Replication Protein A2c Coupled with Replication Protein A1c Regulates Crossover Formation during Meiosis in Rice

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INTRODUCTION

Meiosis is a specialized type of cell division in which one round of DNA replication is followed by two successive rounds of chromosome segregation (meiosis I and II). Meiosis is required for the production of haploid gametes to ensure that the correct chromosome complement is restored upon fertilization. This process is characterized by an extended prophase I where homologous recombination leads to the formation of crossovers (COs), the sites of reciprocal genetic exchange. This process results in chromosomes possessing unique allelic combinations that may be advantageous during evolution. A fundamental feature of meiotic recombination is assurance of the obligate CO (Shinohara et al., 2008). This ensures that each pair of chromosomes exhibits at least one CO, which is required to tether the chromosomes during metaphase I, thus preventing mis-segregation and unbalanced gametes. The number of chiasmata (the cytological sites of a CO) is generally limited per meiosis, so the total number is highly regulated (Higgins et al., 2004).

Based on genetic and molecular studies of recombination in budding yeast (Saccharomyces cerevisiae) and other model organisms, a double-strand break (DSB) repair model has been proposed to elucidate the mechanism of homologous recombination and CO formation (Szostak et al., 1983). Meiotic recombination is initiated by the formation of programmed DNA DSBs catalyzed by a complex containing sporulation 11, a topoisomerase II–like protein and other accessory proteins (Keeney et al., 1997). The DSBs are then resected to form 3′ single-stranded DNA (ssDNA) overhangs that are coated by the strand-exchange proteins RADIATION SENSITIVE 1 (RAD51) and DISRUPTED MEIOTIC cDNA1 (DMC1) to promote interhomolog recombination followed by second-end capture. At selected sites, double Holliday junction recombination intermediates are formed and resolved into COs (Aillers and Lichter, 2001; Hunter and Kleckner, 2001). The majority of meiotic COs are formed by the class I pathway, which ensures the obligate CO. Class I COs are sensitive to interference, a phenomenon whereby the occurrence of one CO reduces the probability of a second CO nearby. By contrast, class II COs are insensitive to interference and arise through different pathways (Bercowitz et al., 2007; Hollingsworth and Brill, 2004).

Class I CO formation is dependent on the collaborative activities of the ZMM proteins (Zip1, Zip2, Zip3, Zip4, Msh4, Msh5, and Mre3) in budding yeast (Börner et al., 2004). ZMM proteins are conserved in eukaryotes, and homologs of these proteins have been identified in plants. Zip1 is the transverse filament component of the synaptonemal complex (Sym et al., 1993). ZYP1 and ZEP1, the homolog of Zip1, have been identified in Arabidopsis thaliana and rice (Oryza sativa), respectively. There was a slight but significant reduction in COs in ZYP1RNAs and an increased number of COs in zep1 (Higgins et al., 2005; Osman et al., 2006; Wang et al., 2010). However, zep1 zip4 and zep1 mer3 double mutants exhibited much lower numbers of COs.
than zip4 and mer3 single mutants (Chen et al., 2005; Mercier et al., 2005; Wang et al., 2009; Shen et al., 2012). Recently, a homolog of yeast Zip3, Human Enhancer of Invasion10 (HEI10), was identified in both Arabidopsis and rice. In both organisms, HEI10 mutants exhibited substantial reductions in the number of COs. In addition, rice HEI10 acts cooperatively with Mer3 to regulate the formation of class I COs (Chelysheva et al., 2012; Wang et al., 2012). In Arabidopsis, two meiosis-specific members of the MutS-homolog gene family (MSH4 and MSH5) were characterized as key factors in class I CO formation (Higgins et al., 2004, 2008). Although several proteins required for class I CO formation have been reported in Arabidopsis and rice, more components need to be identified to elucidate the underlying mechanisms in plants. The establishment and dissolution of sister chromatid cohesion are essential for meiotic homologous recombination. During prophase I, chromatin is organized as co-oriented linear arrays of loop structures, which are tethered at their AT-rich bases called “axis association sites” by preferential binding of specific proteins, including meiotic chromosome axis proteins REC8, HOMOLOG PAIRING1 (HOP1), and Red1 (Blat and Kleckner, 1999; Blat et al., 2002; Glynn et al., 2004). These proteins modulate

Figure 1. Meiosis of Wild-Type and rpa2c Mutant PMCs in Rice.

(A) to (J) Male meiotic chromosome spread was stained with DAPI in the wild type (A) to (E) and rpa2c (F) to (J). Arrows indicate the separated region in (G) and micronuclei of abnormal tetrad in (J). Bars = 5 μm.

(K) to (T) Pairing status of homologous chromosome was examined by FISH using ribosome 5s rDNA probe in the wild type (K) to (O) and rpa2c (P) to (T). FISH foci (pink fluorescence) are indicated by white arrows. DNA was counterstained by DAPI. Arrows in (S) and (T) indicated unbalanced distribution of FISH foci. Bars = 5 μm.

(K) and (P) are leptotene; (A), (F), (L), and (Q) show early pachytene; (G), (M), and (R) show late pachytene; (B) is diplotene; (C), (H), (N), and (S) are diakinesis; (D), (I), (O), and (T) are telophase I; and (E) and (J) are telophase II.

[See online article for color version of this figure.]
the formation and distribution of DSBs along chromosomes (Kugou et al., 2009; Kim et al., 2010). REC8 and HOP1 are conserved across diverse species, and their homologs (At-REC8/At-SYN1/At-DIF1 [three names for the same gene] and Os-REC8 for REC8; ASY1 and PAIR2 for HOP1) have also been identified in Arabidopsis and rice. Depletion of REC8 causes chromosome fragmentation and formation of univalents (Cai et al., 2003; Chelysheva et al., 2005; Shao et al., 2011), and mutation of ASY1 and PAIR2 also results in defects in synopsis and CO formation in Arabidopsis and rice, respectively (Caryl et al., 2000; Nonomura et al., 2006; Sanchez-Moran et al., 2007).

