* double mutant. This line exhibited a further reduction in

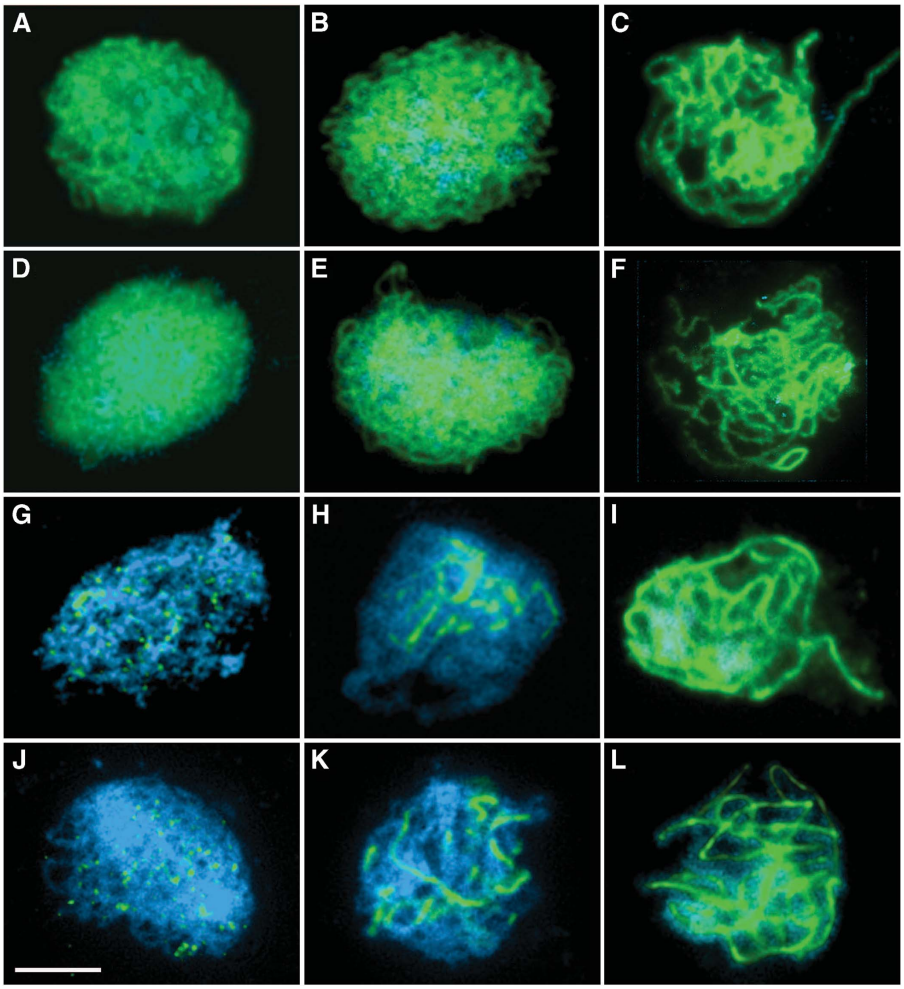

Figure 4 Immunolocalization of AtASY1 (**A–F**) and AtZYP1 (**G–L**) proteins (green) to wildtype (A–C, G–I) and *Atrpa1a* (D–F, J–L) prophase I nuclei. (A, D, G, J) late leptotene/early zygotene. (B, E, H, K) zygotene. (C, F, I, L) pachytene. At each stage wildtype and *Atrpa1a* are indistinguishable, indicating that there are no major defects in axis assembly and synapsis in *Atrpa1a*. Bar, 10 μm.

