Retinoblastoma protein is essential for early meiotic events in *Arabidopsis*

Zhong Chen^{1,4}, James D Higgins^{2,4},
Jeanie Tan Li Hui¹, Jing Li¹,
F Chris H Franklin^{2,*} and
Frédéric Berger^{1,3,*}

¹Temasek Life Sciences Laboratory, Singapore, ²School of Biosciences, University of Birmingham, Birmingham, UK and ³Department of Biological Sciences, National University of Singapore, Singapore

We have analysed the role of RBR (retinoblastoma related), the *Arabidopsis* homologue of the tumour suppressor Retinoblastoma protein (pRb), during meiosis. We characterise the *rbr-2* mutation, which causes a loss of RBR in male meiocytes. The *rbr-2* plants exhibit strongly reduced fertility, while vegetative growth is generally unaffected. The reduced fertility is due to a meiotic defect that results in reduced chiasma formation and subsequent errors in chromosome disjunction. Immunolocalisation studies in wild-type meiocytes reveal that RBR is recruited as foci to the chromosomes during early prophase I in a DNA double-strand-break-dependent manner. In the absence of RBR, expression of several meiotic genes is reduced. The localisation of the recombinases AtRAD51 and AtDMC1 is normal. However, localisation of the MutS homologue AtMSH4 is compromised. Additionally, polymerisation of the synaptonemal complex protein AtZYP1 is abnormal. Together, these data indicate that loss of RBR during meiosis results in a reduction of crossover formation and an associated failure in chromosome synapsis. Our results indicate that RBR has an important role in meiosis affecting different aspects of this complex process. *The EMBO Journal* (2011) 30, 744–755. doi:10.1038/emboj.2010.344; Published online 7 January 2011
Subject Categories: cell cycle; plant biology
Keywords: *Arabidopsis*; meiosis; meiotic recombination; retinoblastoma

Introduction

Meiosis is a specialised cell division in which a single round of DNA replication is followed by two rounds of nuclear division to produce four haploid products. In most species, recombination between homologous pairs of chromosomes is

an essential feature of meiosis. Recombination during prophase I of meiosis results in genetic crossover (CO) formation, which establishes physical links between the homologues that are cytologically visible as chiasmata (Jones, 1984; Jones and Franklin, 2006). The chiasmata, in conjunction with sister chromatid cohesion, which is established during or soon after S phase, facilitates accurate chromosome disjunction at the metaphase I-to-anaphase I transition. At this first division, sister chromatid cohesion is lost along the chromosome arms, but is maintained at the centromeric region. This allows segregation of the homologues, but maintains the inter-sister chromatid links until the second meiotic division.

In budding yeast, recombination is initiated by the formation of DNA double-strand breaks (DSBs) catalysed by the topoisomerase-related protein Spo11 (Keeney *et al*, 1997). The DSBs are then resected to generate 3'-single-stranded tails that interact with the RecA homologues Rad51 and Dmc1 to form nucleoprotein filaments. The filament on one side of the break then invades the homologous duplex DNA of one of the two non-sister chromatids to form a stable single-end invasion intermediate. The displaced DNA strand forms a D-loop that extends as the invading strand is extended. This enables the capture of the 3'-end on the other side of the break. Subsequent ligation of the broken DNA strands results in the formation of the double Holliday junction (dHj) recombination intermediate that is then resolved to form a CO (reviewed in Neale and Keeney, 2006). Studies suggest that meiotic recombination in *Arabidopsis* shares many features in common with that of budding yeast and mammals (Hamant *et al*, 2006), although some differences have emerged during evolution. For instance, DSB formation in *Arabidopsis* involves two Spo11 paralogues, AtSPO11-1 and AtSPO11-2, rather than a single Spo11 protein in yeast (Grelon *et al*, 2001; Stacey *et al*, 2006).

A prominent feature of meiosis is that the chromosomes undergo spatial and structural reorganisation that are dependent on the recombination machinery. At leptotene, the sister chromatids form looped chromatin arrays that are associated by a closely apposed linear protein axis that is elaborated along the base of each looped array. Recombination complexes assemble at Spo11-dependent DSBs that form at sites within chromatin loops that have become spatially associated with the chromosome axes (reviewed in Zickler and Kleckner, 1999). In most organisms, this enables alignment of the homologous chromosomes and requires the activity of the strand-exchange proteins Rad51 and Dmc1 that are recruited to the break site following strand resection. The mechanism of chromosome alignment is likely the same in *Arabidopsis*, as loss of AtDMC1 results in asynapsis, such that only univalent chromosomes are observed at metaphase I (Doutriaux *et al*, 1998; Sanchez-Moran *et al*, 2007). During zygotene, assembly of the synaptonemal complex (SC) occurs. This is a tripartite proteinaceous structure that intimately links the homologous chromosomes along their entire

*Corresponding authors. FCH Franklin, School of Biosciences, University of Birmingham, Birmingham B15 2TT, UK.
Tel.: +44 121 414 5910; Fax: +44 121 414 5925;
E-mail: F.C.H.Franklin@bham.ac.uk or F Berger, Department of Chromatin and reproduction, Temasek Life Sciences Laboratory, 1 Research Link, National University of Singapore, Singapore 117604, Singapore. Tel.: +65 656 872 7818; Fax: +65 656 872 7007;
E-mail: fred@tll.org.sg

⁴These authors contributed equally to this work

Received: 5 July 2010; accepted: 29 November 2010; published online: 7 January 2011

length (Page and Hawley, 2004). Completion of SC formation denotes the onset of pachytene, during which recombination is completed. At the end of pachytene, the SC breaks down and the chromosomes condense during diplotene/diakinesis to reach a maximum condensation at metaphase I before the first meiotic division. This is followed by the second meiotic division, leading to the formation of a tetrad containing four haploid spores. Each spore undergoes two mitotic divisions, leading to the development of mature pollen.

Although meiosis is not a cell cycle, coordination and progression through meiosis requires many of the components of the mitotic cell cycle (Marston and Amon, 2004). Cyclin-dependent kinases (CDKs) have been extensively studied in the context of the mitotic cell cycle in yeast, mammals and plants. They are also required during meiosis (Kishimoto, 2003; McKim and Hawley, 1995). Studies in budding yeast have shown that the CDK, Cdc28, forms a complex with B-type cyclins to promote chromosome replication before the onset of meiosis and has a role in the formation of Spo11-dependent DSBs (Stuart and Wittenberg, 1998; Henderson *et al*, 2006). Cdc28 has also been shown to be required for normal SC formation, indicating that it has an important role in meiotic chromosome morphogenesis (Zhu *et al*, 2010). A *cdk2* knockout mouse was found to be defective in SC formation, indicating that CDKs are also important during meiosis in multicellular organisms (Ortega *et al*, 2003). Relatively little is known about the meiotic role of cell-cycle regulators in plants. *SOLO DANCERS* (*SDS*) is thought to encode a plant-specific cyclin that is required for chromosome synapsis and recombination during prophase I in *Arabidopsis* (Wang *et al*, 2004). A second cyclin, *CYCLIN A1;2*, encoded by *TARDY ASYNCHRONOUS MEIOSIS* (*TAM*) is required for timely and synchronous meiotic progression (Magnard *et al*, 2001). A weak allele of the CDKA (*CDKA;1*), which is the *Arabidopsis* homologue of Cdk2/Cdc28, was shown to affect meiosis (Dissmeyer *et al*, 2007). Plants carrying the *cdka;1* allele appeared to initiate meiosis normally, but cytokinesis occurred after the first meiotic division rather than after the second division. SMG7, an *Arabidopsis* protein that is thought to have a role in non-sense-mediated RNA decay, is proposed to promote exit from meiosis by regulating CDK activity (Riehs *et al*, 2008).

The human tumour suppressor gene retinoblastoma (*Rb*) is an important target for CDK phosphorylation during the cell cycle, wherein it regulates the G1/S transition by acting as a repressor of E2F transcription factors (E2Fs; Friend *et al*, 1986). Activated CDK-cyclin complexes phosphorylate Retinoblastoma protein (pRb) at a later stage of G1 phase causing the protein to lose its binding affinity for E2Fs (Weinberg, 1995). E2Fs then regulate the expression of a variety of genes required for cell-cycle progression and cell differentiation (Dimova and Dyson, 2005). The Rb-E2F pathway is also conserved in plants (Shen, 2002).

In addition to its key cell-cycle regulatory function, pRb also recruits chromatin remodelling factors that exert a broad range of cellular functions distinct from cell-cycle control (Brehm and Kouzarides, 1999), including cell fate regulation (Macaluso *et al*, 2006), senescence (Funayama and Ishikawa, 2007) and apoptosis (Harbour and Dean, 2000). pRb also interacts directly with structural chromatin components such as condensins (Longworth *et al*, 2008) and origin of replication proteins (Ahlander *et al*, 2008). In mice, knockout of *Rb*

function causes excessive proliferation and abnormal placental development, leading to embryonic lethality (Clarke *et al*, 1992; Jacks *et al*, 1992; Lee *et al*, 1992; Wu *et al*, 2003).