Replication protein A (RPA) is an ssDNA binding protein required for multiple processes in eukaryotic DNA metabolism, including DNA replication, DNA repair, and homologous recombination (Wold, 1997; Iftode et al., 1999). RPA is a stable heterotrimeric protein composed of three subunits, RPA1 (~70 kD), RPA2 (~32 kD), and RPA3 (~14 kD) (Wold, 1997; Iftode et al., 1999). RPA is considered to be one of the accessory proteins participating in assembly of the strand-exchange proteins RAD51 and DMC1, and it significantly enhances the strand-exchange activity of Rad51 (McIlwraith et al., 2000). However, when an excess of RPA is added to ssDNA prior to RAD51, strand exchange is inhibited. This inhibition is overcome by recombination mediators, like RAD52, another type of ssDNA binding protein that stimulates RAD51 strand-exchange in the postinvasion steps (Sung et al., 2003). In addition, colocalization of RPA, RAD51, and RAD52 further confirms that RPA participates in these processes in budding yeast (McIlwraith et al., 2000). Furthermore, mutation of RFA1, which encodes the 70-kD RPA subunit, resulted in an overall decrease in meiotic recombination frequency of up to 100-fold in budding yeast (Soustelle et al., 2002).

Animals and yeast generally only possess one each of the three RPA genes, with the exception that humans carry two homologs of RPA32 (Keshav et al., 1995). However, Arabidopsis and rice have multiple genes for most RPA subunits (Shultz et al., 2007). This indicates that different subunit combinations may produce different types of RPA complex, required for different aspects of DNA metabolism. Previous studies have reported that RPA1a is required for class I CO formation, DNA damage response, and telomere length homeostasis but is dispensable for meiotic DSB repair in Arabidopsis (Osman et al., 2009; Takashi et al., 2009). Our previous investigation revealed that RPA1a plays an essential role in DNA repair but may not participate in, or is dispensable for, DNA replication and homologous recombination in rice (Chang et al., 2009). However, the precise functions of RPA2 subunits in DNA metabolism in plants are unclear. Here, we report the functional analysis of RPA2c, a highly transcribed gene during meiosis, encoding a paralog of the RPA2 (~32 kD) subunit in rice. Our study

![Figure 2. Distribution of Bivalents and Chiasmata in rpa2c Mutants.](image)

(A) and (B) Cytological analysis shows the abnormal frequency and distribution of the bivalents and residual chiasmata in the rpa2c mutant at diakinesis/metaphase I. Bars = 5 μm.

(D) and (E) The distribution of chiasmata in rpa2c (D) and the wild type (E). Lines with triangles and circles indicate observed distribution and predicted Poisson distribution, respectively.
revels that RPA2c is essential for promoting wild-type levels of COs. Moreover, this protein is involved in CO formation at a late stage of homologous recombination but not for meiotic and somatic DNA repair in rice. We also present evidence that a large RPA subunit, RPA1c, may partner with RPA2c to promote CO formation.

RESULTS

Defective Meiosis of Rice rpa2c Mutant Causes Complete Sterility

To identify genes that are essential for meiosis in rice, a sterile rpa2c mutant was obtained from our rice T-DNA insertional library (Wu et al., 2003; Zhang et al., 2006). The rpa2c mutant showed normal vegetative growth and floral development but produced smaller anthers that contained starch-lacking and shrunken mature pollen (see Supplemental Figures 1A to 1E online). Reciprocal crosses between rpa2c and the wild type did not produce any seeds (see Supplemental Table 1 online), indicating that both male and female gametogenesis was aborted in rpa2c.

To determine the cause of sterility in rpa2c, chromosome spreads of pollen mother cells (PMCs) at different meiotic stages were prepared for staining with 4,6-diamidino-2-phenylindole (DAPI). In wild-type prophase I, condensed chromatin formed distinct thin chromosome threads at leptotene (see Supplemental Figure 1F online), and synopsis of homologous chromosomes was initiated at zygotene (see Supplemental Figure 1G online). At pachytene, fully synapsed homologous chromosomes were observed (Figure 1A). After further condensation, the homologous chromosomes repelled each other at diplotene but were linked together by chiasmata (Figure 1B). At diakinesis, 12 bivalents were visible (Figure 1C) and then aligned on the equatorial plate at metaphase I, before the homologous chromosomes separated and migrated to opposite poles at anaphase I (Figure 1D). After completion of the second meiotic division, tetrads were finally produced (Figure 1E).

Cytologically, there was no obvious difference between the wild type and rpa2c mutant during leptotene, zygotene, and early pachytene (see Supplemental Figures 1H and 1I online; Figure 1F). However, some precociously separated regions of homologous chromosome were visible at late pachytene (Figure 1G). As the chromosomes condensed during diplotene/diakinesis, univalents (up to 24) were observed in rpa2c mutant nuclei (Figures 1H). During anaphase I and telophase I, random segregation of univalents resulted in unequal distribution of chromosomes in daughter nuclei (Figures 1I), eventually leading to unbalanced gametes and micronuclei at tetrad (Figure 1J). These results suggested that the number of chiasmata were reduced in the rpa2c mutant.

Further confirmation of a meiosis defect in the rpa2c mutant was achieved by fluorescence in situ hybridization (FISH) using rice 5S rDNA to probe the pairing status of homologous chromosomes in PMCs during prophase I. In rice, there is only one repeated 5S rDNA locus on the short arm of chromosome 11 (Zhang et al., 2005). Therefore, two signals should be observed with unpaired chromosomes and only one with paired/synapsed chromosomes. In the wild type and rpa2c mutant, two 5S rDNA signals were observed at leptotene (Figures 1K and 1P) (Zhang et al., 2005). During early pachytene, paired signals were detected in both the wild type (Figure 1L) and the rpa2c mutant (n = 36) (Figure 1Q). During diplotene and diakinesis, 5S rDNA signals were paired continuously in the wild type (Figures 1M and 1N) and then separated because of chromosome segregation at anaphase I (Figure 1O), whereas separated FISH foci were observed during late pachytene in the rpa2c mutant (Figure 1R). Furthermore, unpaired 5S rDNA signals were frequently observed at diplotene and diakinesis as a result of the presence of univalents (Figure 1S), which led to an unbalanced distribution of FISH foci at telophase I (Figure 1T). These results confirmed the reduced number of chiasmata in rpa2c PMCs. Furthermore, it appeared that homologous chromosomes achieved full synopsis in early pachytene but failed to maintain the paired structure at late pachytene in rpa2c.

