CENTRAL REGION COMPONENT1, a Novel Synaptonemal Complex Component, Is Essential for Meiotic Recombination Initiation in Rice

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In meiosis, homologous recombination entails programmed DNA double-strand break (DSB) formation and synaptonemal complex (SC) assembly coupled with the DSB repair. Although SCs display extensive structural conservation among species, their components identified are poorly conserved at the sequence level. Here, we identified a novel SC component, designated CENTRAL REGION COMPONENT1 (CRC1), in rice (Oryza sativa). CRC1 colocalizes with ZEP1, the rice SC transverse filament protein, to the central region of SCs in a mutually dependent fashion. Consistent with this colocalization, CRC1 interacts with ZEP1 in yeast two-hybrid assays. CRC1 is orthologous to Saccharomyces cerevisiae pachytene checkpoint2 (Pch2) and Mus musculus THYROID RECEPTOR-INTERACTING PROTEIN13 (TRIP13) and may be a conserved SC component. Additionally, we provide evidence that CRC1 is essential for meiotic DSB formation. CRC1 interacts with HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS1 (PAIR1) in vitro, suggesting that these proteins act as a complex to promote DSB formation. PAIR2, the rice ortholog of budding yeast homolog pairing1, is required for homologous chromosome pairing. We found that CRC1 is also essential for the recruitment of PAIR2 onto meiotic chromosomes. The roles of CRC1 identified here have not been reported for Pch2 or TRIP13.

INTRODUCTION

In meiosis, homologous recombination is initiated by programmed DNA double-strand break (DSB) formation. Meiotic DSB formation has been most extensively studied in Saccharomyces cerevisiae in which the DSBs are formed by a large complex, with sporulation protein11 (Spo11) as the catalytic subunit (Edlinger and Schölglhofer, 2011). Spo11, a conserved type II topoisomerase, is responsible for catalyzing meiotic DSB formation in various eukaryotes (Edlinger and Schölglhofer, 2011). In addition to Spo11, nine factors (radiation sensitive50, meiotic recombination11 [Mer11], X ray-sensitive2, recombination102, recombination104, recombination114, superkiller8, meiotic recombination2 [Mer2], and meiosis defective4 [Mei4]) were characterized as being subunits of the complex (Edlinger and Schölglhofer, 2011). In contrast with Spo11, the nine factors are poorly conserved at the sequence or functional level, and among them, only the Mei4 orthologs (MEI4 in Mus musculus and PUTATIVE RECOMBINATION INITIATION DEFECT2 [PRD2] in Arabidopsis thaliana) were found to be essential for meiotic DSB formation in plants and mammals (De Muyt et al., 2009; Kumar et al., 2010). MEI4 and PRD2 show enormous sequence divergence from Mei4 and were identified as Mei4 orthologs by a sophisticated in silico study (Kumar et al., 2010).

In plants, besides SPO11, four meiotic DSB-forming factors (PRD1, PRD2, PRD3, and DSB FORMATION [DFO]) in Arabidopsis were identified (De Muyt et al., 2007, 2009; Zhang et al., 2012), but the mechanisms underlying their functions remain elusive. Among them, PRD1 is the ortholog of mouse MEIOSIS DEFECTIVE1, and PRD3 and DFO were considered to be plant-specific proteins. In rice (Oryza sativa), the PRD3 ortholog HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS1 (PAIR1) is essential for homologous chromosome pairing (Nonomura et al., 2004).

In addition to DSB formation, meiotic homologous recombination also requires synaptonemal complex (SC) assembly. The SC mediates the intimate association of homologous chromosomes (homologs) and promotes the posthomology search steps in meiotic recombination. SC assembly starts from early prophase I when a longitudinal protein core, called the axial element (AE), is formed between the two sister chromatids of each chromosome. When chromosomes become synapsed from zygotene to pachytene, the AEs of each homologous pair are physically connected by numerous transverse filaments (TFs) and run parallel to each other. Thus, the SC is a zipper-like structure in which the lateral elements (LEs; corresponding to
the AEIs formed before synapsis) constitute the two parallel backbones and the TFs are similar to the teeth of the zipper (Schmekel and Daneholt, 1995). The region where the TF teeth meet and interdigitate is the central element, corresponding to the electron-dense structure running along and between the two parallel LEIs in electron microscopy examination (Schmekel and Daneholt, 1995).