The *Arabidopsis* genome contains a single Rb-related gene (*RBR*; Ebel *et al*, 2004). The loss of function of *RBR* prevents proper gametogenesis, leading to extremely reduced transmission of *rbr* alleles. *RBR* is also expressed in adult cells; however, analysis of this role has been restricted because of the profound effect that its loss has on embryogenesis. RNA interference using a root-specific promoter allowed down-regulation of *RBR* in *Arabidopsis* roots, revealing that the protein regulates root stem-cell maintenance (Wildwater *et al*, 2005). Ectopic expression and repression of *RBR* transcription in somatic tissues have enabled further progress towards gaining a fuller understanding of the role of the protein during post-embryonic development (Park *et al*, 2005; Desvoyes *et al*, 2006; Wyrzykowska *et al*, 2006; Borghi *et al*, 2010).

Thus far, a role for pRb or *RBR* in meiosis has not been established. The animal germ line is difficult to manipulate. However, establishment of the germ line in plants takes place during post-embryonic development and is, therefore, amenable to genetic dissection (Berger *et al*, 2008). The diploid vegetative phase dominates the life cycle until flowers are produced. Flowers bear sexual organs in which meiosis takes place, and produces haploid structures in which gametes differentiate (Berger *et al*, 2008; Drews and Yadegari, 2002). Thus, despite the fact that loss of *RBR* is gametophyte lethal, it has been possible to investigate the role of *RBR* during both male and female gametogenesis by taking advantage of the fact that half the gametes produced by a heterozygous *rbr* mutant possess a wild-type copy of the gene and the remainder carry a defective allele (Ingouff *et al*, 2006; Johnston *et al*, 2008; Chen *et al*, 2009). As a result, it has been established that the primary defect in both male and female gametogenesis is a failure in cell-cycle regulation, leading to overproliferation of the cells within the developing gametophyte. Consequently, genetic transmission of mutant *rbr* alleles is completely prevented on the female side and strongly reduced on the male side.

In this study, we have been able to take advantage of this reproductive programme to determine the potential role of *RBR* in meiosis. We provide evidence that clearly indicates a crucial role for *RBR* during prophase I of meiosis. Our studies reveal that loss of *RBR* results in reduced CO formation and aberrant chromosome synapsis, leading to chromosome mis-segregation and gamete lethality.

Results

The *rbr-2* allele results in aberrant *RBR* mRNA splicing and is associated with reduced fertility

As loss of *RBR* results in cellular proliferation in the male gametophyte, it was postulated that it would be possible to rescue paternal transmission of an *rbr* mutant in a heterozygous background by downregulation of *CDKA;1*, as this would be expected to restore cellular proliferation in the *rbr* pollen to the wild-type level (Chen *et al*, 2009). When this hypothesis was tested in a *rbr-2/+; cdka;1/+* background, the proportion of viable pollen increased in line with expectation (Chen *et al*, 2009). Subsequently, we found that maternal transmission of *rbr-2* was also rescued by *cdka;1*

Table I Genetic rescue of *rbr-2* transmission by *cdka;1*

Female × male	F1 progeny genotype (%)			n
	Wild type	<i>rbr-2/+</i>	<i>rbr-2/rbr-2</i>	
<i>rbr-2/+</i> × Col	100	0	NA	268
<i>rbr-2/+; cdka;1/+</i> × Col	51.2	48.8	NA	342
Col × <i>rbr-2/+</i>	92.0	8.0	NA	637
Col × <i>rbr-2/+; cdka;1/+</i>	53.0	47.0	NA	430
<i>rbr-2/+</i> × <i>rbr-2/+</i>	79.1	20.9	0	1212
<i>rbr-2/+; cdka;1/+</i> × <i>rbr-2/+; cdka;1/+</i>	29.2	48.6	22.2	370
	Wild type	<i>rbr-3/+</i>	<i>rbr-3/rbr-3</i>	
<i>rbr-3/+</i> × Col	100	0	NA	190
<i>rbr-3/+; cdka;1/+</i> × Col	100	0	NA	187
Col × <i>rbr-3/+</i>	94.3	5.7	NA	280
Col × <i>rbr-3/+; cdka;1/+</i>	93.9	6.1	NA	278
<i>rbr-3/+</i> × <i>rbr-3/+</i>	93.7	6.3	0	380
<i>rbr-3/+; cdka;1/+</i> × <i>rbr-3/+; cdka;1/+</i>	93.9	6.1	0	374

Figure 1 RBR expression in vegetative tissues and in meicytes of *rbr-2* plants. (A) Morphological features of wild-type and homozygous *rbr-2* plants. The vegetative development is similar, but *rbr-2* plant produce flowers that bear reduced amount of pollen. As a consequence, very few pollen grains are deposited on the stigma causing sterility. (B) Western blots for detection of RBR proteins in leaves produced by wild-type and *rbr-2* plants using anti-RBR-C and anti-RBR-N antibodies. The loading controls are shown under the western blots. (C) Expression analysis in buds containing meicytes of genes encoding meiotic proteins and RBR transcripts representing the wild-type unspliced (*RBR-3'*) and both the wild-type and spliced transcripts (*RBR-5'*) produced by *rbr-2*. Scale bar, 8 cm and 1 mm (inset).

(Table I). However, an unanticipated outcome to these experiments was the finding that a quarter of the progeny from *rbr-2/+; cdka;1/+* self-fertilised plants were *rbr-2* homozygous plants. This indicated that *rbr-2* homozygous

seeds survived, irrespective of the presence of CDKA;1 (Figure 1A). This rescue was not observed with the allele *rbr-3* (Table I), which suggested special properties associated with the *rbr-2* allele. Although, the vegetative growth of *rbr-2*

homozygote plants revealed some differences to that of wild-type plants, such as an increase in hypocotyl length and a slight elevation in ploidy level, no dramatic effects were apparent (Figure 1A, Supplementary Figure S1A and B). This was surprising because earlier reports had shown that partial loss of RBR causes hyperproliferation and alters differentiation in various vegetative cell types (Ingouff *et al*, 2006; Park *et al*, 2005; Rossi *et al*, 2003; Sabelli *et al*, 2005; Wildwater *et al*, 2005; Wyrzykowska *et al*, 2006). However, the *rbr-2* plants exhibited a large reduction in fertility (Figure 1A), producing approximately 1–5 seeds per silique, compared with wild-type plants, which produce between 50 and 60 seeds per silique.

Molecular analysis of *rbr-2* revealed the presence of two *RBR* transcripts in vegetative tissues (Supplementary Figure S2A). Expression of the full-length *RBR* product was nearly normal (Supplementary Figure S1C). However, it was reduced in bud tissues, which comprised both vegetative somatic tissues and meiocytes (Figure 1C). DNA sequencing indicated that the novel shorter form resulted from an aberrant splicing event caused by the presence of the T-DNA insert in an intron in the *rbr-2* allele (Supplementary Figure S2B). The T-DNA insert could also be spliced out with intron 12 to generate a transcript identical to the wild-type *RBR* transcript. The alternate form removed exon 12 and the remaining five base pairs from intron 13 (5'-ATAAG-3'), resulting in a truncated *RBR* transcript. The shorter transcript was predicted to produce a truncated RBR protein (RBR_m) devoid of the B-box (Supplementary Figure S2C), which is essential for RBR function (Brehm *et al*, 1998).

Expression of RBR protein in wild-type and *rbr-2* meiocytes during prophase I

The reduced fertility observed in *rbr-2* was consistent with an effect on meiosis. We investigated further the intracellular distribution of RBR during meiosis. To selectively detect the full-length RBR protein, we raised an anti-RBR peptide antibody (Ab), against a sequence within the RBR C-terminal region (RBR-C; see Materials and methods) that was predicted to be absent in the product of the shorter transcript. We also used an Ab (designated as anti-RBR-N) raised against the amino-terminal region (amino acids 1–374; Borghi *et al*, 2010) to detect the expression of the predicted truncated RBR protein together with the full-length RBR protein. We detected a single band of ~125 kDa when protein blots from leaf tissues were probed with anti-RBR-N and anti-RBR-C antibodies (Figure 1B). Although low amounts of mutant-spliced RBR transcripts were detected in leaf tissues (supplementary Figure 2A), the anti-RBR-N Ab did not detect a band corresponding to the truncated RBR protein with the expected lower molecular weight. This suggests that either the amount of truncated protein in leaf tissue is below the detection threshold or that this truncated protein is degraded. It would appear that wild-type RBR and the truncated product are both expressed in vegetative cells with no major development effects (Supplementary Figure S1). In addition, when the truncated transcript was expressed under the *RBR* promoter in transgenic wild-type lines, their vegetative phenotype was indistinguishable from that of the *rbr-2* mutant. In contrast to *rbr-2*, the transgenic plants expressing RBR_m appeared fully fertile (three transgenic lines observed and $n = 10$ siliques per line). This suggested that the truncated

RBR_m protein encoded by the *rbr-2* allele did not negatively compete with RBR when co-expressed in a wild-type background. In summary, vegetative tissues of *rbr-2* plants expressed levels of wild-type RBR that did not affect development.