Residual Chiasmata Are Randomly Distributed in the rpa2c Mutant

Cytological analysis of the rpa2c mutant revealed a high frequency of univalents, indicating a reduction in chiasmata (Figures 2A and 2B). We next performed a quantitative cytological analysis of nuclei at metaphase I to determine the frequency and distribution of chiasmata in the rpa2c mutant. Chiasma counts were based on the observation that rod-shaped bivalents possess one chiasma and ring-shaped bivalents possess a minimum of two chiasmata (Sanchez Moran et al., 2001). The number of bivalents in rpa2c mutant nuclei ranged from 0 to 9 (Figure 2C), and the mean bivalent number was reduced to 4.2 per cell, compared with 12 in the wild type. The significantly reduced number of bivalents was caused by a decrease in number of chiasmata, which ranged from 0 to 11 in rpa2c, and the mean chiasma frequency was 4.98 (n = 70) per cell. Furthermore, the residual chiasma distribution in rpa2c was close to the predicted Poisson distribution (x² = 192.65; P < 0.01; Figure 2E) unlike the wild type, where the mean chiasma frequency (22.7 per PMC; n = 50) deviated significantly from a Poisson distribution (x² = 192.65; P < 0.01; Figure 2E).

In rpa2c, there was a −78% reduction in chiasma frequency compared with the wild type, and the residual chiasmata were numerically randomly distributed. This indicates that formation of class I COs was clearly affected, but we cannot rule out that rpa2c does not affect interference-independent (class II) COs as well.

An RPA2c Knockout Exhibits Meiotic Defects

Analysis of the genomic sequence flanking the T-DNA insertion site in rpa2c showed that it was located in the fourth intron of RPA2c on chromosome 6, which is composed of 10 exons and nine introns, respectively (Figure 3A). Genotyping with a pair of gene-specific primers (2c-F/R) coupled with a T-DNA-specific primer (NTLB5) revealed that the T-DNA insertion cosegregated with the sterile phenotype (Figure 3B). RT-PCR analysis using primer pair 2c-RT1-F/R showed no amplification, but the expected fragment was amplified by primer pair 2c-RT2-F/R in rpa2c (Figure 3C), indicating that the T-DNA insertion caused...
a truncated RPA2c transcript resulting in loss of function. To further confirm that sterility was caused by loss of function of RPA2c, we performed a complementation test by introducing a 7.7-kb genomic fragment including the entire RPA2c gene into the homozygous rpa2c allele callus. Fertility was restored and normal meiosis was observed in anthers of regenerated plants carrying the transgene (Figures 3D to 3J). As a negative control, normal meiosis was observed in anthers of regenerated plants (Figure 3K).

The full-length RPA2c cDNA was isolated using rapid amplification of the cDNA ends (RACE)-PCR. Sequence analysis predicted that RPA2c encodes a 430-amino acid protein and putative subunit of the RPA complex. Conserved domain searches using amino acid sequence of RPA2c against conserved domain database (http://www.ncbi.nlm.nih.gov/cdd/) revealed a DNA binding motif of the cDNA ends (RACE)-PCR. Sequence analysis predicted that RPA2c encodes a 430-amino acid protein and putative subunit of the RPA complex. Conserved domain searches using amino acid sequence of RPA2c against conserved domain database (http://www.ncbi.nlm.nih.gov/cdd/) revealed a DNA binding motif.

Figure 3. Genetic Analysis and Complementation Test of rpa2c.

(A) Schematic structure of RPA2c and T-DNA insertion site. Filled boxes represent exons, and lines represent introns; T-DNA insertion site is located in the fourth intron of rpa2c. 2c-F/R and NTLB5 indicate the PCR primers for genotyping the T-DNA in rpa2c. 2c-RT-1-F/R and 2c-RT-2-F/R indicate the RT-PCR primers for RPA2c expression analysis. LB, left border; RB, right border.

(B) Genotyping of T-DNA insertion in wild-type (WT), heterozygous, and mutant plants.

(C) RT-PCR analysis of RPA2c expression levels in the wild type and rpa2c. Total RNA was extracted from spikelets at the meiosis stage. GAPDH was used as a control.

(D) Positive transgenic plants (left) can set seed normally, whereas negative lines are sterile (right).

(E) A spikelet of a positive transgenic plant (left) and a negative plant (right). Bar = 1 mm.

(F and G) Iodine–potassium iodide solution stained pollen from a positive plant (F) and a negative plant (G). Bars = 100 μm. [See online article for color version of this figure.]

RPA2c Is Preferentially Expressed during Meiosis

The expression pattern of RPA2c was examined in different tissues using quantitative RT-PCR (qRT-PCR). Accumulation of RPA2c transcripts was observed in young panicles but absent or at very low levels in vegetative organs (Figure 4A). The highest expression level of RPA2c was detected in young panicles with lengths of ~4 cm, which contained both male and female meiocytes at early stages (Nonomura et al., 2004). Among various vegetative tissues, RPA2c transcripts were relatively abundant in the shoot apex but were not detectable in the root, culm, sheath, or leaf (Figure 4A). Thus, the expression of RPA2c reaches its peak value during meiosis. To determine the precise expression pattern of RPA2c, RNA in situ hybridization was performed on tissues with high transcript expression. RNA in situ hybridization signals were faint in the shoot apical meristem and in the axillary meristems of young panicles (Figures 4B and 4C). At early anther stages, the RPA2c signals were detected in microsporocytes and the tapetal cell layers (Figure 4D). Subsequently, the signals were detected in meiocytes (Figure 4E) and attenuated by the tetrad stage (Figure 4F). As a negative control, hybridization with an RPA2c sense probe did not reveal detectable signals (Figure 4G). These results suggest that RPA2c is preferentially expressed during meiosis in rice.