Several TF proteins have been identified, including zipper1 (Zip1) in budding yeast (Dong and Roeder, 2000), SYNAPTOMES MAL COMPLEX PROTEIN1 in mice (Meuwissen et al., 1992), crossover suppressor on 3 of Gowen in Drosophila melanogaster (Anderson et al., 2005), SYP proteins in Caenorhabditis elegans (Schild-Prüfert et al., 2011), and ZYP1 in Arabidopsis (Higgins et al., 2005). Although they bear no apparent sequence similarity, these TF proteins exhibit significantly similar structures. All TF proteins identified share a coiled-coil domain in the central region with one globular domain at each end. In rice, a TF protein termed ZEP1 was also identified (Wang et al., 2010).

The TF protein is not the only component in the SC central region. Studies in mice and Drosophila have identified several non-TF proteins (SYNAPTOMES MAL COMPLEX CENTRAL ELEMENT1 [SYCE1], SYCE2, SYCE3, and TESTIS EXPRESSED SEQUENCE12 in mice; Corona in Drosophila) localizing to the SC central element (Costa et al., 2005; Hamer et al., 2006; Schramm et al., 2011; Lake and Hawley, 2012). These non-TF central region proteins are essential for TF assembly within SCs, but their orthologs could not be identified in plants by BLAST searches in the database.

Pachytene checkpoint2 (Pch2), a conserved AAA-ATPase, was first identified as a pachytene checkpoint factor in budding yeast (San-Segundo and Roeder, 1999). Subsequently, the essential role of Pch2 in pachytene checkpoint control was also uncovered in C. elegans and Drosophila (Bhalla and Demburg, 2005; Joyce and McKim, 2009). In mice, the Pch2 ortholog THYROID RECEPTOR-INTERACTING PROTEIN13 (TRIP13) promotes the normal localization of RADS1 on meiotic chromosomes (Roig et al., 2010). In addition, the establishment of normal crossover distribution also requires Pch2/TRIP13 in budding yeast and mice (Zanders and Alani, 2009; Roig et al., 2010).

Here, we identified the rice ortholog of budding yeast Pch2 and mouse TRIP13. To our surprise, we found that rice Pch2/TRIP13 is an SC component and localizes to the central region of SCs. Hence, this rice protein was named CENTRAL REGION COMPONENT1 (CRC1). In addition, we provide evidence that CRC1 is essential for the initiation of meiotic recombination and the recruitment of PAIR2 onto meiotic chromosomes. The roles of CRC1 identified here have not been reported for Pch2 or TRIP13.

RESULTS

Map-Based Cloning of CRC1

The first crc1 mutant was characterized from the japonica rice variety C418. The mutant grew normally at the vegetative stage but was completely sterile (Figures 1A and 1B). I–KI staining showed that the pollen grains were completely nonviable in the mutant (Figures 1C and 1D). Pollinating the mutant flowers with wild-type pollen did not result in seed production, indicating that the mutant is both male and female sterile.

To isolate the target gene, we crossed the heterozygotes with the indica variety Guangluai 4 and used a total of 1487 F2 and F3 segregates showing the sterile phenotype to map the target gene. The gene was preliminarily mapped between the sequence-tagged site (STS) markers S2 and S3 on the long arm of chromosome 4 using 411 F2 segregates, and then further linkage analysis using F3 segregates mapped the gene to a 16-kb segment of the BAC clone AL606595 (see Supplemental Figure 1A online). In this region, there was only one predicted gene (gene ID: 4336167), and a 2-bp deletion at the position 830 to 831 in the gene’s coding sequence was identified in the mutant by sequencing. This mutant allele was named crc1-1. Additionally, three other mutant alleles of CRC1, named crc1-2, crc1-3, and crc1-4, were also identified through map-based cloning (see Supplemental Figure 1B online). Both crc1-2 and crc1-3 were isolated from Yandao 8 (japonica). In crc1-2, a G-to-A transition induces an amino acid substitution of Gly-264 to Arg in the gene’s coding sequence. The crc1-4 mutant was isolated from Zhongxian 3037 (indica), and a G-to-A substitution at the last nucleotide site of the third intron, which results in transcripts lacking the fourth exon, was identified in this mutant. All of the mutants grew normally at the vegetative stage but were completely sterile.

Real-time RT-PCR showed that CRC1 was expressed most highly in young panicles (5 to 7 cm), at lower levels in internodes, roots, and flowering panicles, but not in leaves (see Supplemental Figure 2 online).

An RNA interference (RNAi) experiment of CRC1 generated 74 transgenic lines, 67 of which grew normally at the vegetative stage, but exhibited severely reduced fertility, with seed setting rates lower than 10%.