In buds containing meiocytes from *rbr-2* plants (400–800 µm in length), we detected a lower amount of unspliced wild-type *RBR* transcript than in wild-type buds (Figure 1C). However, most cells within a developing bud are vegetative in nature and only a small proportion are reproductive. Thus, any differences in RBR expression in reproductive cells would be potentially masked. It is also possible that RBR expression levels in wild-type meiocytes is relatively much higher than in surrounding wild-type somatic cells, which would explain the unexpected high impact of *rbr-2* on the level of wild-type RBR transcript. To assess and compare the distribution of RBR protein during meiosis in wild type and *rbr-2*, immunolocalisation studies were conducted on chromosome-spread preparations from pollen mother cells (PMCs) during prophase I using anti-RBR-C antibodies (Figure 2A–E). In wild-type meiocytes, the RBR protein was found to localise along the chromosome axes forming numerous foci (96 ± 7 , $n = 5$) during late G2/early leptotene (Figure 2A). As prophase I progressed, the number of RBR foci decreased, with $38 (\pm 2, n = 5)$ foci detectable at zygotene, which was reduced to $18 (\pm 2, n = 5)$ foci at pachytene (Figure 2B and C). The RBR protein was not detected at later stages (data not shown). When chromosome-spread preparations from *rbr-2* meiocytes were analysed, we could not detect RBR protein (Figure 2D). In contrast, immunolocalisation of RBR on chromosome-spread preparations from wild-type meiocytes using the anti-RBR-N Ab was indistinguishable from that using the RBR-C Ab (Figure 2F–H). When the anti-RBR-N Ab was applied to chromosome spreads from *rbr-2* meiocytes, numerous chromosome-associated foci were detected at early leptotene, and their number reduced as prophase I progressed (Figure 2I). As these foci were not detected with the RBR-C Ab, it seems likely that they correspond to the predicted truncated RBR_m protein encoded by the spliced RBR transcript. In conclusion, in *rbr-2* meiocytes, the truncated RBR_m is by far the major protein encoded by *RBR*, suggesting that *rbr-2* meiocytes essentially produce the spliced *RBR* transcript. As reported above, the expression of truncated RBR_m under the control of pRBR promoter does not cause any phenotype change, suggesting that the absence of wild-type RBR protein is the basis for the reduced fertility in *rbr-2* plants.

Loss of RBR reduces meiotic gene expression

RBR controls transcription, at least in part, by inhibiting E2Fs (Macaluso *et al*, 2006). A large number of *Arabidopsis* genes involved in meiosis contain the consensus E2F-binding sites and might be E2F regulated (Supplementary Table S1). Hence, given the lack of wild-type RBR expression in *rbr-2* meiocytes and reduced fertility, we investigated whether loss of protein affected meiotic gene expression in buds (approximately 400–800 µm in length). In *rbr-2* buds containing meiocytes, the expression of genes important for meiotic events during prophase I, including *AtMSH4* (Higgins *et al*, 2004), *STRUCTURAL MAINTENANCE OF CHROMOSOMES 3* (*SMC3*; Liu *et al*, 2002), *TAM* (Magnard *et al*, 2001) and *AtZYP1a* (Higgins *et al*, 2005) was reduced markedly

Figure 2 Immunolocalisation of RBR and RBR_m in prophase I nuclei. (A–C) Immunolocalisation of RBR in wild-type nuclei with anti-RBR C-terminal Ab (RBR-C). (F–H) Immunolocalisation of RBR in wild-type nuclei with anti-RBR N-terminal Ab (RBR-N). (A, F) Leptotene, (B, G) zygotene, (C, H) pachytene; (D) RBR is not detected in *rbr-2* plants by anti-RBR-C Ab; (I) detection of RBR_m in *rbr-2* plants using anti-RBR-N Ab; (E, J) RBR foci are absent in *Atspo11-1-4* meiotic nuclei, as determined using both anti-RBR-C Ab (E) and anti-RBR-N Ab (J). Scale bar, 10 µm.

(Figure 1C). Although the presence of predicted E2F-binding sites in the promoters of several of these genes (Supplementary Table S1) suggested that RBR may act directly on E2F to regulate the expression of key meiotic proteins, it was also possible that the absence of RBR in meiotic cells had indirect effects on transcription of meiotic genes. This was suggested by the fact that the loss of RBR does not affect the expression of *CDC45* (Stevens *et al*, 2004), although its promoter contains a putative E2F-binding site, but does affect the expression of meiotic genes *AtMSH4*, *AtRAD51* and *AtRAD51C*, the promoter of which does not contain an E2F-binding site (Figure 1C and Supplementary Table S1).

***rbr-2* is defective in CO formation and synapsis**

To confirm that the reduced fertility observed in *rbr-2* was due to an effect on meiosis and to establish its nature, a cytological analysis of chromosome-spread preparations from PMCs of mutant and wild-type plants was carried out (Figure 3). All meiotic stages were observed in *rbr-2*, indicating that loss of RBR does not prevent initiation and progression of the meiotic programme. Nevertheless, a number of meiotic defects were clearly apparent, and it was notable that the size range of the buds containing meiotic cells in prophase I was much greater for *rbr-2* than for wild type. In both cases, buds of ~400 µm length contained meiotic cells at leptotene. In wild type, buds of ~600 µm had completed meiosis and contained only tetrads, but at this stage, *rbr-2* buds still contained cells at zygotene/diplotene. Tetrads were not observed in *rbr-2* buds shorter than 800–900 µm. In addition, several different stages were often observed in meiotic cells from anthers within the same bud, whereas wild-type anthers from the same bud contain meiotic cells at more or less the same meiotic stage (Armstrong and Jones, 2003). This suggested that loss of RBR may affect the rate of meiotic progression. During early prophase I (leptotene), *rbr-2* nuclei were indistinguishable from that of wild type (Figure 3A and F). However, in contrast to wild type, which progressed through zygotene to achieve full chromosome synapsis at pachytene, the majority of *rbr-2* chromosomes appeared to

achieve only partial synapsis, with unpaired chromosome axes clearly visible (Figure 3B and G). This was confirmed by immunolocalisation of the SC transverse filament protein AtZYP1 that marks synapsed chromosomes and AtASY1, a component of the chromosome axes (Higgins *et al*, 2005; Armstrong *et al*, 2002; Sanchez-Moran *et al*, 2007). In wild-type meiotic cells, AtZYP1 polymerisation commenced at zygotene, forming a linear signal between each homologous chromosome pair. By pachytene, the AtZYP1 signal was continuous, denoting formation of the mature SC (Figure 4A). In contrast, the polymerisation of AtZYP1 in the *rbr-2* nuclei was incomplete or occasionally absent (Figure 4B). In the wild-type meiotic cells, AtASY1 dissociates from the axes during pachytene/early diplotene, as the SC breaks down (Figure 4C and E). In *rbr-2* meiotic cells, AtASY1 localisation appeared normal during much of prophase I, but it did not dissociate in a timely manner and persisted through diakinesis (Figure 4D and F).

Wild-type diakinesis is marked by closely apposed homologous chromosome pairs (bivalents) linked by chiasmata, the cytological manifestation of genetic COs (Figure 3C). In contrast, the corresponding *rbr-2* chromosomes appeared to be longer than wild type (mean length 60.06 versus 50.17 µm, respectively; $P < 0.05$) and most lacked chiasmata (Figure 3H). At metaphase I, the wild-type nuclei contained five bivalents (Figure 3D), which following the first and second meiotic divisions formed normal tetrads (Figure 3E). However, at metaphase I, most of the chromosomes in the *rbr-2* nuclei were present as univalents (Figure 3I) that mis-segregated, leading to the formation of four meiotic products, some containing fewer and others more than the wild-type complement of five chromosomes (Figure 3E and J). FISH analysis of metaphase I chromosomes confirmed the reduction of the number of chiasmata in *rbr-2* nuclei to an average of 1.7 ($n = 76$), compared with 9–10 chiasmata typically detected in wild-type nuclei (Supplementary Figure S3A and B; Higgins *et al*, 2004). In addition, close inspection of the *rbr-2* nuclei suggested a tendency for precocious separation of the sister chromatids (Figure 3I). Although, there was no suggestion of a problem with sister chromatid cohesion at diakinesis

Figure 3 Meiotic stages from wild-type and *rbr-2* pollen mother cells. (A–E) Wild type; (F–J) *rbr-2*; (A, F) leptotene, (B, G) pachytene, note the unsynapsed axis in the *rbr-2* mutant (arrows), (C, H) diakinesis, (D, I) metaphase I, (E, J) tetrad stage. Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI). Scale bar, 10 µm.