We also investigated the expression patterns of the other two RPA2 paralogs, RPA2a and RPA2b, in the same set of tissues by qRT-PCR. RPA2a transcripts were detected at high levels in young panicles and reached a peak of abundance in P2 (~4 cm) panicles. In addition, expression of RPA2a was detected in roots, shoot apexes, and culms at relatively low levels and hardly at all in leaves and leaf sheaths (Figure 4H). qRT-PCR results revealed that RPA2a was constitutively expressed in roots, shoot apexes, leaves, leaf sheaths, and panicles at different developmental stages (Figure 4I). These results indicated that RPA2a was preferentially expressed in the shoot apex and young panicles and RPA2b was a constitutively expressed gene.

RPA2c Colocalizes with REC8 and PAIR2 at Premeiotic S/G2 Phase

To investigate the distribution of RPA2c during meiosis in wild-type PMCs, dual immunofluorescence was performed using an antibody in conjunction with antibodies raised against REC8 and PAIR2. REC8 and PAIR2 are established markers of meiotic events in rice (Nonomura et al., 2006; Wang et al., 2009). At S/G2 phase, the discrete REC8 and RPA2c punctate foci appear simultaneously with nearly equal numbers, which almost all colocalize (Figure 5A). PAIR2 foci were first detected at an early stage in PMCs (see Supplemental Figure 3A online), at the same time as RPA2c foci, and the majority of these foci colocalized (see Supplemental Figure 3A online). At early leptotene, REC8 and PAIR2 foci elongated to form linear signals along the chromosomes. Concomitantly, RPA2c foci increased in number and many were contiguous with the REC8 and PAIR2 signals (Figure 5B; see Supplemental Figure 3B online). At late leptotene, RPA2c had a mean number of 470 foci.
REC8 and PAIR2 signals formed thin threads along the chromosomes at zygotene (Figure 5D; see Supplemental Figure 3D online) and then became thicker at early pachytene, indicating chromosome condensation (Figure 5E; see Supplemental Figure 3E online). RPA2c foci always localized to chromosomes, and their number gradually decreased during zygotene and early pachytene (Figures 5D and 5E; see Supplemental Figure 3F online). Finally, only a few foci were detected on the REC8-directed thick synapsed chromosomes at late pachytene and diakinesis (Figure 5F; see Supplemental Figure 3G online). Thus, we concluded that RPA2c associates with chromosomes from premeiotic S-phase to pachytene and discrete RPA2c foci colocalize with REC8 and PAIR2 at S-phase/G2.

Localization of RPA2c Depends on REC8

The identical spatial and temporal distribution of RPA2c and REC8 from S-phase to early prophase I in wild-type PMCs prompted us to further examine the relationship between the two proteins. Dual immunolocalization was performed using the RPA2c antibody in combination with the centromere-specific histone H3 variant (CenH3) (Shao et al., 2011) in PMCs from a loss-of-function REC8 T-DNA insertion line (see Supplemental Figures 4A to 4C online). RPA2c foci were almost absent on chromosomes from interphase to zygotene in rec8, while bright CenH3 signals were clearly observed (Figures 6A to 6C), suggesting that localization of RPA2c depends on loading of REC8.

To investigate whether RPA2c depletion affects the localization of REC8, dual immunolocalization was conducted using the RPA2c and REC8 antibodies in PMC nuclei of rpa2c. Similar to the wild type, REC8 was observed localizing to chromosomes in PMCs of rpa2c at zygotene/early pachytene (Figure 6D), indicating that the loading of REC8 does not depend on RPA2c. As a control, RPA2c was not detected in rpa2c (Figure 6D), reflecting the specificity of the RPA2c antibody.

As we had shown that RPA2c localized to chromosomes almost simultaneously with PAIR2, we next examined whether PAIR2 loading relies on RPA2c. PAIR2 signals were clearly observed at zygotene/pachytene in rpa2c PMCs (Figure 6E),
Figure 5. Colocalization of RPA2c and REC8 through Prophase I.

Dual immunofluorescence using mouse polyclonal antibody against RPA2c (green) and rabbit polyclonal antibody against REC8 (red); chromosome DNA was counterstained with DAPI (blue). Merged images show the overlap of green and red fluorescence. S- to G2-phase (A), early leptotene (B), late leptotene (C), zygotene (D), early pachytene (E), and late pachytene (F). Bars = 5 μm.
RPA2c and MER3 Partially Colocalize during Leptotene

Os-MER3 is the rice homolog of MER3, a member of the ZMM proteins that is essential for class I CO formation in diverse species (Chen et al., 2005; Mercier et al., 2005; Wang et al., 2009). To further investigate the function of RPA2c on CO formation, we performed dual immunostaining using RPA2c and MER3 antibodies. At interphase, a small number of MER3 foci (mean = 23.3, range 19 to 28, n = 5) were observed that did not overlap with RPA2c foci (mean = 99.7, range 82 to 105, n = 5) (Figure 7A). Then, with the increased number of RPA2c and MER3 foci, a few MER3 foci (mean = 11.4, range 8 to 14, n = 5) began to colocalize with RPA2c at early leptotene (Figure 7B). At late leptotene, the number of MER3 foci reached their maximum (mean = 237, range 212 to 264, n = 5), and among those MER3 foci, 66.3% (range 61.8 to 75.3%, n = 5) colocalized with RPA2c foci (Figure 7C). To further examine the effect of RPA2c depletion on MER3 loading, dual immunolocalization was conducted on rpa2c. There was no obvious difference in the mean number of MER3 foci per nucleus at zygotene in the rpa2c mutant (125, n = 5) compared with the wild type (134, n = 5) (Figure 7D). These results indicate that RPA2c probably acts at the same time or later than MER3 in the process of CO formation.

