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BRK1, a Bub1-Related Kinase, Is Essential for Generating Proper Tension between Homologous Kinetochores at Metaphase I of Rice Meiosis[W]

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Bub1 (for budding uninhibited by benzimidazole 1), one of the main spindle checkpoint kinases, acts as a kinetochore scaffold for assembling other checkpoint proteins. Here, we identify a plant Bub1-related kinase 1 (BRK1) in rice (*Oryza sativa*). The *brk1* mutants are sterile due to the precocious separation of sister chromatids at the onset of anaphase I. The centromeric recruitment of SHUGOSHIN1 and phosphorylation of histone H2A at Thr-134 (H2A-pT134) depend on BRK1. Although the homologs can faithfully separate from each other at the end of meiosis I, the uncorrected merotelic attachment of paired sister kinetochores at the early stage of metaphase I in *brk1* reduces the tension across homologous kinetochores, causes the metaphase I spindle to be aberrantly shaped, and subsequently affects the synchronicity of homolog separation at the onset of anaphase I. In addition, the phosphorylation of inner centromeric histone H3 at Ser-10 (H3-pS10) during diakinesis depends on BRK1. Therefore, we speculate that BRK1 may be required for normal localization of Aurora kinase before the onset of metaphase I, which is responsible for correcting the merotelic attachment.

INTRODUCTION

In the mitotic cell cycle, it is only when all chromosomes have appropriately established bipolar attachment to the mitotic spindle that the metaphase-to-anaphase transition can occur (Chen, 2004; Yu and Tang, 2005). Because kinetochores capture microtubules in a stochastic manner, three types of erroneous kinetochore attachments, including monotelic (only one of the sister kinetochores is attached to one spindle pole), syntelic (both sister kinetochores are attached to the same spindle pole), and merotelic (one kinetochore is attached to two opposing spindle poles) attachment, frequently occur (Gregan et al., 2011). Thus, there must be a surveillance mechanism that prevents the precocious separation of sister chromatids until all the improper kinetochore attachments are corrected, ensuring the fidelity of chromosome segregation (Meraldi and Sorger, 2005; Yu and Tang, 2005). The spindle assembly checkpoint (SAC) is such a mechanism that delays anaphase onset when any kinetochore is not properly attached to the spindle fibers or when sister kinetochores are not under tension (Musacchio and Salmon, 2007).

The SAC is mediated by a set of highly conserved proteins, including mitotic arrest-deficient protein 1 (Mad1), Mad2, budding uninhibited by benzimidazole 1 (Bub1), Bub3, Mad3/BubR1, and monopolar spindles 1 (Mps1) (Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996). In response to unattached kinetochores, these proteins collaborate to inhibit the ubiquitin ligase activity of the anaphase-promoting complex/cyclosome (APC/C), through sequestration of Cdc20, an activator of APC/C, thereby delaying sister chromatid separation and exit from mitosis (Peters, 2002; Malmanche et al., 2006). Once all chromosomes are bi-oriented, the SAC will be silenced. At this point, the activated APC/C can polyubiquitinate securin and cyclin, leading to their proteasome-mediated degradation. Destruction of the inhibitory protein securin releases active separase, which triggers sister chromatid separation and anaphase onset by cleaving cohesin. Protein phosphorylation and dephosphorylation are essential regulatory mechanisms in SAC signaling, and the checkpoint kinases play a major role in transducing and amplifying checkpoint signals (Zich and Hardwick, 2010). Mps1, Aurora B (a key member of the chromosomal passenger complex [CPC]), Bub1, and BubR1 are the core spindle checkpoint kinases and are highly conserved among organisms, although the kinase domain of BubR1 is not found in the yeast Mad3 homologs (Malmanche et al., 2006). It has been proposed that phosphorylation in active SAC may directly enrich checkpoint signaling molecules by stabilizing protein–protein interactions and contribute to the building and activation of kinetochore scaffolds (Bub1 and Mad1), which is essential for recruiting the downstream checkpoint proteins Mad3 and Mad2 (Zich and Hardwick, 2010).