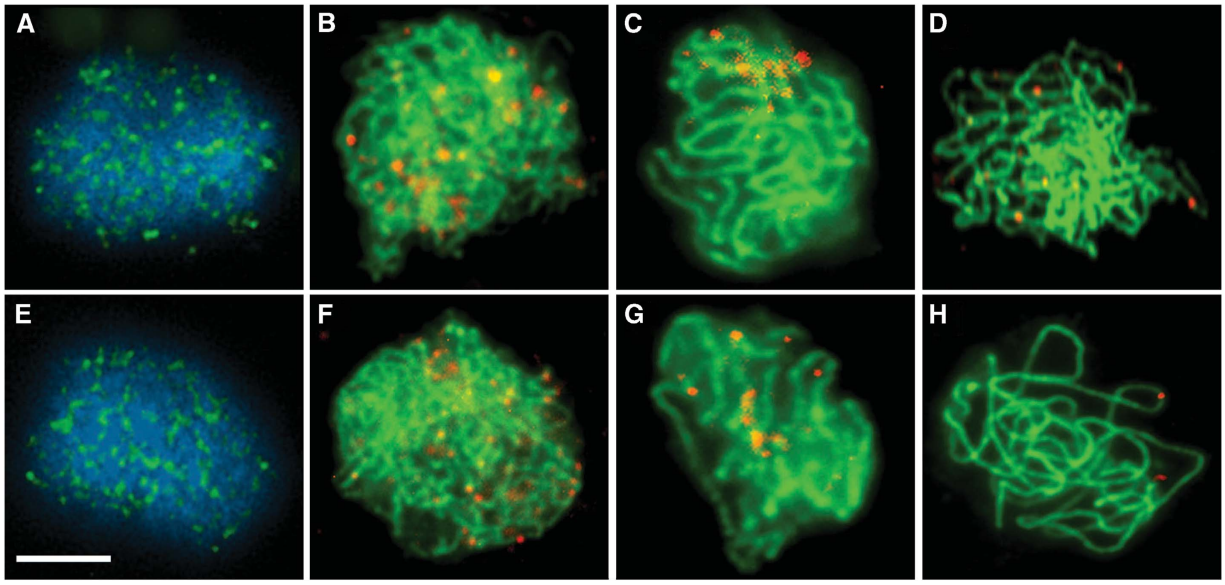

Figure 5 Immunolocalization of strand-exchange and mismatch repair proteins to wild-type (**A**, **B**, **C**, **D**) and *Atrpa1a* (**E**, **F**, **G**, **H**) prophase I nuclei. During early prophase I, wildtype and *Atrpa1a* show similar patterns of AtRAD51 (green) localization (A, E). Wild-type and *Atrpa1a* also show similar patterns of AtMSH4 (red) localization; mid-prophase (B, F), late-prophase (C, G). Late-prophase I *Atrpa1a* nuclei show a marked reduction in numbers of AtMLH1 (red) foci relative to wildtype (H, D respectively). Chromosome axes (green) are labelled using anti-ASY1 Ab in mid-prophase I (B, F); the SC (green) is labelled using anti-ZYP1 Ab in late-prophase I (C, D, G, H). Bar, 10 μm.