Full-Length cDNA Cloning and Deduced Protein Sequence of CRC1

By performing rapid amplification of cDNA ends (RACE) and RT-PCR, we obtained a 1785-bp full-length cDNA of CRC1, which differs remarkably from the cDNA sequence AK106344 provided by the Rice Full-Length cDNA Project. The deduced CRC1 protein, which contains 475 amino acid residues with a central AAA-ATPase domain, is 43.8% identical and 58.7% similar to mouse TRIP13 and 23.1% identical and 34.6% similar to budding yeast Pch2. Multiple sequence alignment of CRC1 with its orthologs revealed that the CRC1/Pch2 proteins were highly conserved within the AAA-ATPase domain (see Supplemental Figure 3 online).

Defects in Meiosis Result in the Sterility of crc1

To explore the reason for the sterility in crc1 mutants, we investigated the meiotic chromosome behavior of pollen mother cells from wild-type and mutant plants. In the wild type, meiotic chromosomes condensed as thin threads at leptotene (Figure 2A). At subsequent stages, the chromosomes continued to condense,
and the thin threads began to synapse at zygotene (Figure 2B). At pachytene, the chromosomes were fully synapsed and present as thick threads (Figure 2C). At diplotene, the synapsis was relieved and chiasmata, which correspond to the crossovers formed at pachytene, were visible. Twelve bivalents could be clearly observed at diakinesis (Figure 2D), and the further condensed bivalents aligned on the equatorial plate at metaphase I (Figure 2E). Reductional segregation of the chromosomes occurred at anaphase I (Figures 2F and 2G), and then the equational segregation of sister chromatids at the second meiotic division produced tetrad spores (Figure 2I).

In crc1-1, the chromosome behavior at leptotene was similar to that in the wild type (Figure 3A). However, abnormal chromosome behavior was observed thereafter. Although the chromosomes condensed normally during prophase I, obvious features of homologous chromosome pairing were not observed from zygotene to pachytene (Figures 3B and 3C). Subsequently, 24 chromosome univalents were clearly observed at diakinesis (Figure 3D), and the univalents were often scattered throughout the entire nucleus at metaphase I (Figure 3E). At anaphase I, the chromosomes were unequally separated, and lagging chromosomes were always observed (Figures 3F and 3G). At the end of meiosis, different numbers of chromosomes and micronuclei were observed in the daughter cells (Figures 3H and 3I). The meiotic chromosomes in sterile CRC1RNAi plants and other crc1 mutants behaved similarly to those in crc1-1 (see Supplemental Figures 4A to 4D online).

**CRC1 Is a Prerequisite for Abnormal Chromosome Entanglement and Fragmentation in rec8 Meiocytes**

REC8, a conserved meiosis-specific component of the cohesin complex, is essential for meiotic DSB repair in various organisms. In rice rec8 meiocytes, chromosome entanglements were observed from diplotene to metaphase I, and chromosome fragments appeared at anaphase I (Figure 4A) (Shao et al., 2011). To investigate the role of CRC1 in meiotic pairing and recombination, the meiosis in the rec8 crc1 double mutant was investigated. In rec8 crc1, the chromosomes during prophase I also exhibited the fluffy appearance observed in the rec8 single mutant. However, the abnormal chromosome entanglement and fragmentation observed in rec8 were not observed in the double mutant (Figure 4A). In rec8 crc1, the chromosomes were clearly...
observed as univalents at the end of prophase I; thereafter, equational segregation of the chromosomes occurred at anaphase I (Figure 4A).

**CRC1 Is Essential for Meiotic DSB Formation**

DSB formation is essential for meiotic chromosome pairing in most eukaryotes, including plants. The elimination of rec8 chromosome entanglement and fragmentation by the crc1 mutation, plus the lack of meiotic pairing in crc1, indicates that CRC1 is very likely a DSB-forming factor. To confirm this speculation, immunostaining was conducted in wild-type and crc1-1 microsporocytes using antibodies against several meiotic proteins of rice, including COM1, DMC1, ZIP4, and MER3. COM1, which is involved in DSB end resection, is essential for rice meiotic recombination (Ji et al., 2012). DMC1 mediates the single-strand invasion in meiotic recombination and is required for rice meiotic pairing (Deng and Wang, 2007). ZIP4 and MER3, two components of the ZMM complex (a protein complex required for crossover formation), are essential for interference-sensitive crossover formation in rice (Wang et al., 2009; Shen et al., 2012). Therefore, the antibodies against COM1, DMC1, ZIP4, and MER3 can be used as markers to monitor the process of meiotic recombination. In immunostaining assays, COM1, DMC1, ZIP4, and MER3 were observed as punctuate foci on wild-type chromosomes during early meiosis, and these foci persisted until pachytene (Figures 5A to 5C and 5G) (Wang et al., 2009; Ji et al., 2012; Shen et al., 2012). By contrast, no signals of COM1, DMC1, ZIP4, or MER3 were observed on the crc1-1 meiotic chromosomes (Figures 5D to 5F and 5J), suggesting that CRC1 is essential for meiotic DSB formation.