Figure 4 Immunolocalisation of meiotic proteins in wild-type and *rbr-2* prophase I nuclei. (A, C, E) Wild-type; (B, D, F) *rbr-2* prophase I nuclei. (A, B) AtZYP1 (green), AtMLH1 (red), DAPI (blue); (C, D) AtASY1 (red), DAPI (blue); (E, F) AtASY1 (red), DAPI (blue) at diakinesis. Scale bar, 10 µm.

esis, this observation could suggest some underlying problem, particularly as the transcription analysis suggested that expression of *AtSMC3*, which encodes one of the subunits of the cohesin complex (Lam *et al*, 2005), was significantly reduced (Figure 1C). However, immunolocalisation on chromosome-spread preparations from *rbr-2* meiocytes using an anti-SMC3 Ab suggested that there was no obvious deficiency of the protein (Supplementary Figure 3E and F). Moreover, we have observed a tendency for sister chromatid separation at metaphase I in other *Arabidopsis*

mutants, such as *Atmsh4* and *Atmsh5*, that have low levels of chiasma frequency, yet presumably normal expression of the cohesion complex (JD Higgins and FCH Franklin, unpublished observation; Supplementary Figure 3C and D). Although, a subtle effect could not be excluded, it would appear there are no major defects in sister chromatid cohesion in *rbr-2*.

Loss of RBR affects recruitment of meiotic proteins

Immunolocalisation studies using a panel of antibodies were conducted to gain further insight into the basis of the meiotic defects identified in *rbr-2*. In *Arabidopsis*, initiation of meiotic recombination is dependent on the formation of DNA DSBs by AtSPO11-1 and AtSPO11-2 (Grelon *et al*, 2001; Stacey *et al*, 2006). DSBs may be detected using an Ab to monitor phosphorylation of histone H2AX (γ H2AX) at the break site (Mahadevaiah *et al*, 2001). In *rbr-2*, we detected γ H2AX foci at wild-type levels (>100) in early prophase I (Figure 5A and B). This indicated that RBR was not required for DSB formation. However, the formation of RBR foci observed in wild-type meiocytes (Figure 2A–C and F–H) was absent in the *Atspo11-1-4* mutant, which does not form DSBs (Sanchez-Moran *et al*, 2007; Figure 2E and J). This suggests that formation of RBR foci is dependent on the formation of DSBs.

During crossing over in budding yeast, the meiotic-specific recombinase Dmc1, either in conjunction with or independently of the general recombinase Rad51, catalyses invasion of homologous DNA sequences on one of the non-sister chromatids by the single-stranded DNA from one side of the DSB (Neale and Keeney, 2006). Although, it has not been demonstrated biochemically, analysis of the corresponding *Arabidopsis* proteins AtDMC1 and AtRAD51 would suggest that this process is conserved in plants (Couteau *et al*, 1999; Doutriaux *et al*, 1998; Sanchez-Moran *et al*, 2007). Immunolocalisation of AtDMC1 and AtRAD51 in chromosome spreads from wild-type and *rbr-2* meiocytes at leptotene were indistinguishable revealing, in both cases, the accumulation of >100 axis-associated foci (Figure 5C–F).

In *Arabidopsis*, wild-type levels of chiasma formation are dependent on the combined activity of the MutS homologues AtMSH4 and AtMSH5 (Higgins *et al*, 2004; Higgins *et al*,

Figure 5 DNA double-strand break formation and localisation of early recombination proteins in wild-type and *rbr-2* nuclei. (A, C, E, G) Wild-type; (B, D, F, H) *rbr-2* nuclei. (A, B) Immunolocalisation of γ H2AX at prophase I indicates that DNA double-strand break formation is normal in *rbr-2*; (C, D) localisation of AtRAD51 is normal during early prophase I; (E, F) localisation of AtDMC1 is normal during early prophase I. (G, H) Localisation of AtMSH4 reveals a drastic reduction of foci in *rbr-2*. Scale bar, 10 μ m.

2008b). Immunolocalisation of AtMSH4 on chromosome-spread preparations from wild type revealed numerous (>100) foci that were first detectable at late leptotene (Figure 5G). These then gradually reduced in number throughout zygotene, with only a few foci (<10) persisting until mid-pachytene (Higgins *et al*, 2004). The number of AtMSH4 foci detected in *rbr-2* meiocytes at late leptotene was drastically reduced. In the majority of nuclei (12/22) AtMSH4 foci were entirely absent, with fewer than 50 detected in each of the remaining nuclei inspected (Figure 5H).

In wild-type *Arabidopsis*, it appears that the sites of COs are marked by the MutL homologue, AtMLH1, which is believed to promote CO formation (Wang *et al*, 1999). Dual immunolocalisation in wild-type meiocytes of AtMLH1 together with AtZYP1 reveals 9–11 AtMLH1 foci associated with a fully synapsed SC at pachytene (Figure 4A). In *rbr-2*, the number of AtMLH1 foci was typically in the range 1–3 (Figure 4B). These co-localised to the short stretches of linear AtZYP1 that were present in the mutant. Thus, the reduction

in AtMLH1 in *rbr-2* corresponded with the reduction in chiasmata observed at metaphase I in the mutant.

The number and turnover of RBR foci observed in wild-type meiocytes during prophase I was similar to that previously observed for AtDMC1 and AtMSH4 (Higgins *et al*, 2004; Sanchez-Moran *et al*, 2007). It was of interest to determine whether there was evidence of co-localisation between RBR and the recombination pathway proteins. Dual immunolocalisation of RBR and AtDMC1 in wild-type meiocytes at leptotene showed numerous foci corresponding to each protein associated with the chromosomes at this stage. In many cases, the foci appeared to co-localise (co-localisation $44\% \pm 3$, $n = 10$) or show a close association, but this was incomplete (Figure 6A–D). Dual labelling of RBR and AtMSH4 revealed a similar overlap of foci at leptotene (co-localisation $43\% \pm 2$, $n = 10$), but this reduced to only 19% ($\pm 4.6\%$, $n = 5$) at zygotene (Figure 6E–H). Co-localisation controls suggested that $\sim 40\%$ of these could be random associations.

Loss of RBR compromises both Class I and Class II COs

Previous studies have shown that CO formation in *Arabidopsis*, as in budding yeast, occurs via at least two pathways. Class I COs, which account for $\sim 85\%$ of the total, are dependent on AtMSH4 and exhibit CO interference, the phenomenon whereby COs do not occur in close proximity to each other (Higgins *et al*, 2004; Jones and Franklin, 2006). The remaining Class II COs, some of which are dependent on AtMUS81, do not exhibit CO interference (Higgins *et al*, 2008a). Although, CO formation is dramatically reduced in *rbr-2*, it is not absent. Indeed, the mean CO frequency at 1.7 is similar to that observed in *Atmsh4* (Higgins *et al*, 2004, 2008a,b). This could imply that RBR is primarily involved in the formation of Class I COs. To clarify whether RBR is implicated in Class I COs or both Class I and II, we constructed an *Atmsh4/rbr-2* double-knockout line. Chiasma counts on metaphase I chromosome-spread preparations from homozygous mutants revealed that the mean chiasma frequency was significantly reduced compared with the *Atmsh4* single mutant (0.45 versus 1.29, $n = 115$; $P < 0.001$). This indicates that RBR is important for normal levels of both Class I and Class II COs.

Discussion

The isolation of a homozygous *rbr-2* line has enabled us to investigate the role of RBR during meiosis. Our studies have revealed that loss of RBR results in a reduction in the formation of meiotic COs and a failure of chromosome synapsis, leading to a dramatic reduction in fertility.

RBR_m is specifically expressed in *rbr-2* meiocytes

Although the wild-type RBR protein was expressed in vegetative cells in the *rbr-2* line, only the truncated RBR_m form was detected in meiocytes. DNA sequence analysis suggested that this was due to an aberrant splicing event. Cell-type-specific splicing is common in vertebrates (Matlin *et al*, 2005), but has rarely been observed in plants. There is extensive evidence of meiosis-specific splicing in other species such as budding yeast, fission yeast and mouse (Engbrecht *et al*, 1991; Juneau *et al*, 2007; Averbeck *et al*, 2005; Bellani *et al*, 2010). As yet, there is no direct evidence

Figure 6 Dual immunolocalisation of RBR with recombination with AtDMC1 and AtMSH4 in wild-type prophase I nuclei. (A–D) RBR and AtDMC1 (leptotene); (E–H) RBR and AtMSH4 (zygotene); (A) AtDMC1, (B) RBR, (C) DAPI, (D) merge, (E) AtMSH4, (F) RBR (G), DAPI, (H) merge. Scale bar, 10 µm.

that alternative splicing might have a role during plant meiosis. However, in *Arabidopsis*, mutations in genes encoding essential components of the spliceosome affect gametogenesis, which supports the existence of splicing activity unique to gametes in plants (Gross-Hardt *et al*, 2007; Moll *et al*, 2008). Although the expression of RBR_m arises from an aberrant splicing event, it does perhaps highlight some differences in the activity of the splicing machinery in meiocytes compared with vegetative cells.