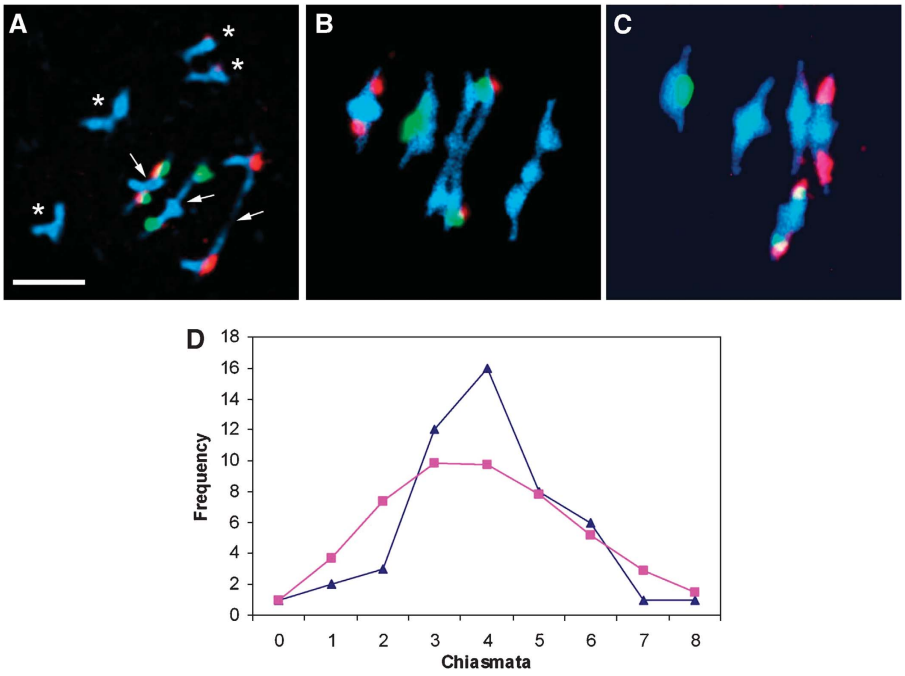

Figure 6 Metaphase I nuclei of *Atrpa1a* (**A**, **B**) and wild-type (**C**) pollen mother cells following FISH to label the positions of *5S* rDNA (red) and *45S* rDNA (green) loci. (A) A nucleus with four univalents (asterisks) and three chiasmata (arrows); a proximal chiasma in chromosome 4 and single distal chiasmata in chromosomes 2 and 5. (B) A nucleus with seven chiasmata; a chiasma in each arm of chromosomes 2 and 5 and single distal chiasmata in chromosomes 1, 3 and 4. (C) A typical wild-type nucleus with 10 chiasmata. (**D**) Observed (triangles) and Poisson-predicted (squares) distributions of chiasma numbers per cell for *Atrpa1a*. Bar, 10 μm.

fertility compared with *Atrpa1a*, setting fewer seed (mean = 4.85/silique; 8.65% of wild-type) and forming shorter siliques (mean = 7.22 mm; 45.58% of wild-type). These figures are consistent with published data for *Atmsh4* (Higgins *et al*, 2004). Cytological analysis of the chiasma frequency in metaphase I chromosome spread preparations revealed a reduction in chiasma frequency to a mean of 1.08 ($n = 50$). Comparison with the residual chiasma frequency (mean = 1.2, $n = 50$) and distribution in an *Atmsh4* mutant grown under identical conditions revealed that there was no significant difference ($P = 0.597$).

Although class I COs are significantly reduced in *Atrpa1a*, they are not entirely absent. Simply subtracting the mean chiasma frequencies for *Atrpa1a* and the *Atrpa1a/Atmsh4* double mutant would suggest that about 2–3 class I COs per cell occur in the absence of AtRPA1a. This is consistent with the immunolocalization studies, which revealed the presence of a few AtMLH1 foci in *Atrpa1a* nuclei at pachytene. In a previous analysis of an *Atmlh3* mutant, we demonstrated that loss of the protein results in a significant reduction in CO formation. Evidence suggests that CO imposition is lost such that a majority (~70%) of class I CO-designated recombination intermediates are resolved as non-COs (Jackson *et al*, 2006). Based on this, it would be predicted that the chiasma frequency in an *Atrpa1a/Atmlh3* double mutant would be intermediate between *Atrpa1* and the *Atrpa1a/Atmsh4* double mutant. We therefore investigated if this was the case. Chiasma counts from metaphase I chromosome spread preparations from an *Atrpa1a/Atmlh3* double mutant gave a mean chiasma frequency of 1.76 ($n = 50$). This figure is significantly lower than the chiasma frequency in either the *Atrpa1a* or *Atmlh3* mutant (mean = 3.92, $n = 50$) ($P < 0.0001$; $P < 0.0001$, respectively), but significantly greater than that in *Atmsh4* and *Atrpa1a/Atmsh4* ($P < 0.01$; $P < 0.01$, respectively). Hence, as predicted, it appears that absence of AtMLH3 activity results in a loss of CO imposition affecting a large proportion, but not all, of the AtMSH4-dependent class I COs that occur in the *Atrpa1a* mutant.