RPA1c Colocalizes with RPA2c to Promote CO Formation

The RPA heterocomplex is implicated in a wide range of cellular activities associated with DNA metabolism. We next investigated which RPA subunit might interact with RPA2c to participate in meiotic recombination in rice. Our previous study revealed that RPA1a is mainly involved in meiotic and somatic DNA DSB repair processes, but may not be associated with homologous recombination (Chang et al., 2009). Here, using qRT-PCR, we determined that RPA1c is also preferentially expressed during meiosis (see Supplemental Figure 6 online). We therefore speculated that RPA1c may partner with RPA2c to promote CO formation. To test this hypothesis, we first performed dual immunostaining of RPA1c and RPA2c in wild-type PMCs. From interphase to leptotene, RPA1c foci appeared together with RPA2c foci. Most importantly, 86.8% ± 3.1% of RPA1c foci (n = 5) colocalized with RPA2c foci at leptotene (Figures 8A to 8C).

Subsequently, we generated a number of RPA1c RNA interference (RNAi) lines to examine whether RPA1c is required for CO formation. Random selection of four RNAi positive transgenic lines with very low seed set exhibited knockdown expression of RPA1c based on qRT-PCR results (Figures 8D and 8E). To examine the function of RPA1c in meiosis, we analyzed chromosome spreads of PMCs at different meiotic stages in RNAi line S31. From leptotene to pachytene, no obvious difference was observed between S31 RNAi and the wild type (Figures 8F to 8H). However, at diakinesis, ~78% of inspected PMC nuclei (n = 139) had reduced numbers of bivalents ranging from 0 to 8 with a mean bivalent number of 4.4 per PMC (Figures 8I and 8J). The mean number of chiasma per cell in RPA1cRNAi was 4.8 (n = 50) compared with 22.7 in the wild type (n = 50), representing a decrease of ~79%. In addition, the distribution of residual chiasmata did not deviate from the predicted Poisson distribution (x^2 = 7.61; P > 0.1; Figure 8K). These results reveal that RPA1c is also required for class I CO formation in rice.
DISCUSSION

RPA2c and RPA1c Are Specifically Involved in Meiotic CO Formation

In yeast and most animals, only one copy of each RPA subunit is thought to exist, which is involved in DNA repair, DNA replication, and recombination. However, multiple copies of RPA subunits occur in Arabidopsis and rice. In Arabidopsis, there are five paralogs of RPA1 and two paralogs each of RPA2 and RPA3 (Shultz et al., 2007). RPA1a is reportedly required for recombination but not for meiotic DNA repair (Osman et al., 2009). Another study showed that the Arabidopsis rpa1b mutant is sensitive to DNA mutagens methyl methane sulfonate (MMS) and UV-B irradiation but grows normally under normal conditions (Ishibashi et al., 2005). These results imply that RPA subunits have evolved with diverse functions regulating various aspects of DNA metabolism. A similar situation has been observed in rice, in which there are three copies of RPA1 (RPA1a, RPA1b, and RPA1c) and RPA2 (RPA2a, RPA2b, and RPA2c) plus a single RPA3. These subunits can specifically combine with each other to produce three types of RPA complex (Ishibashi et al., 2006). However, the function of these RPA genes was unknown until we provided evidence that RPA1a is involved in meiotic and somatic DNA repair, but not CO formation, by analysis of an RPA1a T-DNA insertional mutant (Chang et al., 2009). Here, we characterized RPA2c, an RPA subunit preferentially expressed during meiosis. Phenotypic analysis of an rpa2c mutant revealed a reduction in the number of bivalents and a defect in chiasma formation. Moreover, no chromosome fragments were observed in the rpa2c mutant, implying that meiotic DNA DSBs are repaired. In addition, the rpa2c mutant was insensitive to DNA mutagen treatments (see Supplemental Figure 7 online). We also verified that RPA1c is expressed during meiosis, and knockdown of RPA1c resulted in meiotic defects similar to those in the rpa2c mutant. Thus, Os-RPA1c might be the counterpart of At-RPA1a, which modulates CO formation, although Os-RPA1a actually has the highest sequence identity with At-RPA1a. Thereby, we deduced that the RPA complex containing RPA2c-RPA1c is specifically involved in meiotic CO formation but not in meiotic and somatic DSB repair. Previous reports showed that RPA1b

Figure 7. Immunolocalization of RPA2c and MER3 of the Wild Type and rpa2c.

Dual immunolocalization using mouse polyclonal antibody against RPA2c (green) and rabbit polyclonal antibody against MER3 (red) at early leptotene (A), late leptotene (B), zygotene (C) in wild-type PMCs, and at zygotene (D) in rpa2c. Chromosome was counterstained with DAPI (blue). Merged images show the overlap of green and red fluorescence. Bars = 5 μm.
and \textit{RPA2a} are preferentially expressed in proliferating rice tissues, such as suspension cells, root tips, and young leaves, but are weakly expressed in mature leaves (Ishibashi et al., 2001). Moreover, \textit{RPA1b} is preferentially expressed in proliferating rice tissues and its expression is increased in the intercalary meristem under submergence or treatment with gibberellic acid. \textit{RPA1b} expression in rice precedes that of the DNA replication marker gene histone H3, indicating that it is involved in normal DNA replication and cell proliferation (van der Knaap et al., 1997; Ishibashi et al., 2001). We also identified an \textit{RPA2a} T-DNA–tagged line from our rice T-DNA insertion library, and a quarter of the seeds derived from the

\textbf{Figure 8.} Colocalization of \textit{RPA2c} and \textit{RPA1c}.