RBR is essential for inter-homologue recombination and synapsis

It would appear that early events in the meiotic pathway occur normally in *rbr-2*. As RBR is a negative regulator of the G1/S transition in mitotic cells (Friend *et al*, 1986), and hence most probably in meiocytes, the loss of RBR activity is not expected to prevent meiotic S phase. DSB formation occurs at or near wild-type levels, and there is no evidence of DNA fragmentation in *rbr-2*. This suggests that despite reduced CO formation, the DSBs are efficiently repaired, either through non-CO recombination or via repair using a sister chromatid as the repair template. Our studies indicate that loss of RBR does not prevent meiotic progression, but it may delay progression through prophase I. In many organisms, mutations in recombination proteins lead to the activation of checkpoints that result in meiotic arrest or apoptosis (Roeder and Bailis, 2000). Whether similar checkpoints existed in *Arabidopsis* was initially a matter of debate, as many recombination mutants complete meiosis. However, mutants lacking AtTOP3 α and AtRMI1/BLAP75, which are implicated in the dissolution of dHJs, undergo arrest at telophase I (Chelysheva *et al*, 2008; Hartung *et al*, 2008). Mutants defective in recombination pathway proteins such as AtMSH4 and AtMLH3 show a significant delay in prophase I progression (Higgins *et al*, 2004; Jackson *et al*, 2006). Despite a reduction in CO formation in these mutants, there is no evidence of chromosome fragmentation, indicating that DSBs are eventually repaired. Extrapolating from studies in budding yeast, it has been proposed that an intra-prophase I

surveillance system that detects recombination defects may delay prophase I progression in *Arabidopsis* until DSB repair is complete (Boerner *et al*, 2004; Jackson *et al*, 2006). Thus, it seems likely that the delayed prophase I progression in *rbr-2* is due to a recombination defect rather than a direct consequence of the loss of RBR.

In *Arabidopsis*, inter-homologue recombination requires the activity of the meiosis-specific recombinase AtDMC1. Loss of the protein results in a complete absence of chiasmata. No chromosome fragmentation is observed at metaphase I. This suggests that the AtSPO11-induced DSBs are efficiently repaired using the sister chromatid as the repair template (Couteau *et al*, 1999; Doutriaux *et al*, 1998; Sanchez-Moran *et al*, 2007). Following strand-exchange and stable single-end invasion, subsequent CO formation requires the activity of AtMSH4 (Higgins *et al*, 2004). In *rbr-2* meiocytes, AtDMC1 localisation appears normal, while AtMSH4 foci are reduced or absent. Formation of the SC is also compromised with little polymerisation of the transverse filament protein AtZYP1. Loss of AtDMC1 results in a complete failure of SC formation (Couteau *et al*, 1999; Doutriaux *et al*, 1998; Sanchez-Moran *et al*, 2007). In contrast, *Atmsh4* forms an intact SC, although with some delay (Higgins *et al*, 2004). Thus, in this respect, *rbr-2* resembles *Atdmc1* rather than *Atmsh4*. Together, these observations suggest that RBR has a critical role at some early stage in the recombination pathway following DSB formation and AtDMC1 loading, but before subsequent AtMSH4 loading. This is supported by our finding that RBR is essential for normal levels of both Class I and Class II COs.

The effect of *rbr* on meiotic gene transcription is probably not the basis of the meiotic defect in *rbr-2*

In contrast to mice (Satyanarayana and Kaldis, 2009; Susiarjo *et al*, 2009), very few plant cell-cycle genes have been found to have roles in meiosis, probably due to the overlapping function of these genes in mitosis and meiosis (Mercier and Grelon, 2008). Although RBR affect the expression of meiotic genes under the control of a promoter containing a putative E2F-binding factor, RBR appears to activate their transcription.

However, a direct control of gene expression by the E2F pathway is usually repressive in somatic tissues (Park *et al*, 2005; Desvoyes *et al*, 2006) and in reproductive tissues (Jullien *et al*, 2008). In addition, the meiotic genes that are controlled by RBR and E2F are not overexpressed in vegetative tissues that overexpress E2F (Vandepoele *et al*, 2005), which suggests that additional transcriptional controls specific to meiotic tissues are required. The fact that *rbr-2* also affects the expression of meiotic genes that do not possess a predicted E2F-binding site in their promoter, further supports the idea that the effect of *rbr-2* on meiotic gene expression is probably independent from a direct control by E2F.

Although the expression of AtMSH4 and AtZYP1 is reduced in *rbr-2*, they are nevertheless transcribed at over 60% of the wild-type level. Heterozygous lines carrying a null allele of a wide range of *Arabidopsis* meiotic genes are fully fertile, suggesting that the pathway is resilient to reductions in gene expression. This is supported by a study of *AtSPO11-1* that found that the gene was expressed at ~60% of wild-type level in a heterozygous *Atspo11-1-4^{-/+}* mutant, yet this had no apparent reduction in DSB formation or subsequent chiasma frequency (Roberts, 2010). Of the meiotic genes included in the analysis of transcription, the cohesin *AtSMC3* was most reduced in *rbr-2*. However, immunolocalisation of AtSMC3 appeared normal; while this is not quantitative, it does suggest that the protein is not substantially depleted.

While there was a tendency for precocious separation of sister chromatids at metaphase I, there was no obvious loss of chromosome cohesion in *rbr-2* meiocytes at diakinesis. Precocious sister chromatid separation has been observed in the maize mutant *desynaptic* (Maguire, 1978). It is thought that, in this mutant COs are formed at normal levels, but there is a failure to maintain chiasmata because of an underlying cohesion problem. However, there are obvious defects in both recombination and synapsis in *Arabidopsis rbr-2*, which were not observed in the maize mutant *desynaptic*. This suggests that the basis of the reduced chiasma frequency in these mutants is different. Furthermore, precocious sister chromatid separation is also observed in recombination pathway mutants such as *Atmsh4* and *Atmsh5* (Supplementary Figure 3C and D), in which expression of the cohesin complex is presumably normal. In summary, the level of reduction in meiotic gene expression reported here may be in itself not sufficient to account for the *rbr-2* phenotype. Nevertheless, it is potentially a contributing factor, particularly as the expression of several meiotic genes is reduced in parallel.

A link between RBR and recombination

The fact that foci corresponding to RBR and recombination pathway proteins are localised to the chromosomes during early prophase I could suggest that RBR foci form at or in the vicinity of recombination complexes, particularly as they form about the same number of foci at leptotene following and dependent on AtSPO11-1-mediated DSB formation. A study in budding yeast has shown that the CDK, Cdc28, forms foci during early meiotic prophase I, coinciding with the formation of Rad51 foci (Zhu *et al*, 2010). However, the level of co-localisation was relatively low at 34%. Determining the degree to which there might be a direct spatial relationship between RBR and recombination complexes containing AtDMC1 and AtMSH4 was subject to

the limited resolution afforded by immunofluorescence microscopy using fixed material combined with high numbers of foci present on the chromosomes during early prophase I. Any co-localisation of the RBR and AtMSH4 at zygotene appeared limited, which would support the immunolocalisation studies in *rbr-2*. Although this suggests that the role of RBR precedes AtMSH4 function in relation to CO formation, further analysis will be required to resolve this issue.

Current evidence suggests that the key elements of the core recombination machinery are conserved between plants and budding yeast (Hamant *et al*, 2006). As the latter does not possess an Rb homologue, a direct biochemical interaction between RBR and the recombination machinery is perhaps unlikely, regardless of any spatial relationship. However, studies in budding yeast show that meiotic recombination occurs in the context of chromatin-associated complexes that are juxtaposed to the chromosome axes. Perturbation of the structural organisation of the chromosomes or their underlying axes can adversely influence stable inter-homologue exchange (Kleckner, 2006; Storlazzi *et al*, 2010). In *Arabidopsis*, loss of the chromosome axis protein AtASY1 has been shown to result in the destabilisation of AtDMC1 foci, resulting in a dramatic reduction in CO formation (Sanchez-Moran *et al*, 2007). That loss of pRb could directly affect chromosome organisation is supported by the finding that the *Drosophila* Rb family member RBF1 interacts with the condensin II complex and is essential for proper segregation of mitotic chromosomes (Longworth *et al*, 2008). It is therefore of potential significance that in the *rbr-2* mutant, the chromosomes at diakinesis are significantly longer than those in wild-type meiocytes at the same stage. In addition, while initial localisation of the chromosome axis protein AtASY1 appears normal in the *rbr-2* meiocytes, its depletion from the axes is delayed until diakinesis. Thus, RBR may influence chromosome structure, possibly in the vicinity of recombination sites, to promote the formation of COs. The fact that a few COs can still occur may reflect that, in some cases, DSBs occur at sites where localised modification to the chromosome organisation is not essential. It may be significant that the C-terminus deletion in RBR corresponds to a region that, in other members of the pRb family, interacts with proteins that are important for chromatin remodelling/modification, such as SWI/SNF, histone deacetylases and histone methyltransferases (Longworth and Dyson, 2010).