Together, the data from the analysis of the double mutant lines are consistent with the proposal that the reduction in COs/chiasmata in *Atrpa1a* is primarily due to a detrimental effect on the formation of class I COs and that loss of the protein has no obvious effect on the formation of the class II COs.

Discussion

Arabidopsis possesses several RPA1 paralogues

Unlike the situation in budding yeast and mammals, inspection of the *Arabidopsis* genome reveals five *RPA1* paralogues (Shultz *et al*, 2007). During a survey of the meiotic proteome of *B. oleracea*, a close relative of *Arabidopsis*, we identified peptides corresponding to one of these, AtRPA1a (Sanchez-Moran *et al*, 2005). Subsequent analysis of a homozygous *Atrpa1a* mutant indicated that the gene was not required for vegetative growth, but was essential for normal levels of fertility. A previous study primarily focusing on *RPA1* paralogues in rice had also included a preliminary analysis of *Atrpa1a*, from which the authors concluded that loss of *Atrpa1a* was lethal due to a failure to recover *Atrpa1a* homozygotes. Similarly, they reported that attempts to use RNA interference to knockdown AtRPA1a resulted in lethality (Ishibashi *et al*, 2005). Although the basis of this discrepancy is uncertain, several factors could account for it. In the earlier study only limited numbers of *Atrpa1a* progeny were analysed. Also, the *Atrpa1a* lines were obtained from different

sources. The earlier study used seed from ABRC, whereas the line used in this study was obtained later from NASC. Hence, it is likely that our material had been grown through more generations. It is possible that earlier generations contained an additional linked T-DNA insertion that may have affected an essential gene, which combined with a limited analysis may account for the failure to identify homozygous lines. The RNAi studies may have been confounded by the construct downregulating other members of the *AtRPA1a* family as 'off-targets'. This phenomenon is reported to be a potential problem (Xu *et al*, 2006). Regardless of the underlying explanation, our complementation analysis study confirmed that the basis for the meiotic phenotype was due to the loss of AtRPA1a. Moreover, an *Atrpa1a* homozygote is now available from NASC and our subsequent analysis of an additional independent allele GABI_406D08 confirms the meiotic phenotype.

The finding that *Arabidopsis* contains several *RPA1* paralogues is reminiscent of previous observations with *AtSPO11*. With the exception of *Arabidopsis*, all organisms analysed so far possess a single *SPO11* gene. In the case of *Arabidopsis*, three *SPO11* paralogues have been identified (Hartung and Puchta, 2000; Grelon *et al*, 2001). Two of these, *AtSPO11-1* and *AtSPO11-2*, have been shown to work in conjunction to initiate meiotic DSB formation (Grelon *et al*, 2001; Stacey *et al*, 2006; Hartung *et al*, 2007). One general factor that could in part account for these duplications is that *Arabidopsis* is thought to have had a tetraploid ancestor and that its extant form retains duplicated regions encompassing around 60% of the genome (AGI, 2000).

DNA double-strand break repair is not dependent on AtRPA1a

RPA is implicated in several activities during homologous recombination. *In vitro* studies using purified RPA from budding yeast have led to the suggestion that *in vivo* RPA binds to ssDNA prior to RAD51, which then displaces it to form a functional nucleoprotein complex that can mediate strand-exchange. This process is impaired when the RPA complex is prepared using the recombination-deficient *rfa1-t11* allele due to the displacement of the mutant form of RPA by RAD51 occurring more slowly than normal (Kantake *et al*, 2003). Other analyses of the *rfa1-t11* and *rfa1-t48* mutants have revealed that although DSB formation is normal and strand-resection to form a 3′ single-stranded tail occurs in a timely fashion, the 5′ ends of the DNA at the break sites undergo hyper-resection indicative of a failure in the repair process (Soustelle *et al*, 2002). More recently a study of repair following HO endonuclease-induced DSB formation at the MAT locus in an *rfa1-t11* background indicates that the mutant cells are unable to undergo strand-exchange, despite apparently normal Rad51 loading. It is suggested that RPA may bind to the displaced D-loop, thus stabilizing it and preventing reversal of the strand-exchange reaction (Wang and Haber, 2004). This observation is consistent with conclusions of an earlier biochemical study that RPA stabilized Rad51-mediated strand-exchange intermediates through sequestration of the displaced DNA strand (Eggler *et al*, 2002).