Bub1 is a Ser/Thr protein kinase that was first identified in a screen for budding yeast mutants that are sensitive to benomyl, a spindle poison (Hoyt et al., 1991; Roberts et al., 1994). Bub1 contains three main domains: a conserved N-terminal domain that regulates its kinetochore localization, an intermediate, nonconserved domain that is required for Bub3 binding, and

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a C-terminal catalytic Ser/Thr kinase domain (Klebig et al., 2009). Bub1 is not only required for the centromeric localization of Cenp-F, BubR1, Cenp-E, and Mad2 but is also essential for correct chromosome congression on the metaphase plate (Johnson et al., 2004; Meraldi and Sorger, 2005). The kinase domain of Bub1 is essential for targeting shugoshin, which plays a critical role in protecting centromeric cohesin from cleavage by separase during meiosis I, to the centromere to ensure the fidelity of chromosome segregation (Kitajima et al., 2004; Fernius and Hardwick, 2007). Recently, it was reported that Bub1 can phosphorylate histone H2A at Ser-121 in fission yeast or Thr-120 in human cells and that this is important for centromeric localization of shugoshin (Kawashima et al., 2010).

Among those kinds of erroneous kinetochore-microtubule connections, merotelic attachment is inadequately monitored by the SAC-dependent arrest because it can still generate tension, although incorrectly, and does not produce unattached kinetochores (Musacchio and Salmon, 2007). Therefore, eukaryotes must employ specific mechanisms for correcting merotelic attachment, in which the inner centromere-localized Aurora B kinase is mainly involved (Lampson and Cheeseman, 2011). In the absence of proper tension across kinetochore pairs (e.g., syntelic or merotelic kinetochore attachment), Aurora B is able to reach and phosphorylate its kinetochore substrates, which leads to destabilization of the erroneous kinetochore-microtubule attachment. Once proper amphitelic attachment is established, the spindle pulling force separates the kinase from its outer kinetochore substrates, stabilizing the correct connection (Fuller et al., 2008; Liu et al., 2009).

Different from mitosis, meiosis is a specialized cell division for sexual reproduction, in which one round of chromosome replication is followed by two rounds of cell division. To guarantee fidelity of homologous chromosome separation in meiosis I, sister kinetochores of each homolog must form an integral unit by adopting a side-by-side rather than mitotic back-to-back conformation to attach to microtubules emanating from the same spindle pole, called mono-orientation (Yokobayashi and Watanabe, 2005). In this manner, proper tension can be generated when homologous pairs are attached to opposite poles of the meiosis I spindle. The mechanism by which the SAC is modified and its core kinases cooperate to regulate the generation of proper tension across the paired sister kinetochores and finally ensure the synchronicity of homolog separation in meiosis I has hitherto been unclear.

Here, we employed map-based cloning to identify the *Bub1-RELATED KINASE1* (*BRK1*) gene in rice (*Oryza sativa*), which encodes a Ser/Thr kinase. Like yeast Bub1, BRK1 is also essential for the centromeric recruitment of SHUGOSHIN1 (SGO1) and histone H2A-T134 phosphorylation. In addition, we found that the plant Bub1 is required for correcting the merotelic attachment of paired sister kinetochores during metaphase I, which can attenuate the tension across homologous kinetochore pairs and affect the synchronicity of homolog separation at the onset of anaphase I. As the phosphorylation of inner centromeric histone H3-S10 at diakinesis is abolished in *brk1*, the erroneous kinetochore-microtubule attachment in the absence of BRK1 may result from abnormal localization of Aurora kinase at the early stage of metaphase I.

RESULTS

Identification of *brk1* Mutants and Isolation of the Gene

A completely sterile mutant was identified from the self-fertilized progeny of an *indica* cultivar rice variety, Zhongxian 3037, induced by $^{60}Co\sim\gamma$-ray radiation. The mutant plant grew normally during the vegetative stage but presented as sterile after flowering. When evaluated by staining with 1% I_2-KI solution, its pollen grains were inviable (see Supplemental Figure 1 online). Among the progeny of self-fertilized heterozygous plants of the mutant, 133 plants had normal fertility and 41 plants were completely sterile (segregation ratio of 3:1, $\chi^2 = 0.123$, $P > 0.05$), implying that the sterility phenotype is controlled by a single recessive gene. The mutant was named *brk1-1*. When pollinated with wild-type pollen, the mutant plants did not set any seeds, demonstrating that megaspore development was also affected.