Meiotic DSBs trigger the phosphorylation of histone H2AX from leptotene to early zygotene in various eukaryotes (Dickey et al., 2009). Therefore, to further confirm that CRC1 is essential for rice meiotic recombination (Ji et al., 2012). DMC1 mediates the single-strand invasion in meiotic recombination and is required for rice meiotic pairing (Deng and Wang, 2007). ZIP4 and MER3, two components of the ZMM complex (a protein complex required for crossover formation), are essential for interference-sensitive crossover formation in rice (Wang et al., 2009; Shen et al., 2012). Therefore, the antibodies against COM1, DMC1, ZIP4, and MER3 can be used as markers to monitor the process of meiotic recombination. In immunostaining assays, COM1, DMC1, ZIP4, and MER3 were observed as punctuate foci on wild-type chromosomes during early meiosis, and these foci persisted until pachytene (Figures 5A to 5C and 5G) (Wang et al., 2009; Ji et al., 2012; Shen et al., 2012). By contrast, no signals of COM1, DMC1, ZIP4, or MER3 were observed on the crc1-1 meiotic chromosomes (Figures 5D to 5F and 5J), suggesting that CRC1 is essential for meiotic DSB formation.
for meiotic DSB formation, an antibody specifically recognizing the rice phospho-histone H2AX (γH2AX) was raised in rabbits, and immunostaining with the antibody was conducted in microsporocytes. The results revealed numerous chromatin-associated γH2AX dot and patchy signals during early prophase I in the wild type (Figure 4B). In crc1-1, however, no γH2AX signals were observed in meiocytes at the corresponding stages (Figure 4B). Collectively, these results indicate that CRC1 is essential for meiotic DSB formation.

**CRC1 Is Essential for the Recruitment of PAIR2 onto Meiotic Chromosomes**

PAIR2, the rice ortholog of budding yeast Hop1 and mouse HORMAD1 and -2, is essential for homologous chromosome pairing (Nonomura et al., 2006). In budding yeast and mice, the normal chromosome localization of Hop1, HORMAD1, and HORMAD2 requires Pch2/TRIP13 (Börner et al., 2008; Wojtas et al., 2009). Therefore, immunostaining for PAIR2 was conducted in wild-type and crc1-1 microsporocytes. The result showed that PAIR2 preferentially associated presynaptic chromosome axes in the wild type (Figure 5H) (Nonomura et al., 2006; Wang et al., 2009). In crc1-1, however, no PAIR2 signals were observed (Figure 5K), indicating that CRC1 is essential for the chromosome recruitment of PAIR2.

Immunostaining for another chromosome axis-associated protein, PAIR3, was also conducted. Similar to the wild type, the antibody against PAIR3 labeled the chromosome axes during prophase I in crc1-1 (Figures 5I and 5L).

**CRC1 Interacts with PAIR1 in Vitro**

We then tried to identify CRC1-interacting partners to help elucidate the roles of CRC1. Therefore, yeast two-hybrid (Y2H) assays between CRC1 and proteins including PAIR1, PAIR2, PAIR3, SPO11-1, and SPO11-4 were conducted. The results

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**Figure 3. Meiosis in the crc1-1 Mutant.**

(A) Leptotene.
(B) Zygotene.
(C) Pachytene.
(D) Diakinesis.
(E) Metaphase I.
(F) Anaphase I.
(G) Late anaphase I.
(H) Telophase I.
(I) Telophase II.
Bars = 5 μm.
revealed an interaction between CRC1 and PAIR1 (Figure 4C). The self-interaction of CRC1 was also detected in this system (Figure 4C). To further validate these interactions, we conducted in vitro glutathione S-transferase (GST) pull-down assays. In the assays, MBP-CRC1 showed specificity to GST-PAIR1 and GST-CRC1 (Figure 4D), consistent with the results of the Y2H assays. The interactions suggest that CRC1 and PAIR1 act as a complex to promote meiotic DSB formation.