In *Arabidopsis* the failure to repair meiotic DSBs is associated with a variety of mutants, such as *Atrad51*, *Atmre11* and *Atmnd1*, that are defective in early steps of the homologous recombination pathway (Li *et al*, 2004; Puizina *et al*, 2004; Kerzendorfer *et al*, 2006). This is manifested by chromosome fragmentation at metaphase I. In the case of *Atrpa1a* no evidence of fragmentation was detected. Hence, it is clear that in contrast to the recombination-deficient *rfa1* alleles in budding yeast, *AtRPA1a* is not essential for meiotic DSB repair. This suggests that one or more of the remaining *AtRPA1* paralogues present in *Arabidopsis* may undertake a role during the early stages of the recombination pathway. It is also conceivable that AtRPA1a is also normally active during these early steps, possibly in conjunction with one of the other AtRPA1 proteins, but in its absence this role can be fulfilled by another member of the AtRPA1 family.

Analysis of an Atrpa1a mutant suggests a role in CO formation

Analysis of budding yeast mutants has revealed an important role for RPA1 in the early stages of meiotic recombination. However, immunolocalization studies in mouse have indicated that the protein is present throughout much of prophase I, thereby implying that RPA may play a further role in the later stages of the recombination pathway (Plug *et al*, 1998; Moens *et al*, 2007). Consistent with this, recent *in vitro* biochemical studies of the RPA protein from the budding yeast mutant *rfa1-t11* have revealed that the protein is defective in promoting Rad52-mediated strand annealing and second-end capture following D-Loop formation (Sugiyama *et al*, 2006).

Our studies revealed that the chronology of AtRPA1a localization in *Arabidopsis* is essentially the same as that of RPA in mouse. The protein is present throughout much of prophase I. Loss of AtRPA1a coincides with the removal of AtMSH4 foci and appearance of AtMLH1, but by the time AtMLH1 forms foci on pachytene chromosomes, AtRPA1a is no longer associated with the chromatin. Thus it seems likely that AtRPA1a is not required to maintain the interaction of the MutL heterocomplex with the CO intermediate.

Studies in chromosome spread preparations of *Atrpa1a* meiocytes indicated no discernable effect on the localization of AtRAD51 compared with wildtype. Similarly the distribution of AtMSH4 foci was indistinguishable from that in wildtype. This was accompanied by apparently normal elaboration of the chromosome axes and SC based on the localization of AtASY1 and AtZYP1, respectively. Complete polymerization of the SC in *Atrpa1a* is indicative that the AtRPA1a protein is not essential for early stages in the recombination pathway. This is based on previous studies that *Arabidopsis* meiotic mutants affecting DSB formation and resection and strand-exchange are asynaptic, whereas mutants deficient in proteins such as AtMSH5 and AtMLH3 which are required later in the recombination pathway undergo full synapsis (Couteau *et al*, 1999; Jones *et al*, 2003; Jackson *et al*, 2006; Higgins *et al*, 2008b). However, our analysis revealed that although the number and chronology of AtMSH4 foci was normal, a significant deficiency in the number of AtMLH1 foci compared with wildtype was apparent. This implies a defect in the formation of COs and was substantiated by our observation that the chiasma frequency at metaphase I in *Atrpa1a* is significantly reduced. These data provide direct evidence that AtRPA1a plays a key role at a late stage in the processing of recombination pathway intermediates to form COs.