We isolated *BRK1* using a map-based cloning strategy. The mapping population was constructed by crossing heterozygous $BRK1^{+/-}$ to a *japonica* cultivar Zhonghua 11. *BRK1* was first mapped to the long arm of chromosome 7 through linkage analysis and further narrowed to a 94.7-kb region. Within this region, one candidate gene (*Os07g32480*), annotated as the mitotic checkpoint Ser/Thr protein kinase BUB1 by the Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice), was identified. Its cDNA sequence, which was redefined by real-time PCR and rapid amplification of cDNA ends (RACE) PCR, is significantly longer than the predicted one at the N terminus. The cDNA is 2068 bp long with an open reading frame of 1683 bp, and the gene has 14 exons and 13 introns (Figure 1A). When this candidate gene from the *brk1-1* mutant was sequenced, we found an A-to-T point mutation within its exon 4 relative to the wild type, which introduced a premature termination codon (TAG) (Figure 1A). Additionally, a single *Tos17*-insertion mutant of *BRK1*, *brk1-2*, was identified by screening the public insertion line collections. Sequence analysis of PCR products from this line confirmed that *Tos17* was inserted into *BRK1* exon 9 (Figure 1A). The homozygous *brk1-2* mutant also appeared normal during the vegetative stage but had reduced fertility in the flowering phase (seed set was 32.78%). To further confirm that the sterile phenotype of *brk1* mutants indeed resulted from the mutant gene, RNA interference (RNAi) experiments were performed, specifically targeted against *BRK1*. Among the 36 $BRK1^{RNAi}$ transgenic plants, 25 displayed the sterile phenotype.

Real-time PCR analysis showed that *BRK1* is transcribed most abundantly in the panicles and at a relatively low level in leaves and roots. Additionally, we found that the transcript level of *BRK1* is dramatically reduced in *brk1-1* and $BRK1^{RNAi}$ mutants but only modestly downregulated in *brk1-2* (see Supplemental Figure 2 online). Therefore, defective BRK1 is responsible for the sterile phenotype observed in the mutants.

Kinase Activity Analysis of BRK1 in Vitro

It has been predicted through bioinformatic methods that BRK1 (EEC82122.1 and EEE67244.1 from *O. sativa indica* and *O. sativa japonica*, respectively) is the closest plant homolog of

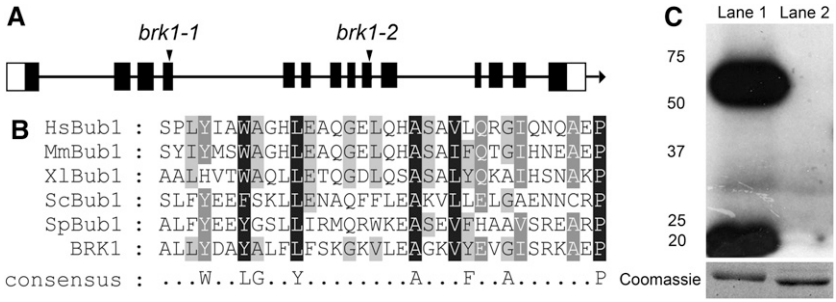

Figure 1. BRK1 Is a Bub1-Related Ser/Thr Kinase.

(A) Schematic representation of *BRK1*. Black boxes and lines indicate exons and introns, respectively, and untranslated regions are shown in white boxes. The triangles indicate the mutant site and the location of the *Tos17* insertion in *brk1-1* and *brk1-2*, respectively.

(B) Multiple sequence alignment of the TPR domain in rice BRK1 with Bub1 of human (Hs), mouse (Mm), frog (Xl), budding yeast (Sc), and fission yeast (Sp). Identical and similar residues are indicated by black and gray boxes, respectively. The TPR consensus is shown at the bottom.

(C) BRK1 displays kinase activity in vitro. Lane 1, the BRK1 kinase domain can phosphorylate itself (57.8 kD) and the MPB substrate (18.4 kD). Lane 2, the mutant BRK1 kinase domain (K287R) loses its kinase activity. Bottom panel shows a Coomassie blue–stained gel illustrating similar levels of the two samples and the mutant BRK1 kinase domain (K287R) is stable.