(A) to (C) Dual immunolocalization using mouse polyclonal antibody against \textit{RPA1c} (green) and rabbit polyclonal antibody against \textit{RPA2c} (red); chromosome DNA was counterstained with DAPI (blue). Merged images show the overlap of green and red fluorescence. Interphase (A), early leptotene (B), and late leptotene/zygotene (C). Bars = 5 μm.

(D) Fertility of five independent T0 \textit{RPA1c}-suppressed lines. The data shown are the means ± se of three main panicles.

(E) The relative transcript levels of \textit{RPA1c} in \textit{RPA1c}-suppressed lines. Total RNA was extracted from spikelets 4 to 6 cm in length. Rice \textit{Ubiquitin5} was used as an internal control. The data shown are the means ± se of three independent experiments.

(F) to (I) PMCs meiotic chromosome spreads were stained with DAPI in line S31 at leptotene (F), zygotene (G), pachytene (H), and diakinesis (I). Bars = 5 μm.

(J) The frequency of bivalent number per meiocyte in \textit{RPA1c}-suppressed line S31.

(K) The distribution of chiasmata in line S31. Lines with triangles and circles indicate observed distribution and predicted Poisson distribution, respectively.
heterozygous T-DNA insertion plant failed to germinate. Our preliminary data suggest that RPA1b and RPA2a may be required for DNA replication. However, comprehensive and precise composition of each type of RPA and the respective functions of the RPA subunits need to be further elucidated in rice and Arabidopsis.

**Role of RPA2c in Meiotic Recombination**

In budding yeast and mouse, RPA was identified as an important factor in the latter stages of meiotic recombination based on its immunolocalization throughout most of prophase I. It was further reported that RPA may promote Rad52-mediated strand annealing and second-end capture after D-loop formation. Similarly, an Arabidopsis RPA1a mutant (rpa1a) is defective in a late stage of recombination, and RPA1a was suggested to play a role in second-end capture. In this study, we have shown that rice RPA2c is critical for promoting class I CO’s during meiosis. Chromosome pairing and synapsis appeared unaffected in rpa2c compared with the wild type, based on DAPI staining of chromosome spreads and counting FISH signals at early pachytene. However, unlike the distribution of RPA in other species, RPA2c in rice associates with chromatin at S-phase until pachytene based on dual immunostaining of RPA2c with PAIR2 and REC8. However, early RPA2c localization did not appear to be required for CO formation. Previous studies have revealed that at least two classes of CO exist in budding yeast, Arabidopsis, and rice. In this study, rpa2c mutants exhibited a reduction in chiasmata to ~22% and loss of the obligate CO. Moreover, residual chiasmata fit a Poisson distribution, indicating that they are random in number from an apparent loss of CO control. Thus, these results suggest that RPA2c is required for the majority or all of COs but may not have an effect on class II COs. MER3 encodes a DNA helicase containing a DEXH box domain that unwinds duplex DNA in the 3’ to 5’ direction (Nakagawa et al., 2001). It is postulated that MER3 could stabilize nascent interactions via DNA heteroduplex extension to promote capture of the second DNA end, leading to the formation of the double Holliday junctions (Mazina et al., 2004). In our study, the majority of MER3 foci colocalized with RPA2c, and wild-type levels of MER3 foci appear in the rpa2c background. This suggests that RPA2c acts in parallel or downstream of MER3 in CO formation. In addition, taking into account the studies of RPA’s role in a late stage of recombination in yeast and Arabidopsis as well as highly conserved RPA among species, it seems reasonable to speculate that RPA2c is required for second-end capture in rice. Another obvious defect in chromosomal behavior in rpa2c is precocious separation of homologous chromosomes during middle/late pachytene. The meiotic defects of rpa2c are highly similar to the phenotype of rice mer3 null mutant (Wang et al., 2009). Considering that cohesion of homologous chromosomes is largely maintained by cohesin at pachytene and that RPA2c colocalizes with the cohesion subunit REC8, we therefore speculate that RPA2c plays a role in maintaining the paired state of homologous chromosomes until their arms separate normally at diakinesis in rice.

**The Meiotic RPA Complex May Assemble at Axis-Associated ssDNA**

REC8 is a conserved meiotic cohesion complex subunit present in most model organisms that plays a key role in different meiotic process. In budding yeast, the meiosis-specific cohesion component REC8 binds preferentially to AT-rich regions (axis association sites) during the development of axis structures to produce co-oriented linear arrays of loops (Zickler and Kleckner, 1999; Blat et al., 2002). In Arabidopsis and maize (Zea mays), homologs of REC8 are required for homologous pairing and recombination, largely by influencing the chromatin structure (Bhatt et al., 1999; Cai et al., 2003; Chelysheva et al., 2005; Golubovskaya et al., 2006). Rice REC8 was recently identified, and its depletion resulted in a lack of homologous pairing and failure to load key rice meiotic proteins, including PAIR2, PAIR3, MER3, and ZEP1, onto the chromosomes (Wang et al., 2009, 2011; Shao et al., 2011). Therefore, it is reasonable to consider that REC8 may provide a platform for premeiotic recombination apparatus assembly. Here, we show colocalization of discrete REC8 and RPA2c foci that appeared at the S/G2 phase. It is possible that these are nonspecific aggregations of the proteins or may have a functional role. RPA2c-containing RPA could bind to axis association sites at S/G2 phase determined by REC8. This is further supported by the overlapping localization of RPA2c and PAIR2, a rice ortholog of HOP1 (Nonomura et al., 2006). Because RPA is a ssDNA binding protein and loading of RPA2c on chromosomes depends on REC8, we further speculate that AT-rich axis association sites are easily accessed for the loading of REC8 on chromatin during premeiotic S-phase and then may produce ssDNA regions in situ, and this is the prerequisite for the consequent loading of RPA2c to this region.