CRC1 Localizes to the Central Region of SCs

To investigate the spatial and temporal localization of CRC1, a polyclonal antibody was raised against the full-length sequence of CRC1, and immunostaining with the antibody was conducted in rice microsporocytes. In this assay, CRC1 first appeared as punctuate foci on chromosomes at leptotene (Figure 6A). At zygotene, the cells began to show linear CRC1 signals along the chromosome axes (Figure 6B). At early pachytene, CRC1 was observed as linear signals along the entire chromosome axes, and these linear signals were slightly narrower than the REC8 signals at this stage (Figure 6C). At late pachytene or early diplotene, when REC8 was present as double thread signals on each chromosome, CRC1 signals were situated in the center between and along the REC8 signals (Figure 6D), indicating that CRC1 localizes to the SC central region. The CRC1 protein was rapidly removed from the chromosomes at diplotene, and at diakinesis, no CRC1 signals were observed on the chromosomes (Figure 6E).

Similar immunostaining signals were observed when we used polyclonal antibodies against the N-terminal 171 residues of CRC1 or against the CRC1 C-terminal 175 residues. The CRC1 immunostaining signals were not detected in mitotic cells or in crc1 mutants.

CRC1 and ZEP1 Colocalize to SC Central Region in a Mutually Dependent Fashion

The pattern of CRC1 localization was similar to that of ZEP1. Therefore, dual immunostaining for CRC1 and ZEP1 was conducted in the wild type. The result revealed complete colocalization of CRC1 and ZEP1 from leptotene to pachytene (Figure 7A). Immunostaining for CRC1 and ZEP1 was also conducted in zep1 and crc1-1, respectively. CRC1 was not detected on zep1 meiotic chromosomes, nor was ZEP1 detected in crc1-1 (Figure 7B), indicating that CRC1 and ZEP1 colocalize to the SC central region in a mutually dependent fashion.

Figure 4. CRC1 Is Essential for Meiotic DSB Formation.

(A) Comparison of meiotic chromosome behavior in rec8 and rec8 crc1. Bars = 5 μm.

(B) Immunostaining for γH2AX (red) in the wild type and crc1-1 at zygotene. Chromosomes were stained with 4,6-diamidino-2-phenylindole (DAPI; blue). REC8 signals (green) were used to indicate the meiocytes. Bars = 5 μm.

(C) CRC1 interacts with itself and PAIR1 in Y2H assays. The interactions were verified by the growth of yeast on selective medium (SD-Leu-Trp-Ura-His with 5 mM 3-AT) and the blue color of yeast in X-Gal assays. BD, bait vector; AD, prey vector; 3-AT, 3-amino-1,2,4-triazole.

(D) Pull-down assays. Lane 1, purified maltose-binding protein (MBP) tag alone; lane 2, purified MBP-CRC1 alone; lane 3, MBP-CRC1 incubated with resin-bound GST tag; lane 4, MBP-CRC1 incubated with resin-bound GST-PAIR1; lane 5, MBP tag incubated with resin-bound GST-PAIR1; lane 6, MBP tag incubated with resin-bound GST-CRC1; lane 7, MBP-CRC1 incubated with resin-bound GST-CRC1; lane 8, MBP tag incubated with resin-bound GST tag.
Here, the meiosis in zep1 crc1 double mutant was also investigated. In zep1, the homologous chromosomes can pair, but cannot synapse, with each other (Wang et al., 2010). In zep1 crc1, however, no homologous chromosome pairing was observed (Figure 7C), consistent with the essential role of CRC1 in meiotic recombination initiation.

**CRC1 Interacts with ZEP1 in Yeast**

Subsequently, Y2H assays were conducted between CRC1 and ZEP1. The result revealed an interaction between CRC1 and ZEP1 (Figure 7D). The TF proteins were reported to be aligned in head-to-head dimers, with the C terminus positioned in the LE and the N terminus in the central element, to bridge the parallel LES (de Boer and Heyting, 2006). Thus, the domain of ZEP1 responsible for interacting with CRC1 may define the ultrastructural position of CRC1 within SCs. Therefore, Y2H assays between CRC1 and the three ZEP1 domains were conducted. The results showed that CRC1 only interacted with the N-terminal domain (residues 1 to 70) of ZEP1 (see Supplemental Figure 5 online), consistent with the SC central region localization of CRC1.

Figure 5. Immunostaining Detection of Several Meiotic Proteins in the Wild Type and crc1-1.

(A) to (C), (G), and (H) Staining for COM1, DMC1, ZIP4, MER3, and PAIR2 (green) at early zygotene in the wild type.