Bub1 from animals and yeast (Karpov et al., 2010). We aligned the BRK1 sequence with Bub1 sequences from human (*Homo sapiens*), mouse (*Mus musculus*), frog (*Xenopus laevis*), budding yeast (*Saccharomyces cerevisiae*), and fission yeast (*Schizosaccharomyces pombe*) (see Supplemental Figure 3 online). We found that the N terminus of BRK1 is more than 300 amino acids shorter at the N terminus compared with its homologs in other organisms. The N-terminal region of mammalian Bub1 is important for spindle checkpoint and metaphase chromosome congression (Klebig et al., 2009). We did identify a relatively conserved tetratrichopeptide repeat (TPR) motif (amino acids 126 to 159) at the N terminus of BRK1, which is known to be essential for Bub1 binding with Blinkin and its kinetochore localization (Figure 1B) (Kiyomitsu et al., 2007). No conserved Gle2 binding sequence (GLEBS) motif was found in BRK1, which is required for its association with Bub3 (Bolanos-Garcia and Blundell, 2011). The kinase domain of BRK1 (amino acids 257 to 514) is highly conserved, which allowed us to predict that BRK1 is also a Ser/Thr protein kinase.

To assay BRK1 kinase activity in vitro, we expressed the kinase domain (amino acids 208 to 560) of BRK1 fused with a SUMO tag in an *Escherichia coli* expression system. Because Lys-287 has been shown to be essential for catalytic activity in many protein kinases, we also generated a site-directed mutation of Lys-287 to Arg (K287R) to inactivate the kinase domain, which was used as a negative control. Using an in vitro kinase assay, we found that the kinase domain of BRK1 can autophosphorylate, which is a common feature of many protein kinases (Figure 1C). In addition, it could also strongly phosphorylate myelin basic protein (MBP), which is often used as a nonspecific substrate for many different protein kinases (Figure 1C). By contrast, the mutant kinase domain with K287R could neither phosphorylate itself nor MBP (Figure 1C). Therefore, these results firmly establish that BRK1 possesses Ser/Thr kinase activity.

Precocious Separation of Sister Chromatids during Anaphase I Causes Defective Meiosis in *brk1* Mutants

To determine whether the sterility of *brk1-1* was caused by meiotic defects, we examined pollen mother cells at different meiotic stages from both the wild type and the *brk1-1* mutant. During prophase I, chromosomal behavior in *brk1-1* was quite similar to the wild type (Figures 2A and 2E). Whereas at the onset of anaphase I in *brk1-1*, although homologous chromosomes could separate equally, the two sister chromatids of each chromosome started to disassociate from each other (Figure 2F). During telophase I in *brk1-1*, the sister chromatids of most chromosomes completely separated, and this precocious dissociation resulted in scattered chromatids that failed to line up on the equatorial plate during metaphase II (Figure 2G). Consequently, sister chromatids were unequally separated at anaphase II in *brk1-1*, and the lagging chromosomes finally led to the formation of micronuclei during the tetrad stage (Figure 2H).

We also investigated meiotic chromosome behavior in both *brk1-2* and *BRK1*^{RNAi} plants. In *brk1-2*, among the 70 daughter cells at metaphase II, 41 of them had preseparated chromatids resembling those in *brk1-1* (see Supplemental Figures 4C and 4D online), while the other 29 cells were similar to the wild-type meiocytes (see Supplemental Figure 4B online), which indicates that *brk1-2* is a weak allele. However, the chromosomal behavior in *BRK1*^{RNAi} meiocytes was quite similar to that in *brk1-1* (see Supplemental Figures 4E and 4F online). Therefore, the premature separation of sister chromatids at anaphase I in the *brk1* mutants resulted in unequal chromosome segregation during the second meiotic division and accordingly caused their sterile phenotypes.