**METHODS**

**Plant Materials and Growth Conditions**

The rice (Oryza sativa ssp japonica cv Zhonghua11) T-DNA insertion lines 03Z11G173 (rpa2c), 03Z11G174 (rec8), and 03Z11G171 (zep1) were obtained from the Rice Mutant Database (Wu et al., 2003; Zhang et al., 2006; http://rmd.ncpgr.cn/), and 03Z11G175 (pair3) was reported in our previous study (Yuan et al., 2009). Mutant lines were planted in paddy fields of Huazhong Agricultural University during the normal growing season in Wuhan, China, and in a greenhouse during the winter. All transgenic plants were grown under similar growth conditions.

**Complementation Test**

For the functional complementation test, an ~7.7-kb genomic DNA fragment containing the entire RPA2C coding region and 2-kb upstream and downstream regions, respectively, was amplified by primer pair RPA2C-01-RPA2C-02 using KOD Plus DNA polymerase (TOYOBO) and cloned into the binary vector pCAMBIA2301 (Cambia). A construct confirmed by complete sequencing was introduced into Agrobacterium tumefaciens EHA105 and transformed into callus with homozygous rpa2c genotype as the recipient as previously described (Wu et al., 2003). Homozygous callus of rpa2c was selected by genotyping of callus culture induced from seeds harvested from RPA2C/rpa2c heterozygous plants. An empty pCAMBIA2301 vector was used as a negative control.

**Generation of RPA1c RNAi Transgenic Plants**

A 317-bp cDNA fragment of RPA1c was amplified by PCR using RPA1c-DS-F/R primers (see Supplemental Table 2). The PCR product was cloned into the SpeI-SacI and KpnI-BamHI sites of vector pDS1301(Yuan et al.,...
Flanking Sequence Isolation and Genotyping of T-DNA Insertion Sites of Several Lines

The flanking sequence of T-DNA in rpa2c was isolated by thermal asymmetric interlaced PCR according to a previous description (Li et al., 1995; Zhang et al., 2006). Genotyping of the T-DNA insertion in rpa2c was determined by PCR using the T-DNA left border primer NTLB5 coupled with gene-specific primers on both sides of the insertion: 2c-F/R. A similar method was used for genotyping rec8 by primers REC8-F/R and NTLB5, and for genotyping zep1 by primers ZEP1-F/R and TRB2. All primers for genotyping, RT-PCR, qRT-PCR, and vector construction are listed in Supplemental Table 2 online.

RT-PCR and qRT-PCR Analysis for Gene Expression Levels

RT-PCR was performed according to a previous description (Li et al., 2011). Briefly, total RNA was isolated using TRIzol reagent (Invitrogen) and reverse transcribed by SSIII (Invitrogen) with oligo(dT) primer, and the cDNA was used as template for RT-PCR and qRT-PCR. The qRT-PCR was performed in optical 96-well plates that included SYBR Premix ExTaq and Rox Reference DyeII (Takara) on a PRISM7500 PCR instrument (Applied Biosystems). Primers 2c-RT1-F/R and 2c-RT1-F/R were used for detection of RPA2c transcripts; primer pairs RPA1c-QRT-F/R, RPA2a-QRT-F/R, and RPA2b-QRT-F/R were used for examining RPA1c, RPA2a, and RPA2b transcripts respectively; and primers REC8RT-F and REC8RT-R, ZEPRT-F and ZEPRT-R were used for detection of REC8 and ZEP1 transcripts, respectively. All reactions were performed in three separate experiments. For determining the full-length transcripts of RPA2c, 5'-RACE and 3'-RACE were performed using rice young panicle total RNA and the SMART RACE cDNA amplification kit (Clontech) according to the manufacturer’s instructions. All primers are listed in Supplemental Table 2 online.

Antibody Production

For preparation of RPA2c polyclonal antibody, a 501-bp DNA fragment encoding a 167-amino acid peptide of RPA2c (residues 180 to 346), which is specific among rice RPA2 paralogs, was amplified from young rice panicle cDNA using primers RPA2cAb-F/R and was cloned into pET28a (Novagen) with EcoRI-XhoI. Polyclonal antibodies against PAIR2, PAIR3, REC8, MER3, and ZEP1 were prepared according to previous reports (Wang et al., 2009, 2011). Briefly, a 300-bp fragment encoding 100–amino acid peptides of PAIR2 (residues 510 to 609) was amplified from young panicle cDNA using primers PAIR2Ab-F/R and cloned into pGEX-4T-1 (Amersham) with EcoRI-XhoI. For PAIR3, its partial coding fragment (residues 248 to 393) was amplified from panicle cDNA by primers PAIR3Ab-F/R. The PCR product was cloned into EcoRI-XhoI sites of pGEX-4T-1. For REC8, a 747-bp DNA fragment encoding amino acids 139 to 387 of REC8 was amplified from panicle cDNA using primers REC8Ab-F/R and was inserted into EcoRI-XhoI sites of expression vector pGEX-4T-1. For MER3, a 468-bp DNA fragment encoding amino acids 981 to 1136 of MER3 was amplified from panicle cDNA using primers MER3G-F/R and then cloned into BamHI-XhoI sites of pGEX-4T-1. For ZEP1, the ZEP1 partial coding sequence (residues 451 to 638) was amplified from panicle cDNA by primers ZEPAb-F/R. The PCR product was cloned into EcoRI-XhoI sites of pET28a (Novagen). The insert fragment in each construct was verified to ensure no base change by sequencing using an ABI 3730 DNA analyzer (Applied Biosystems). These constructs were transformed into BL21 (DE3) cells (Novagen) using an Electroporator 2510 (Eppendorf). The recombinant His-tagged peptides were induced at 30°C with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h and purified using Nickel nitritriacetic acid agarose (Qiagen). Polyclonal antibodies of these proteins were raised in rabbits and mouse, respectively. The specificity of RPA2c polyclonal antibodies was tested by protein gel blot analysis (see Supplemental Figures 8A and 8B online). Briefly, 30 μg of total protein extract from young panicles was separated on a 10% SDS-PAGE gel and then transferred to a polyvinylidene difluoride membrane. Diluted primary antibodies (rabbit RPA2c antibody, 1:1000 dilution; mouse RPA2c antibody, 1:500 dilution; rabbit RPA1c antibody, 1:500 dilution) and horseradish peroxidase—conjugated secondary antibodies (1:5000 dilution) were used for hybridization.