(D) to (F) and (J) to (K) Staining for COM1, DMC1, ZIP4, MER3, and PAIR2 (green) at early zygotene in crc1-1.

(I) and (L) PAIR3 signals (green) at late zygotene in the wild type (I) and crc1-1 (L).

REC8 signals (red) were used to indicate the meiotic chromosome axes. Bars = 5 μm.
DISCUSSION

CRC1 May Be a Conserved SC Component

Although SCs are highly conserved at the ultrastructural level, their components identified are poorly conserved at the sequence level. Here, we identified CRC1, a novel SC central region component. CRC1 is highly orthologous to budding yeast Pch2 and mouse TRIP13. Thus, it may be a conserved SC component.

Pch2 is a protein that was thought to have close relationships with synapsis (Wu and Burgess, 2006). Its orthologs exist...
extensively in organisms known to undergo synaptic meiosis but are absent from asynaptic organisms (e.g., *Schizosaccharomyces pombe*, *Aspergillus nidulans*, and *Tetrahymena thermophila*) (Wu and Burgess, 2006), suggesting that Pch2 is highly associated with synapsis during evolution. In budding yeast, Pch2 functions in a pachytene checkpoint pathway that specifically monitors aberrant SC intermediates (Wu and Burgess, 2006; Ho and Burgess, 2011), and the mechanism underlying this role remains elusive. The essential role of Pch2 in the synapsis checkpoint control was also characterized in *C. elegans* (Bhalla and Dernburg, 2005). The identification of CRC1 as an SC component provides a valuable insight into the role of CRC1 in the synapsis-monitoring checkpoint control. Pch2 also may be an SC component and thus functions as a primary sensor to signal the state of SC assembly, thereby promoting progress through or arrest at the pachytene stage.

**Figure 7.** CRC1 Is a Novel SC Component.

(A) CRC1 (green) colocalizes with ZEP1 (red) during SC assembly. Chromosomes were stained with DAPI (blue). Bars = 5 μm.
(B) Immunostaining for CRC1 (green) and ZEP1 (green) in *crc1-1* and *zep1* microsporocytes. REC8 signals (red) were used to indicate the chromosome axes. Bars = 5 μm.
(C) Comparison of meiotic chromosome behavior in *zep1* and *zep1 crc1*. Bars = 5 μm.
(D) CRC1 interacts with ZEP1 and itself in Y2H assays. The interactions were verified by the growth of yeast on selective medium (SD-Leu-Trp-Ura-His with 5 mM 3-AT) and the blue color of yeast in X-Gal assays. BD, bait vector; AD, prey vector.

**CRC1 Is Essential for Meiotic Recombination Initiation and PAIR2 Recruitment onto Chromosomes**

In meiosis, DSB formation is catalyzed by the conserved Spo11 protein. In addition to Spo11, a suite of regulatory and accessory factors are also essential for the DSB formation. In budding yeast, nine factors form a large complex with Spo11 to promote the Spo11-mediated DSB formation (Edlinger and Schlögelhofer, 2011). Here, we provide evidence that CRC1 is essential for meiotic DSB formation. CRC1 interacts with PAIR1, a plant-specific DSB-forming factor, in vitro, suggesting that CRC1 and PAIR1 act as a complex to promote the DSB formation. In Y2H assays, we did not detect the interactions between SPO11 (including SPO11-1 and SPO11-4) and PAIR1 or between SPO11 and CRC1, and the exact functional relationship between SPO11 and the PAIR1-CRC1 complex remains to be determined.
PAIR2, the rice ortholog of budding yeast Hop1 and Arabidopsis ASYNAPTIC1 (ASY1), is essential for the chromosome pairing in meiosis (Nonomura et al., 2006). The budding yeast Hop1 functions in meiotic DSB formation, and significantly reduced DSB level was observed in the absence of Hop1 (Mao-Draayer et al., 1996). In addition, Hop1 also plays an essential role in establishing the interhomolog rather than interister chromatid recombination (Niu et al., 2009). In Arabidopsis, ASY1 also plays a key role in establishing the interhomolog recombination, but meiotic DSBs are formed independently of ASY1 (Sanchez-Moran et al., 2007). In this study, we found that CRC1 is essential for the recruitment of PAIR2 onto meiotic chromosomes. This result suggests that CRC1 is also involved in the early steps of meiotic recombination and perhaps promotes the interhomolog recombination through the recruitment of PAIR2.