BRK1 Localizes to Kinetochores during Both Meiosis and Mitosis

To study the localization of BRK1 during meiosis, we performed dual immunostaining of wild-type pollen mother cells using a

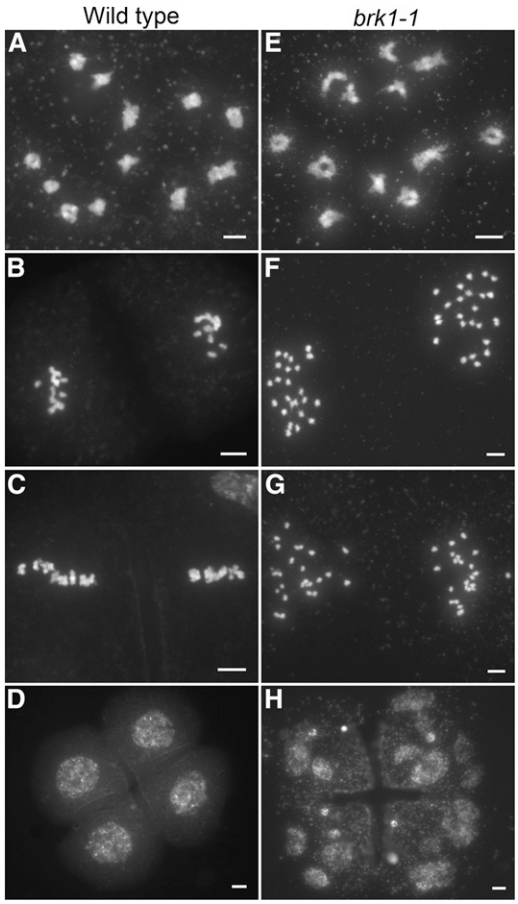

Figure 2. Meiotic Chromosome Behavior in the Wild Type and *brk1-1* Mutant.

(A) to **(D)** The wild type.
(E) to **(H)** *brk1-1*.
(A) and **(E)** Diakinesis. Prophase I in *brk1-1* is normal.
(B) and **(F)** Anaphase I. Sister chromatids precociously dissociate from each other in *brk1-1*.
(C) and **(G)** Metaphase II. The prematurely separated sister chromatids are scattered in the central region of each *brk1-1* daughter cell and cannot align properly on the equatorial plate.
(D) and **(H)** Tetrad. The lagging chromosomes finally result in the formation of micronuclei.
Chromosomes stained with 4',6-diamidino-2-phenylindole (DAPI). Bars = 5 μm.

polyclonal antibody against BRK1 raised from rabbit together with antibodies against REC8, the rice homolog of Rec8, and CENH3, the rice centromere-specific H3 histone, raised in mice (Shao et al., 2011).

BRK1 was first detected as weak foci at centromeres during premeiotic interphase (Figure 3A), with the signals gradually becoming stronger through prophase I (Figures 3B to 3F). With the bivalents lining up on the equatorial plate at metaphase I, BRK1 extended outwards beyond CENH3 (Figure 3G). We also found that BRK1 persisted at kinetochores throughout meiosis II (Figures 3H to 3J).

In addition, immunolocalization of BRK1 was conducted in somatic cells of wild-type rice root tips to show its distribution during mitosis. We observed that BRK1 associated with kinetochores from interphase to telophase (Figures 4A to 4F). Moreover, BRK1 localized to the outer plate of each kinetochore at metaphase (Figure 4D). The dissociation of BRK1 from the kinetochore was also independent of kinetochore-microtubule attachment (see Supplemental Figure 5 online). Therefore, BRK1