How might loss of AtRPA1a disrupt CO formation?

Studies have demonstrated that the MutS heterodimer Msh4/Msh5 and the MutL heterodimer Mlh1/Mlh3 are essential for the formation of meiotic COs (Hoffmann and Borts, 2004; Kolas and Cohen, 2004). Biochemical studies of purified human hMSH4/hMSH5 have led to a model whereby the complex forms a sliding clamp structure that encircles the two homologous DNA duplexes in progenitor dHjs to stabilize the formation of the dHj intermediate (Snowden *et al*, 2004, 2008). It is proposed that the MutL proteins impose resolution of the dHJs as COs, presumably by influencing which DNA strands are cut and religated during resolution, but details of this process have yet to be determined (Wang *et al*, 1999). Based on the *Atrpa1a* phenotype, it seems feasible that loss of AtRPA1a compromises either maturation of the unligated progenitor dHj or alternatively, the protein may be required for efficient interaction between the MutL proteins and the dHj intermediate. In both instances this would lead to the reduction in the number of AtMLH1 foci and chiasmata that is observed in the mutant.

In the model for hMSH4/hMSH5 action, it is proposed that the complex binds duplex DNA adjacent to the D-loop, prior to branch migration and second-end capture (Snowden *et al*, 2004). Taking into account the *in vitro* studies using the RPA complex from *rfa1-t11* (Sugiyama *et al*, 2006) and assuming that, despite the apparent lack of an obvious RAD52 homologue (Iyer *et al*, 2002), strand-annealing and second-end capture during meiosis in *Arabidopsis* are substantially the same as in budding yeast, then one may predict that loss of AtRPA1a could dramatically reduce the efficiency of this step (Figure 7). In *Atrpa1a* localization of AtMSH4 appears normal, but subsequent localization of AtMLH1 and AtMLH3 is reduced. This could reflect the fact that the heterodimer is compromised in its progression from the initial interaction to the formation of a mature dHj-associated complex. However, if this explanation is correct, then our data indicate that loss of AtRPA1a does not completely block the AtMSH4-dependent class I CO pathway. This is based on the observation that the residual CO/chiasma frequency in *Atrpa1a* is significantly higher than that in a *Atmsh4* mutant. Importantly, it seems that the 'extra' chiasmata in *Atrpa1a* are residual class I COs as they are absent in an *Atmsh4/Atrpa1a* double mutant, which forms the same number of chiasmata as *Atmsh4*. How might one account for the fact that the residual chiasma frequency in *Atrpa1a* is greater than in *Atmsh4* as it implies that a proportion of the CO-designated intermediates still proceed to dHjs in the absence of AtRPA1a? One possible scenario is that the proposed role for AtRPA1a in second-end capture is partially fulfilled by another of the AtRPA1 family, all of which are transcribed to some extent in stamen tissue (www.genevestigator.ethz.ch/gv/index.jsp). If so, then it would account for the phenotype of the *Atmsh4/Atrpa1a* double mutant. Despite the capability of forming at least a proportion of the normal complement of progenitor dHJs in the absence of AtMSH4, the mutant would not be able to process them further. Hence, only the class II, AtMSH4-independent, non-interference sensitive COs would remain, just as in the *Atmsh4* single mutant. A corollary of this is that it would be predicted that an *Atrpa1a/Atmlh3* double mutant would form fewer COs/chiasmata than an *Atrpa1a* mutant. The rationale for this is that class I AtMSH4-dependent recombination intermediates require the activity of AtMLH3 in conjunction with AtMLH1 to ensure their resolution as COs. In the absence of AtMLH3, COs/chiasmata exhibit a Poisson distribution indicating that normal control is lost such that only a random subset of these CO-designated class I intermediates give rise to COs. Hence, in an *Atrpa1a/Atmlh3* mutant the class II COs would be unaffected, but the residual class I-designated COs would resolve at random to either COs or non-COs as in the *Atmlh3* mutant. Overall, this would lead to a reduction in the number of chiasmata at metaphase I. This is exactly what was observed in this investigation.