A link between chromosome organisation and recombination progression has been proposed (Kleckner *et al*, 2004). In this model, meiotic (and mitotic) chromosome conformation undergoes cyclic periods of expansion and contraction, which relate to the status of the chromatin and the underlying axes. In the case of meiosis, these stress/relief transitions are coupled to distinct stages in CO formation, including single-end invasion, to form stable strand-exchange products. The defects observed in *rbr-2* are consistent with the mechanisms proposed by such a model. DSB formation is not affected, as RBR does not appear to participate in this event. However, subsequent stress-dependent CO recombination may be compromised because of defects in chromosome/axis organisation arising from loss of RBR, which interferes with programmed stress/relief transitions of the meiotic chromosomes. Interestingly, it has been demonstrated that the transcriptional co-activator, mediator and cohesin interact to provide a link between the gene transcription machinery

and chromatin architecture (Kagey *et al*, 2010). Furthermore, in *Drosophila*, RBF1 interacts with the origin recognition complex and chromatin to directly influence DNA replication (Ahlander *et al*, 2008). Hence, it is conceivable that RBR may function in an analogous manner to couple meiotic recombination with the cell cycle.

Conclusions

Our study indicates that RBR is important for normal levels of meiotic COs and SC formation in *Arabidopsis*. Whether the meiotic defects in *rbr-2* are attributable to one or more of these will require further investigation. At present there is little evidence as to whether an Rb function in meiosis is widely conserved. E2F-binding elements in genes controlling meiosis have been identified in mice (Kehoe *et al*, 2008). In addition, the loss of *Rb* function in the nematode *Caenorhabditis elegans* causes sterility of unknown origin (Cui *et al*, 2004). Despite the paucity of experimental evidence, given the similarity in meiotic control shared between plants and animals, it seems likely that this role of pRb is conserved in other multicellular organisms.

Materials and methods

Experimental materials

The wild-type ecotype Columbia (Col-0) was provided by the Nottingham *Arabidopsis* Stock Centre. The *Arabidopsis thaliana* mutant alleles (Columbia accession) used in this study were *rbr-2* (SALK_002946; SALK collection) and *cdka-1* (SALK_106809; SALK collection). After 3 days at 4°C in dark, seeds were germinated and grown on soil. Plants were cultured in a growth chamber under short days (8 h of light at 20°C/16 h of dark at 16°C; 60–70% relative humidity) until rosettes were formed. Plants were transferred to long days at 20°C (16 h of light/8 h of dark) to induce flowering and grown until seeds were harvested.

RNA extraction and RT-PCR

Sample tissues were collected from *Arabidopsis* plants and frozen in liquid nitrogen immediately. Tissues were ground by Tissue-lyser machine (Qiagen) and total RNA was prepared using the RNeasy mini kit (Qiagen). DNase treatment was done on 2 mg of total RNA using the Dnase-free kit (Ambion). For reverse transcription, 500 ng of total RNA was incubated at 42°C for 1 h with 200 U of murine leukemia virus reverse transcriptase (New England Biolabs) in a 20 µl reaction mixture containing 4 mM oligo (dT) primer, RT reaction buffer, 1 mM deoxynucleotide triphosphate and 40 U of recombinant RNasin ribonuclease inhibitor (Promega). The reaction was terminated by incubation at 90°C for 10 min. (Primers used are listed in Supplementary Table S2.)

Quantitative real-time RT-PCR

Real-time PCR assays were performed using Power SYBR Green PCR Master Mix (Applied Biosystems). A volume of 0.25 µl of the reverse transcript product was used to perform each PCR reaction. Amplification reaction was carried out using specific primers at a concentration of 0.2 mM in a 10 µl reaction mixture. The specificity of the amplification was determined by running a step of dissociation curve analysis. Thermal cycling parameters were 2 min at 50°C, 10 min at 95°C and 50 cycles of 15 s at 95°C and 60 s at 60°C. Three technical replicates were carried out for each sample. The PCR reaction and quantitative measurements were achieved with 7900HT Fast Real-Time PCR System SDS2.3 and RQ

manager 1.2 software (Applied Biosystems). The ΔC_t was calculated using *ACT11* as endogenous control. Relative quantisation (RQ) values were calculated by the $2^{-\Delta C_t}$ method ($RQ = 2^{-\Delta C_t}$). RQ values represent the average of three biological replicates. (Primers used are listed in Supplementary Table S2.)

Western blotting

RBR polyclonal Ab recognising RBR-C was produced from rabbit immunised with synthesised peptide of the amino acid sequence: CNRLNNSSSNRKRTL. The cysteine residue was employed for single point, site-directed conjugation to KLH. RBR was quantified by means of SDS-PAGE followed by western blotting. The extracted total protein content of the cleared supernatant was measured (protein assay kit; Bio-Rad) and equal amounts of total protein were loaded. Proteins were separated on a 10% polyacrylamide gel and transferred to PVDF membrane. Polyclonal Ab diluted in the ratio 1:5000 was applied followed by an anti-mouse/rabbit IgG-POD coupled to HRP and visualised by BM Chemiluminescence Western Blotting Kit (Roche).

Cytological procedures

The cytological methods were carried out as previously described (Higgins *et al*, 2004). The following antibodies were used in this study: anti-RBR-C terminal (rabbit, 1/200 dilution), anti-RBR N-terminal (Borghi *et al*, 2010), anti-AtASY1 (rabbit/rat, 1/500 dilution), anti-AtMSH4 (rabbit, 1/500 dilution), anti-AtZYP1 (rabbit/rat, 1/500 dilution), anti-AtDMC1 (rabbit 1/500 dilution), anti-AtRAD51 (rabbit, 1/500 dilution), anti-AtMLH1 (rabbit/rat, 1/200 dilution) and anti-γH2AX (ser 139, Upstate Biotechnology, catalogue no. 07-164; rabbit, 1/100 dilution; Higgins *et al*, 2004; Higgins *et al*, 2005; Mercier *et al*, 2003). To confirm the specificity of anti-RBR-C Ab, comparative immunolocalisation was conducted on wild type and *rbr-2*. This confirmed that the signal present in wild type was absent in the mutant (Figure 2A–D). FISH on metaphase I chromosomes was carried out using the 45S rDNA and 5S rDNA probes, as previously described (Higgins *et al*, 2004). Microscopy was conducted using a Nikon 90i Fluorescence Microscope (Tokyo, Japan). Image capture and analysis was carried out using NIS Elements F software (Nikon, Tokyo, Japan).

Statistical procedures

The statistical procedures were carried out as described previously (Higgins *et al*, 2004).

Supplementary data

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

Acknowledgements

We are most grateful to Wilhelm Gruissem (ETH, Zurich) for providing the N-terminal RBR antibody. We thank Philipp Kaldis and Stephen Cohen for critical reading of the manuscript and the reviewers for their constructive comments. FB and ZC were funded by the Temasek Life Sciences Laboratory. ZC was funded by the Singapore Millenium Foundation. The laboratory of FCHF is funded by the Biological and Biotechnology Research Council, UK. ZC contributed all genetic and molecular analysis of the *rbr-2* homologous mutant line. JDH contributed the cytological and immunological analysis of meiotic defects. JTLH performed the cell sorting analysis. JL contributed the Q-PCR. FB and FCHF conceived the project, participated in data analyses and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Ahlander J, Chen XB, Bosco G (2008) The N-terminal domain of the *Drosophila* retinoblastoma protein Rbf1 interacts with ORC and associates with chromatin in an E2F independent manner. *PLoS One* 3: e2831
- Armstrong SJ, Caryl AP, Jones GH, Franklin FC (2002) Asy1, a protein required for meiotic chromosome synapsis, localises to axis-associated chromatin in *Arabidopsis* and Brassica. *J Cell Sci* 115: 3645–3655