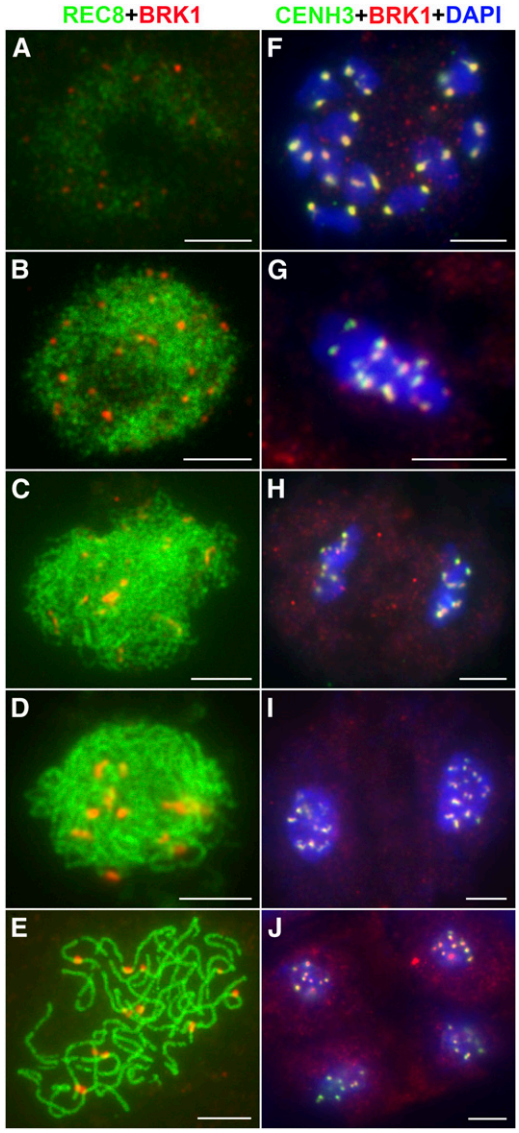

Figure 3. The Localization of BRK1 during the Wild-Type Meiosis.

(A) to **(E)** Premeiotic interphase to early prophase I. BRK1 (red) is first detected at centromeres during premeiotic interphase **(A)**, and then its signals become stronger at leptotene **(B)**, zygotene **(C)**, early pachytene **(D)**, and late pachytene **(E)**. REC8 (green) was used as the chromosome marker.
(F) to **(J)** Diakinesis to tetrad. BRK1 (red) persists at centromeres throughout the whole meiotic process: diakinesis **(F)**, metaphase I **(G)**, anaphase I **(H)**, prophase II **(I)**, and tetrad **(J)**. CENH3 (green) indicates the centromeres.
Chromosomes stained with DAPI (blue). Bars = 5 μm.

associated with the kinetochore stably at the outer plate throughout both meiosis and mitosis.

BRK1 Is Essential for the Centromeric Localization of SGO1

Because the Bub1-dependent localization of SGO1 to centromeres is conserved among eukaryotes and the defective centromeric cohesion of sister chromatids in *brk1* mutants resembles that in *sgo1* (Wang et al., 2011), we speculated that the distribution of SGO1 would also be affected by the absence of BRK1. To verify this, we examined the immunolocalization of BRK1 and SGO1 during both meiosis and mitosis in the wild type, *sgo1-1*, and *brk1-1* mutants.

During the wild-type meiosis, SGO1 was first recruited to the chromosome at early leptotene, when it began to colocalize with BRK1 at the centromere. As SGO1 localized to the pericentromeric regions, its foci were always broader than the BRK1 signals (Figure 5A). With the dissociation of SGO1 from the centromere, this colocalization was terminated during telophase I. In meiocytes from the *sgo1-1* mutant, the recruitment of SGO1 to centromeres from nucleoli was blocked (Wang et al., 2011), while the localization of BRK1 still resembled that observed in the wild type, which indicated that the meiotic distribution of BRK1 is independent of SGO1 (Figure 5B). Neither BRK1 nor SGO1 could be detected during meiosis in *brk1-1* and *BRK1^{RNAi}*, which demonstrated that BRK1 was required for the centromeric localization of SGO1 (Figure 5C; see Supplemental Figure 6 online). In *brk1-2* meiocytes, BRK1 signals were hard to detect and SGO1 was dispersed in the nucleus rather than recruited to centromeres (see Supplemental Figure 6 online). Moreover, similar results as observed in the wild type, *sgo1-1*, and *brk1-1* meiosis I were also detected in the corresponding mitotic somatic cells (Figures 5D to 5F). Therefore, we conclude that the centromeric recruitment of SGO1 is dependent on BRK1 during both meiosis and mitosis.