CRC1 Has Different Functions from Pch2 and TRIP13

The budding yeast Pch2 and mouse TRIP13 are highly orthologous to rice CRC1, but the roles of CRC1 identified here have not been reported for Pch2 or TRIP13.

CRC1 is essential for meiotic recombination initiation. However, it is known that Pch2 and TRIP13 are not essential for meiotic recombination initiation. In budding yeast pch2 mutant strains, meiotic recombination is slightly delayed, but meiotic pairing appears to be normal, and recombination repair can be completed (Wu and Burgess, 2006; Börner et al., 2008). Therefore, the mutant strains exhibit normal spore viability. In Trip3 knockout mice, meiotic recombination is initiated normally but cannot be completed (Roig et al., 2010).

In rice, CRC1 is essential for the recruitment of PAIR2 onto meiotic chromosomes. However, this is not the case for Pch2 or TRIP13. In budding yeast and mice, the chromosome axis-associated HORMA-domain proteins (budding yeast Hop1 and mouse HORMAD1 and HORMAD2) preferentially localize to presynaptic chromosome axes, and with the SC assembly, they are significantly depleted from the synapsed regions of chromosomes (Börner et al., 2008; Wojtasz et al., 2009). Pch2 and TRIP13 are essential for the depletion of the HORMA-domain proteins, but not for the recruitment of these proteins (Börner et al., 2008; Wojtasz et al., 2009; Roig et al., 2010). The distribution of Hop1, HORMAD1, and HORMAD2 along the presynaptic chromosome axes is normal in the pch2/Trip13 mutants.

METHODS

Plant Materials

All crc1 lines were isolated from mutants induced by 60Co γ-ray irradiation. To obtain the rec8/crc1 and zep1/crc1 double mutants, the crc1-1/crc1 plants were crossed with rec8i/crc1 and zep1i/crc1; subsequently, genotyping of the F1 and F2 progeny was conducted to select the double mutants. The rec8 and zep1 mutants used in this study were named Osrec8-1 and zep1-1, respectively, in previous reports (Wang et al., 2010; Shao et al., 2011). All of the plants were grown in paddy fields.

Map-Based Cloning of the Target Gene

To map the target gene, STS and cleaved-amplified polymorphic sequence markers were developed based on the genomic sequence difference between Nipponbare (japonica) and 9311 (indica) according to the data published at http://www.ncbi.nlm.nih.gov/. Among them, R1 and R3 were cleaved-amplified polymorphic sequence markers, and the others were STS markers (see Supplemental Table 1 online). The PCR products of R1 and R3 primers were digested with Nhel and Ncol, respectively, to determine the polymorphism. The crc1-2/crc1, crc1-3/crc1, and crc1-4/crc1 plants were crossed with Longtepui (indica), Guangluai 4 (indica), and Nipponbare (japonica), respectively, to produce the mapping populations.

Full-Length cDNA Cloning of CRC1

RNA extraction was conducted using the TRizol reagent (Invitrogen). Reverse transcription and RACE were performed using the SMARTer RACE cDNA amplification kit (Clontech) (see Supplemental Table 2 online for the gene-specific primers used in this experiment). Gene-specific primers RS-1 and RS-2, combined with the universal primers provided in the kit, were used to perform 5’ RACE PCR. For 3’ RACE PCR, gene-specific primers R3-1 and R3-2 were used along with the universal primers. PCR using primers ORF-F and ORF-R was performed to amplify the open reading frame. The PCR products were cloned into the PMD18-T vector (TakaRa) and sequenced. The sequences were then spliced together to obtain the full-length cDNA sequence.

Real-Time RT-PCR for Transcript Expression Assay

Total RNA was extracted individually from the roots, internodes, leaves, young panicles (5 to 7 cm), and flowering panicles of C418. Reverse transcription was performed using the SuperScript III reverse transcriptase (Invitrogen). Real-time RT-PCR analysis was performed using the Bio-Rad CFX96 real-time PCR instrument and EvaGreen (Biotium). The RT-PCR was conducted with gene-specific primers CRC1RT-F and CRC1RT-R for CRC1, and Actin-F and Actin-R for ACT6 (see Supplemental Table 2 online for the primer sequences).

RNAi Knockdown of CRC1

A fragment of the CRC1 coding sequence was amplified using the primers CRC1i-1F and CRC1i-1R (see Supplemental Table 2 online for the primer sequences). The RNAi vector (pCam23A) construction and transformation were performed as previously described (Wang et al., 2009; Huang et al., 2010).