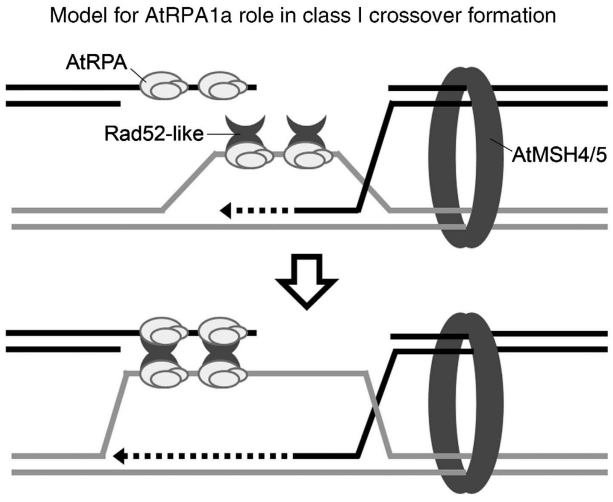

Figure 7 A model for AtRPA1a during second-end capture. We propose that an RPA complex containing AtRPA1a coats the D-loop and ssDNA tail of the resected second strand from the DSB site to mediate second-end capture through a RAD52-like protein (based on Sugiyama *et al*, 2006). In the absence of AtRPA1a this step is compromised leading to a reduction in CO formation.

It is possible that the primary effect of loss of AtRPA1a is on the MutL complex rather than the MutS complex. On the balance of evidence this is perhaps less likely, but cannot yet be excluded. Nevertheless, if this is the case, then it is most likely that initial loading of AtMLH1/AtMLH3 is destabilized. This is based on observations in mouse and *Arabidopsis* which demonstrate that loading of the MutL proteins occurs at the time that the RPA complex is dissociating from the chromatin, such that very few mixed complexes are detected (Moens *et al*, 2007). However, the fact that *Atrpa1a* nuclei are observed containing up to 5 AtMLH1 foci reveals that this step cannot be totally dependent upon AtRPA1a.

The finding that there is no significant difference in the chiasma frequency of *Atmsh4/Atrpa1a* compared with *Atmsh4* indicates that the formation of class II interference-independent COs is not dependent on AtRPA1a activity and strongly suggests that the predominant role of AtRPA1a is in class I CO formation. However, we cannot entirely exclude the possibility that AtRPA1a may play a role in class II CO formation. Recent studies indicate that class II COs in *Arabidopsis* are only partially dependent on Mus81. We estimate that AtMUS81 participates in about a third of class II COs, approximately 5% of the total COs (Higgins *et al*, 2008a). This suggests that there are at least two pathways leading to class II COs in *Arabidopsis*. Thus, if AtRPA1a was required for only a proportion of class II COs and accepting that there could be some redundancy with other members of the AtRPA1 family, the overall reduction in CO frequency

would be very small and unlikely to be detectable by chiasma counts.

Materials and methods

Plant material and nucleic acid extraction

A. thaliana ecotype Columbia (0) was used for wild-type analysis. T-DNA insertion lines SALK_017580 and GABI_406D08 were obtained from NASC for mutant analysis (Alonso, 2003). Plants were grown, material harvested and nucleic acid extractions were performed as described earlier by Higgins *et al* (2004).

T-DNA insertion site mapping

The T-DNA insertion site of SALK_017580 was amplified with primers LBa1 5′-TGGTTCACGTAGTGGGCCATCG-3′ and RPA-R1 5′-CAACCCTGTTCGGAGGCG-3′. The PCR product was cloned into pDrive (Qiagen) and sequenced. Pairs of primers were used to determine whether the plants were homozygous or heterozygous for the T-DNA insertion. Primers RPA-F1 5′-GAGTATTCGTGGC TATGTATTTGG-3′ and RPA-R1 were used to amplify the wild-type genomic region of SALK_517580 and primers LBa1 and RPA-R1 were used to amplify the region where the T-DNA had inserted.