- Armstrong SJ, Jones GH (2003) Meiotic cytology and chromosome behaviour in wild-type *Arabidopsis thaliana*. *J Exp Bot* **54**: 1–10
- Averbeck N, Sunder S, Sample N, Wise JA, Leatherwood J (2005) Negative control contributes to an extensive program of meiotic splicing in fission yeast. *Mol Cell* **18**: 491–498
- Bellani MA, Boateng KA, McLeod D, Camerini-Otero RD (2010) The Expression profile of the major mouse SPO11 isoforms indicates that SPO11 beta introduces double strand breaks and suggests that SPO11 alpha has an additional role in prophase in both spermatocytes and oocytes. *Mol Cell Biol* **30**: 4391–4403
- Berger F, Hamamura Y, Ingouff M, Higashiyama T (2008) Double fertilization—caught in the act. *Trends Plant Sci* **13**: 437–443
- Boerner GV, Kleckner N, Hunter N (2004) Crossover/noncrossover differentiation, synaptonemal complex formation, and regulatory surveillance at the leptotene/zygotene transition of meiosis. *Cell* **117**: 29–45
- Borghi L, Gutzat R, Futterer J, Laizet Y, Hennig L, Grussem W (2010) *Arabidopsis* RETINOBLASTOMA-RELATED is required for stem cell maintenance, cell differentiation, and lateral organ production. *Plant Cell* **22**: 1792–1811
- Brehm A, Kouzarides T (1999) Retinoblastoma protein meets chromatin. *Trends Biochem Sci* **24**: 142–145
- Brehm A, Miska EA, McCance DJ, Reid JL, Bannister AJ, Kouzarides T (1998) Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature* **391**: 597–601
- Chelysheva L, Vezon D, Belcram K, Gendrot G, Grelon M (2008) The *Arabidopsis* BLAP75/Rmi1 homologue plays crucial roles in meiotic double-strand break repair. *PLoS Genet* **4**: e1000309
- Chen Z, Hafidh S, Poh SH, Twell D, Berger F (2009) Proliferation and cell fate establishment during *Arabidopsis* male gametogenesis depends on the Retinoblastoma protein. *Proc Natl Acad Sci USA* **106**: 7257–7262
- Clarke AR, Maandag ER, van Roon M, van der Lugt NM, van der Valk M, Hooper ML, Berns A, te Riele H (1992) Requirement for a functional Rb-1 gene in murine development. *Nature* **359**: 328–330
- Couteau F, Belzile F, Horlow C, Grandjean O, Vezon D, Doutriaux MP (1999) Random chromosome segregation without meiotic arrest in both male and female meiocytes of a dmc1 mutant of *Arabidopsis*. *Plant Cell* **11**: 1623–1634
- Cui M, Fay DS, Han M (2004) lin-35/Rb cooperates with the SWI/SNF complex to control *Caenorhabditis elegans* larval development. *Genetics* **167**: 1177–1185
- Desvoyes B, Ramirez-Parra E, Xie Q, Chua NH, Gutierrez C (2006) Cell type-specific role of the retinoblastoma/E2F pathway during *Arabidopsis* leaf development. *Plant Physiol* **140**: 67–80
- Dimova DK, Dyson NJ (2005) The E2F transcriptional network: old acquaintances with new faces. *Oncogene* **24**: 2810–2826
- Dissmeyer N, Nowack MK, Pusph S, Stals H, Inze D, Grini PE, Schnittger A (2007) T-loop phosphorylation of *Arabidopsis* CDKA; 1 is required for its function and can be partially substituted by an aspartate residue. *Plant Cell* **19**: 972–985
- Doutriaux MP, Couteau F, Bergounioux C, White C (1998) Isolation and characterisation of the RAD51 and DMC1 homologs from *Arabidopsis thaliana*. *Mol Gen Genet* **257**: 283–291
- Drews GN, Yadegari R (2002) Development and function of the angiosperm female gametophyte. *Annu Rev Genet* **36**: 99–124
- Ebel C, Mariconti L, Grussem W (2004) Plant retinoblastoma homologues control nuclear proliferation in the female gametophyte. *Nature* **429**: 776–780
- Engbrecht J, Voelkelmeiman K, Roeder GS (1991) Meiosis-specific RNA splicing in yeast. *Cell* **66**: 1257–1268
- Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert DM, Dryja TP (1986) A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* **323**: 643–646
- Funayama R, Ishikawa F (2007) Cellular senescence and chromatin structure. *Chromosoma* **116**: 431–440
- Grelon M, Vezon D, Gendrot G, Pelletier G (2001) AtSPO11-1 is necessary for efficient meiotic recombination in plants. *EMBO J* **20**: 589–600
- Gross-Hardt R, Kagi C, Baumann N, Moore JM, Baskar R, Gagliano WB, Jurgens G, Grossniklaus U (2007) LACHESIS restricts gametic cell fate in the female gametophyte of *Arabidopsis*. *PLoS Biol* **5**: e47
- Hamant O, Ma H, Cande WZ (2006) Genetics of meiotic prophase I in plants. *Annu Rev Plant Biol* **57**: 267–302
- Harbour JW, Dean DC (2000) Rb function in cell-cycle regulation and apoptosis. *Nat Cell Biol* **2**: E65–E67
- Hartung F, Suer S, Knoll A, Wurzel-Wildersinn R, Puchta H (2008) Topoisomerase 3alpha and RMI1 suppress somatic crossovers and are essential for resolution of meiotic recombination intermediates in *Arabidopsis thaliana*. *PLoS Genet* **4**: e1000285
- Henderson KA, Kee K, Maleki S, Santini PA, Keeney S (2006) Cyclin-dependent kinase directly regulates initiation of meiotic recombination. *Cell* **125**: 1321–1332
- Higgins JD, Armstrong SJ, Franklin FCH, Jones GH (2004) The *Arabidopsis* MutS homolog AtMSH4 functions at an early step in recombination: evidence for two classes of recombination in *Arabidopsis*. *Genes Dev* **18**: 2557–2570
- Higgins JD, Buckling EF, Franklin FCH, Jones GH (2008a) Expression and functional analysis of AtMUS81 in *Arabidopsis* meiosis reveals a role in the second pathway of crossing-over. *Plant J* **54**: 152–162
- Higgins JD, Sanchez-Moran E, Armstrong SJ, Jones GH, Franklin FCH (2005) The *Arabidopsis* synaptonemal complex protein ZYP1 is required for chromosome synapsis and normal fidelity of crossing over. *Genes Dev* **19**: 2488–2500
- Higgins JD, Vignard J, Mercier R, Pugh AG, Franklin FCH, Jones GH (2008b) AtMSH5 partners AtMSH4 in the class I meiotic crossover pathway in *Arabidopsis thaliana*, but is not required for synapsis. *Plant J* **55**: 28–39
- Ingouff M, Jullien PE, Berger F (2006) The female gametophyte and the endosperm control cell proliferation and differentiation of the seed coat in *Arabidopsis*. *Plant Cell* **18**: 3491–3501
- Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA, Weinberg RA (1992) Effects of an Rb mutation in the mouse. *Nature* **359**: 295–300
- Jackson N, Sanchez-Moran E, Buckling E, Armstrong SJ, Jones GH, Franklin FCH (2006) Reduced meiotic crossovers and delayed prophase I progression At-MLH3-deficient *Arabidopsis*. *EMBO J* **25**: 1315–1323
- Johnston AJ, Matveeva E, Kirioukhova O, Grossniklaus U, Grussem W (2008) A dynamic reciprocal RBR-PRC2 regulatory circuit controls *Arabidopsis* gametophyte development. *Curr Biol* **18**: 1680–1686
- Jones GH (1984) The control of chiasma distribution. *SEB Symp* **38**: 293–320
- Jones GH, Franklin FC (2006) Meiotic crossing-over: obligation and interference. *Cell* **126**: 246–248
- Jullien PE, Mosquna A, Ingouff M, Sakata T, Ohad N, Berger F (2008) Retinoblastoma and its binding partner MSI1 control imprinting in *Arabidopsis*. *PLoS Biol* **6**: e194
- Juneau K, Palm C, Miranda M, Davis RW (2007) High-density yeast-tiling array reveals previously undiscovered introns and extensive regulation of meiotic splicing. *Proc Natl Acad Sci USA* **104**: 1522–1527
- Kagey MH, Newman JL, Bilodeau S, Zhan Y, Orlando DA, van Berkum NL, Ebmeier CC, Goossens J, Rahl PB, Levine SS, Taajes DJ, Dekker J, Young RA (2010) Mediator and cohesin connect gene expression and chromatin architecture. *Nature* **467**: 430–435
- Keeney S, Giroux CN, Kleckner N (1997) Meiosis-specific DNA double strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. *Cell* **88**: 375–384
- Kehoe SM, Oka M, Hankowski KE, Reichert N, Garcia S, McCarrey JR, Gaubatz S, Terada N (2008) A conserved E2F6-binding element in murine meiosis-specific gene promoters. *Biol Reprod* **79**: 921–930
- Kishimoto T (2003) Cell-cycle control during meiotic maturation. *Curr Opin Cell Biol* **15**: 654–663
- Kleckner N, Zickler D, Jones GH, Dekker J, Padmore R, Henle J, Hutchinson J (2004) A mechanical basis for chromosome function. *Proc Natl Acad Sci USA* **101**: 12592–12597
- Kleckner N (2006) Chiasma formation: chromatin/axis interplay and the role(s) of the synaptonemal complex. *Chromosoma* **115**: 175–194
- Lam WS, Yang XH, Makaroff CA (2005) Characterization of *Arabidopsis thaliana* SMC1 and SMC3: evidence that AtSMC3 may function beyond chromosome cohesion. *J Cell Sci* **118**: 3037–3048
- Lee EY, Chang CY, Hu N, Wang YC, Lai CC, Herrup K, Lee WH, Bradley A (1992) Mice deficient for Rb are nonviable and