Antibody Production

The anti-CRC1 antibodies were raised in mice against GST-fused proteins. The DMC1 polyclonal antibody was generated in mice against GST-fused DMC1 protein. The antibodies against REC8, ZEP1, PAIR2, PAIR3, COM1, MER3, and ZIP4 were previously described (Wang et al., 2009, 2011; Wang et al., 2010; Ji et al., 2012; Shen et al., 2012). To produce the rice (Oryza sativa) γH2AX antibody, a rabbit polyclonal antibody was raised against a KLH conjugated peptide (DIGSAP[S]QE, designated H2AX-C-pS134) of the rice H2AX (gene ID: 4333939) and then the antibody was affinity purified first with H2AX-C (DIGSASQEF) and then with H2AX-C-pS134 to obtain the antibody that specifically recognizes the phosphorylated Ser-134 of rice H2AX.

Meiotic Chromosome Preparation

Fresh young panicles were fixed in Carnoy’s solution (ethanol:glacial acetic acid, 3:1), and anthers at the proper developmental stage were then squashed in an acetocarmine solution. After being frozen in liquid nitrogen for 2 min, the slides with squashed meiocytes were dehydrated through an ethanol series (70, 90, and 100%). Chromosomes on the slides were then counterstained with DAPI in an antifade solution (Vector Laboratories).
Chromosome images were captured under the Zeiss A2 fluorescence microscope with a micro-charged-coupled device camera.

**Immunofluorescence**

After being fixed in 4% (w/v) paraformaldehyde for 30 min at room temperature, fresh young panicles were soaked in PBS solution. Anthers at the proper developmental stage were then squashed on slides. After soaking in liquid nitrogen for 2 min, the slides were dehydrated through an ethanol series (70, 90, and 100%). Then, the slides with squashed meiocytes were used in immunostaining with antibody combinations diluted 1:500 in TNB buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.5% blocking reagent). After incubation in a humid chamber at 37°C for 4 h, the slides were washed three times with PBS solution. Texas red-conjugated goat anti-rabbit antibody and fluorescein isothiocyanate-conjugated sheep anti-mouse antibody (1:100) were then added to the slides. Finally, after incubation in the humid chamber at 37°C for 30 min and then three rounds of washes with PBS, the slides were counterstained with DAPI in the antifade solution.

**Y2H Assays**

The Y2H assays were performed using the ProQuest two-hybrid system with Gateway Technology (Invitrogen). Plasmids pDEST32 and pDEST22 were used as the bait and prey vectors, respectively. The yeast strain used here was MaV203.

**Pull-Down Assays**

The PAIR1 coding sequence was cloned into PGEX4T-2 (Amersham), and the CRC1 coding sequence was cloned into PEGX4T-2 and PMAL-C5X (NEB), Escherichia coli BL21 (DE3) cells harboring the empty or recombinant vectors were cultured in Luria-Bertani medium at 37°C to reach the exponential phase, and the protein expression was then induced by adding 0.1 μM isopropyl-β-D-thiogalactopyranoside to the cultures that would be further cultured at 16°C for about 10 h. Then, the cell lysates containing ~15 to 50 μg soluble GST-fused proteins or GST tag were incubated with 120 μL 50% Glutathione Sepharose 4B (GE Healthcare) at 4°C for 1 h. Thereafter, the Glutathione Sepharose 4B was washed three times with PBS solution. Then, the cell lysates containing ~15 to 50 μg soluble MBP-fused proteins or MBP tag were incubated with the Glutathione Sepharose 4B at 4°C for 1 h. After washing the Glutathione Sepharose 4B five times with PBS solution, the proteins were eluted with 100 μL elution buffer (50 mM Tris-HCl and 10 mM reduced glutathione, pH 8.0). Then, the proteins were subjected to protein gel blotting analysis using an anti-MBP antibody (EarthOx).

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under the accession numbers: CRC1, KF245924; COM1, AK119314; DMC1, AB079874; ZIP4, BAD82367; MER3, FJ009126; H2AX, NP_001051106; PAIR1, AB158462; PAIR2, AB109238; PAIR3, FJ449712; SPO11-1, GU170363; SPO11-4, GU177866; REC8, AY371049; ZEP1, GU479042; TRIP13, NP_081458; PCH2, (2005). A conserved checkpoint complex protein 1 (SYCP1) are at the centre of meiosis. J. Cell Sci. 118: 2755–2762.


CENTRAL REGION COMPONENT1, a Novel Synaptonemal Complex Component, Is Essential for Meiotic Recombination Initiation in Rice
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