Construction of the AtRPA1a complementation plasmid

Primers RPA-COMP-F 5′-CCACTAGTAAGCTTCTCCCGCAAAATTCA GC-3′ and RPA-COMP-R 5′-CCACTAGTTAATATAATAGTGTACTAAA CTCGAGCTTGC-3′ were used to amplify the entire *AtRPA1a* coding sequence with flanking 5′ and 3′ UTR regions from cDNA clone pda 11728 (Riken GSC/BRC). The PCR product was cloned into the binary vector pPF408 (Siaud *et al*, 2004) using *Spe*I sites incorporated into the primers. The construct was confirmed by sequencing.

Plant transformation

The binary plasmid construct was introduced into *Agrobacterium tumefaciens* LBA 4404 and plants transformed as described earlier (Higgins *et al*, 2004). Transformed plants were selected by spraying with glufosinate-ammonium (0.1 g/l), on appearance of the first true leaves, followed by two more sprayings at 8-day intervals.

Antibody production

Primers RPA-SUB-N 5′-CCGCTAGCCCGGTGAGTTTGACTCCGAAC-3′ and RPA-SUB-170 5′-CCCTCGAGAGATGGCCTGAAGCTTGGAGTA TTG-3′ were used to amplify a 510 bp fragment encoding a 170aa region comprising residues 2 to 171 of *AtRPA1a* from cDNA clone pda 11728 (Riken GSC/BRC). The PCR product was cloned into the expression vector pET21b (Novagen) using *Nhe*I and *Xho*I sites incorporated into RPA-SUB-N and RPA-SUB-170, respectively. Recombinant His-tagged protein was isolated from *E. coli* BL21 (Novagen) under native conditions using Ni-agarose following the manufacturer's protocol (QIAGEN). Polyclonal antiserum against the recombinant protein was raised in rat (ISL, Paignton, UK).

Peptide antiserum was raised in rabbit against the 20aa sequence ETDTEAQKTFSGTGNIPPPN (residues 116–135) conjugated to KLH (Sigma-Genosys Ltd.).

Both antibodies were used at a dilution of 1/500. Specificity was established by application to the *Atrpa1a* deletion mutant as described in the Results section.

Nucleic acid sequencing

Nucleotide sequencing was carried out by the Functional Genomics Unit, School of Biosciences, University of Birmingham, UK.

Cytological procedures

The cytological methods were carried out as described earlier (Higgins *et al*, 2004). Besides anti-AtRPA1a (described above), the following antibodies were used in this study: anti-ASY1 (rabbit/rat, 1/500 dilution), anti-MSH4 (rabbit, 1/500 dilution), anti-ZYP1 (rabbit/rat, 1/500 dilution), anti-AtRAD51 (rabbit, 1/500 dilution), anti-AtMLH1 (rabbit, 1/200 dilution), and anti-MLH3 (rabbit/rat, 1/200 dilution) (Mercier *et al*, 2003; Higgins *et al*, 2004, 2005). FISH on metaphase I chromosomes was carried out using the 45*S* rDNA and 5*S* rDNA probes. Microscopy was conducted using a Nikon Eclipse T300 Microscope (Tokyo, Japan). Image capture and image analysis was done using SmartCapture 2 (Digital Scientific, Cambridge, UK).

Statistical procedures

Observed and Poisson-expected numbers of chiasmata per cell were tested for agreement using a chi-squared (χ^2) test. For the purposes of this test, classes were grouped such that none of the expected values fell below five (Lancaster, 1966). The number of degrees of freedom was given by the number of classes (after grouping) minus two, due to the necessity of estimating a parameter, μ, the mean. Analysis of variance was used to compare chiasma frequency between mutant lines.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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