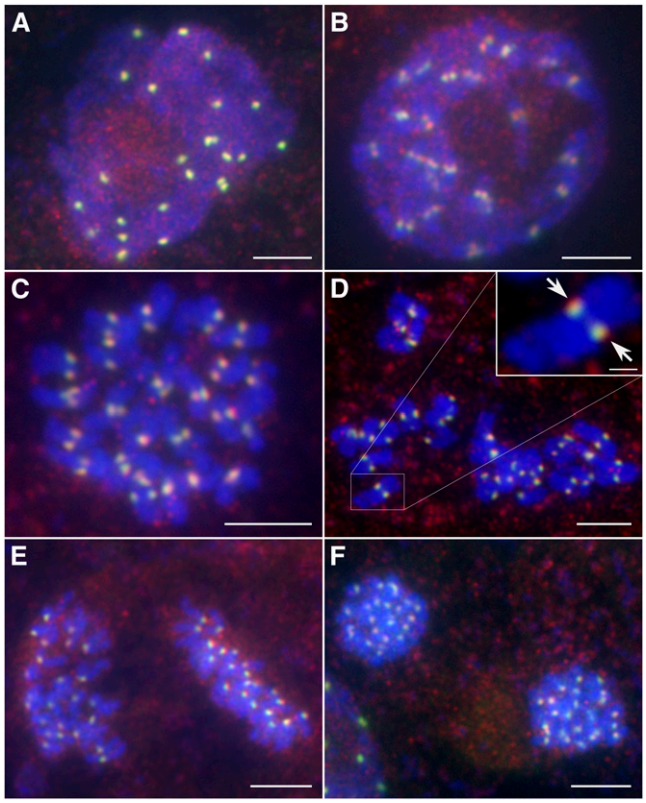

Figure 4. The Localization of BRK1 during Wild-Type Mitosis.

Dual immunolocalization of BRK1 (red) and CENH3 (green) during various mitotic stages of root meristem cells in the wild type. Chromosomes stained with DAPI (blue). Bars = 5 μm.
(A) Interphase. BRK1 can be detected at centromeres during interphase.
(B) and **(C)** Prophase and prometaphase, respectively.
(D) Metaphase. The magnified image of individual chromosomes shows that BRK1 localizes to the outer plates of kinetochores indicated by arrows. Bar in the magnified image = 1 μm.
(E) and **(F)** Telophase and anaphase, respectively.

BRK1 Is Required for Phosphorylation of Rice H2A-T134 during Both Meiosis and Mitosis

It has been reported that Bub1 can phosphorylate Ser-121 and Thr-120 of H2A in yeast and human cells, respectively (Kawashima et al., 2010). Recently, it has been reported that the Bub1 phosphorylation site on H2A is relatively conserved in plants and its phosphorylation is specific to centromere regions (Dong and Han, 2012). Rice Thr-134 of H2A corresponds to the Thr at positions 120 and 133 in human and maize (*Zea mays*), respectively. Therefore, we raised a polyclonal antibody against a synthetic peptide [LPKK(pT)AEKAA, amino acids 130 to 139] in rice H2A from rabbit, with the Thr-134 labeled by a phosphoryl group. ELISA and dot blot assays demonstrated that the antibody specifically recognizes the phosphorylated Thr-134 of the H2A peptide. To verify whether Bub1 is responsible for H2A phosphorylation in plants, dual immunostaining experiments were performed individually on meiocytes and root meristem cells from the wild type, *brk1-1*, and *sgo1-1* using antibodies against H2A-pT134 and CENH3.

In the wild type, H2A-pT134 entirely colocalized with CENH3 in both meiotic and mitotic cells (Figures 6A and 6D), showing that H2A-T134 is specifically phosphorylated at the centromeric regions. In *sgo1-1*, the distribution of H2A-pT134 was quite similar to in the wild type (Figures 6B and 6E). However, no H2A-pT134 immunosignal was detected in the *brk1-1* mutant (Figures 6C and 6F), indicating that H2A-T134 phosphorylation was abolished in the absence of BRK1. We can therefore conclude that BRK1 is essential for H2A-T134 phosphorylation in rice. Weak H2A-pT134 signals were observed at centromeres in *brk1-2* meiocytes (see Supplemental Figure 6 online), which suggests that *brk1-2* maintains residual kinase activity.