Rabbit and mouse CenH3 polyclonal antibodies were prepared according to a previous report (Nagaki et al., 2004). Briefly, a peptide consisting of the 19 N-terminal amino acids of rice CenH3 was synthesized and conjugated with a Cys (ARTKHPPAVRPQKCLV C-amide). Polyclonal antibodies of this peptide were raised in rabbits and mouse, respectively.

Meiotic Chromosome Spread Preparation

Young panicles of rice were fixed in Carnoy’s solution (ethanol:glacial acetic, 3:1, v/v). Anthers containing PMCs undergoing meiosis were chopped with a razor in aceto-carmine solution on slides and then covered by cover slips. After removing the cover slips in liquid nitrogen, the slides were dehydrated for 2 min in each ethanol series of 70, 90, and 100%, respectively. Finally, chromosomes spreads on air-dried slides were counterstained with DAPI in an antifade solution (Vector), and the images were captured by DM4000B microscope with a DFC480 digital camera system (Leica) or by an AX10 microscope (Zeiss) with a digital charge-coupled device camera ORCA-R2 C10600 (Hamamatsu).

FISH and Immunofluorescence

FISH on meiotic chromosome spreads was performed as previously described (Jiang et al., 1995). The 866-bp DNA fragment comprising three copies of the 5S rDNA coding sequence was amplified from rice genomic DNA with primers ORYSS-F and ORYSS-R and cloned into pGEM-T easy vector (Promega). The sequence-confirmed construct (pGEM-T-ORYSS) was labeled with Digoxigenin-11-dUTP using Nick Translation Mix (Roche). Chromosomes spreads were counterstained with DAPI in an antifade solution (Vector).

For immunofluorescence, young panicles of rice were harvested and fixed in 4% (w/v) paraformaldehyde for 30 min at room temperature.
PMCs at the proper meiotic stage were squashed on slides with LB01 lysis buffer (15 mM Tris-HCl, pH 7.5, 2 mM Na2-EDTA, 0.5 mM spermine, 4 HCl, 80 mM KCl, 20 mM NaCl, and 0.1% Triton X-100). After cellular debris was removed, air-dried slides were dehydrated in an ethanol series of 70, 90, and 100%. Thereafter, immunostaining was performed in a humid chamber at 37°C for 4 h, and slides were incubated with different antibody combinations diluted 1:200 in 1× PBS containing 1% BSA. After three rounds of washing in 1× PBS, slides were incubated with Texas red-conjugated goat anti-rabbit antibody and fluorescein isothiocyanate-conjugated goat anti-mouse antibody (1:1000 dilution; Jackson ImmunoResearch Laboratories) for 1 h, and then the chromosomes spreads were counterstained with DAPI in an antifade solution (Vector). Both in FISH and immunofluorescence assays, primary images were captured by fluorescence microscopy with a DFC480 digital camera system (Leica) or by an AX10 microscope (Zeiss) with a digital charge-coupled device camera ORCA-R2 C10600 (Hamamatsu). The final images were merged using Adobe Photoshop 7.0 software.

**Mitomycin C and Methyl Methanesulfonate Treatments and UV-C Exposure**

Mitomycin C and methyl methanesulfonate treatments and UV-C exposure were performed as described previously (Chang et al., 2009). Briefly, seeds harvested from heterozygous T-DNA plants (RPA2c/rpa2c) were surface sterilized and germinated on solid half-strength Murashige and Skoog medium. After genotyping of seedlings, the length of each leaf per plant was measured in culture solution with various concentrations of Mitomycin C or methyl methanesulfonate (Sigma-Aldrich). Leaf growth per plant was chosen as the index of sensitivity. For UV-C irradiation, 70-d-old seedlings were harvested from heterozygous T-DNA plants (rpa2c/ZEP1 in Meiocytes of Wild-Type PMCs of Rice). Leaf growth per plant was chosen as the index of sensitivity. For UV-C irradiation, 70-d-old seedlings were inoculated with MMS, and UV-C Irradiation.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: RPA2c, Os06g0693300; RPA1c, Os05g0111000; REC8, AY371049; PAIR2, AB109238; CenH3, Ay438639; ZEP1, GU479042; PAIR3, FJ449711; and MER3, FJ008126.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Characterization of the rpa2c Mutant.

**Supplemental Figure 2.** Molecular Features of RPA2c.

**Supplemental Figure 3.** Immunolocalization of RPA2c and PAIR2 in Wild-Type PMCs of Rice.

**Supplemental Figure 4.** Characterization of Two Mutant Lines, 03Z211EU54 (rec8) and 03Z211LG71 (zep1).

**Supplemental Figure 5.** Immunolocalization of REC8, PAIR3, and ZEP1 in Meiocytes of rpa2c Mutant and RPA2c in pair3 and zep1 Mutants.

**Supplemental Figure 6.** Expression Profile of RPA1c.

**Supplemental Figure 7.** rpa2c Is Insensitive to DNA Mutagens MMC, MMS, and UV-C Irradiation.

**Supplemental Figure 8.** Protein Gel Blot with RPA2c and RPA1C Polyclonal Antibodies.

**Supplemental Table 1.** Reciprocal Crosses between rpa2c and the Wild Type.

**Supplemental Table 2.** Primers for Genotyping, RT-PCR and Plasmid Construction.

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**AUTHOR CONTRIBUTIONS**

X.L., Y.C., and C.W. conceived this project and designed all research. Y.C. identified the rpa2c mutant. X.L. performed the experiments with some assistance from Y.C., X.X., C.Z., and X.H.L.; X.L., J.D.H., and C.W. wrote the article.

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Replication Protein A2c Coupled with Replication Protein A1c Regulates Crossover Formation during Meiosis in Rice
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