- show defects in neurogenesis and haematopoiesis. *Nature* **359**: 288–294
- Liu CM, McElver J, Tzafrir I, Joosen R, Wittich P, Patton D, Van Lammeren AA, Meinke D (2002) Condensin and cohesin knockouts in *Arabidopsis* exhibit a titan seed phenotype. *Plant J* **29**: 405–415
- Longworth MS, Dyson NJ (2010) pRb, a local chromatin organizer with global possibilities. *Chromosoma* **119**: 1–11
- Longworth MS, Herr A, Ji JY, Dyson NJ (2008) RBF1 promotes chromatin condensation through a conserved interaction with the Condensin II protein dCAP-D3. *Genes Dev* **22**: 1011–1024
- Macaluso M, Montanari M, Giordano A (2006) Rb family proteins as modulators of gene expression and new aspects regarding the interaction with chromatin remodeling enzymes. *Oncogene* **25**: 5263–5267
- Magnard JL, Yang M, Chen YC, Leary M, McCormick S (2001) The *Arabidopsis* gene tardy asynchronous meiosis is required for the normal pace and synchrony of cell division during male meiosis. *Plant Physiol* **127**: 1157–1166
- Maguire M (1978) Evidence for separate genetic control of crossing over and chiasma maintenance in maize. *Chromosoma* **65**: 173–183
- Mahadevaiah SK, Turner JM, Baudat F, Rogakou EP, de Boer P, Blanco-Rodriguez J, Jasin M, Keeney S, Bonner WM, Burgoyne PS (2001) Recombinational DNA double-strand breaks in mice precede synapsis. *Nat Genet* **27**: 271–276
- Marston AL, Amon A (2004) Meiosis: cell-cycle controls shuffle and deal. *Nat Rev Mol Cell Biol* **5**: 983–997
- Matlin AJ, Clark F, Smith CW (2005) Understanding alternative splicing: towards a cellular code. *Nat Rev Mol Cell Biol* **6**: 386–398
- McKim KS, Hawley RS (1995) Chromosomal control of meiotic cell division. *Science* **270**: 1595–1601
- Mercier R, Armstrong SJ, Horlow C, Jackson NP, Makaroff CA, Vezon D, Pelletier G, Jones GH, Franklin FCH (2003) The meiotic protein SWI1 is required for axial element formation and recombination initiation in *Arabidopsis*. *Development* **130**: 3309–3318
- Mercier R, Grelon M (2008) Meiosis in plants: ten years of gene discovery. *Cytogenet Genome Res* **120**: 281–290
- Moll C, von Lyncker L, Zimmermann S, Kagi C, Baumann N, Twell D, Grossniklaus U, Gross-Hardt R (2008) CLO/GFA1 and ATO are novel regulators of gametic cell fate in plants. *Plant J* **56**: 913–921
- Neale MJ, Keeney S (2006) Clarifying the mechanics of DNA strand exchange in meiotic recombination. *Nature* **442**: 153–158
- Ortega S, Prieto I, Odajima J, Martin A, Dubus P, Sotillo R, Barbero JL, Malumbres M, Barbacid M (2003) Cyclin-dependent kinase 2 is essential for meiosis but not for mitotic cell division in mice. *Nat Genet* **35**: 25–31
- Page SL, Hawley RS (2004) The genetics and molecular biology of the synaptonemal complex. *Annu Rev Cell Dev Biol* **20**: 525–558
- Park JA, Ahn JW, Kim YK, Kim SJ, Kim JK, Kim WT, Pai HS (2005) Retinoblastoma protein regulates cell proliferation, differentiation, and endoreduplication in plants. *Plant J* **42**: 153–163
- Riehs N, Akimcheva S, Puizina J, Bulankova P, Idol RA, Siroky J, Schleiffer A, Schweizer D, Shippen DE, Riha K (2008) *Arabidopsis* SMG7 protein is required for exit from meiosis. *J Cell Sci* **121**: 2208–2216
- Roberts NY (2010) Investigating the control of homologous chromosome pairing and crossover formation in meiosis of *Arabidopsis thaliana*. PhD Thesis, University of Birmingham, UK
- Roeder GS, Bailis JM (2000) The pachytene checkpoint. *Trends Genet* **16**: 395–403
- Rossi V, Locatelli S, Lanzanova C, Boniotti MB, Varotto S, Pipal A, Goralik-Schramel M, Lusser A, Gatz C, Gutierrez C, Motto M (2003) A maize histone deacetylase and retinoblastoma-related protein physically interact and cooperate in repressing gene transcription. *Plant Mol Biol* **51**: 401–413
- Sabelli PA, Dante RA, Leiva-Neto JT, Jung R, Gordon-Kamm WJ, Larkins BA (2005) RBR3, a member of the retinoblastoma-related family from maize, is regulated by the RBR/E2F pathway. *Proc Natl Acad Sci USA* **102**: 13005–13012
- Sanchez-Moran E, Santos JL, Jones GH, Franklin FC (2007) ASY1 mediates AtDMC1-dependent interhomolog recombination during meiosis in *Arabidopsis*. *Genes Dev* **21**: 2220–2233
- Satyanarayana A, Kaldis P (2009) Mammalian cell-cycle regulation: several Cdk, numerous cyclins and diverse compensatory mechanisms. *Oncogene* **28**: 2925–2939
- Shen WH (2002) The plant E2F-Rb pathway and epigenetic control. *Trends Plant Sci* **7**: 505–511
- Stacey NJ, Kuromori T, Azumi Y, Roberts G, Breuer C, Wada T, Maxwell A, Roberts K, Sugimoto-Shirasu K (2006) *Arabidopsis* SPO11-2 functions with SPO11-1 in meiotic recombination. *Plant J* **48**: 206–216
- Stevens R, Grelon M, Vezon D, Oh J, Meyer P, Perennes C, Domenichini S, Bergounioux C (2004) A CDC45 homolog in *Arabidopsis* is essential for meiosis, as shown by RNA interference-induced gene silencing. *Plant Cell* **16**: 99–113
- Storlazzi A, Gargano S, Ruprich-Robert G, Falque M, David M, Kleckner N, Zickler D (2010) Recombination proteins mediate meiotic spatial chromosome organization and pairing. *Cell* **141**: 94–106
- Stuart D, Wittenberg C (1998) CLB5 and CLB6 are required for premeiotic DNA replication and activation of the meiotic S/M checkpoint. *Genes Dev* **12**: 2698–2710
- Susiarjo M, Rubio C, Hunt P (2009) Analyzing mammalian female meiosis. *Methods Mol Biol* **558**: 339–354
- Vandepoele K, Vlieghe K, Florquin K, Hennig L, Beemster GT, Gruijssem W, Van de Peer Y, Inze D, De Veylder L (2005) Genome-wide identification of potential plant E2F target genes. *Plant Physiol* **139**: 316–328
- Wang TF, Kleckner N, Hunter N (1999) Functional specificity of MutL homologs in yeast: evidence for three Mlh1-based heterocomplexes with distinct roles during meiosis in recombination and mismatch correction. *Proc Natl Acad Sci USA* **96**: 13914–13919
- Wang YX, Magnard JL, McCormick S, Yang M (2004) Progression through meiosis I and meiosis II in *Arabidopsis* anthers is regulated by an A-type cyclin predominately expressed in prophase I. *Plant Physiol* **136**: 4127–4135
- Weinberg RA (1995) The retinoblastoma protein and cell cycle control. *Cell* **81**: 323–330
- Wildwater M, Campilho A, Perez-Perez JM, Heidstra R, Blilou I, Korthout H, Chatterjee J, Mariconti L, Gruijssem W, Scheres B (2005) The RETINOBLASTOMA-RELATED gene regulates stem cell maintenance in *Arabidopsis* roots. *Cell* **123**: 1337–1349
- Wu L, de Bruin A, Saavedra HI, Starovic M, Trimboli A, Yang Y, Opavska J, Wilson P, Thompson JC, Ostrowski MC, Rosol TJ, Woollett LA, Weinstein M, Cross JC, Robinson ML, Leone G (2003) Extra-embryonic function of Rb is essential for embryonic development and viability. *Nature* **421**: 942–947
- Wyrzykowska J, Schorderet M, Pien S, Gruijssem W, Fleming AJ (2006) Induction of differentiation in the shoot apical meristem by transient overexpression of a retinoblastoma-related protein. *Plant Physiol* **141**: 1338–1348
- Zhu Z, Mori S, Oshiumi H, Matsuzaki K, Shinohara M, Shinohara A (2010) Cyclin-dependent kinase promotes formation of the synaptonemal complex in yeast meiosis. *Genes Cells* **15**: 1036–1050
- Zickler D, Kleckner N (1999) Meiotic chromosomes: integrating structure and function. *Annu Rev Genet* **33**: 603–754