Depletion of BRK1 Reduces the Tension across Homologous Kinetochores during Metaphase I

Bub1 has been reported to be required for proper chromosome congression during metaphase of human cell and the high fidelity of chromosome segregation in normal fission yeast mitosis (Bernard et al., 1998; Meraldi and Sorger, 2005). We accordingly

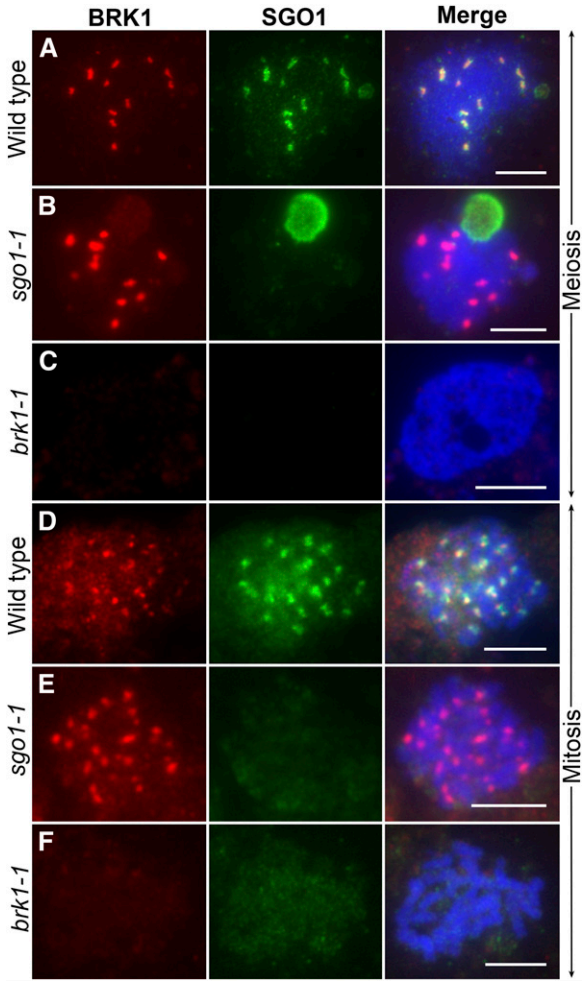

Figure 5. BRK1 Is Required for the Centromeric Recruitment of SGO1.

(A) and **(D)** BRK1 and SGO1 colocalize at centromeres during zygotene **(A)** and prometaphase **(D)** in the wild type.
(B) and **(E)** Zygotene and prometaphase in *sgo1-1*, respectively. BRK1 could be normally recruited to centromeres, but SGO1 is not.
(C) and **(F)** Zygotene and prometaphase in *brk1-1*, respectively. Neither BRK1 nor SGO1 is detected in the *brk1-1* mutant.
Chromosomes stained with DAPI (blue). Bars = 5 μm.

compared the chromosome behavior from metaphase to anaphase of both meiosis I and mitosis in *brk1-1* with those in the wild type and the *sgo1-1* mutant. In the wild type, 12 bivalents could line up orderly on the equatorial plate during metaphase I, and their centromeric regions were markedly stretched out by microtubules (Figure 7A). All the homologous chromosomes separated simultaneously from each other and migrated toward opposite poles at the onset of anaphase I. In the wild-type somatic cell, chromatid pairs congressed correctly to the spindle equator at metaphase and then the segregation of sister chromatids was also synchronous at anaphase (Figure 7A). The chromosome alignment and separation in meiosis I and mitosis of the *sgo1-1* mutant were close to that in the wild type (Figure 7A). By contrast, in the *brk1-1* mutant, we found that the 12 bivalents could not align correctly on the spindle equator during metaphase I, so the metaphase plate was not as obvious as that in the wild type (Figure 7A). Furthermore, the chromatin of homologous centromeres could not be drawn out toward the opposite spindle poles. Although all the homologs could finally be drawn apart toward the opposite poles in the absence of BRK1, the separation of homologous chromosomes at the onset of anaphase I in *brk1-1* was not as synchronous as that in the wild type (Figure 7A). Little difference was detected in the mitotic chromosome congression and segregation between the *brk1-1* mutant and the wild type. Therefore, BRK1 is required for the correct alignment of bivalents and the synchronous separation of homologs during meiosis I.

As mentioned above for the defects in *brk1-1* metaphase I, we suspected that the tension across homologous kinetochores induced by microtubules might be reduced in *brk1-1*. Since the distance between two sister kinetochores can be used to indicate

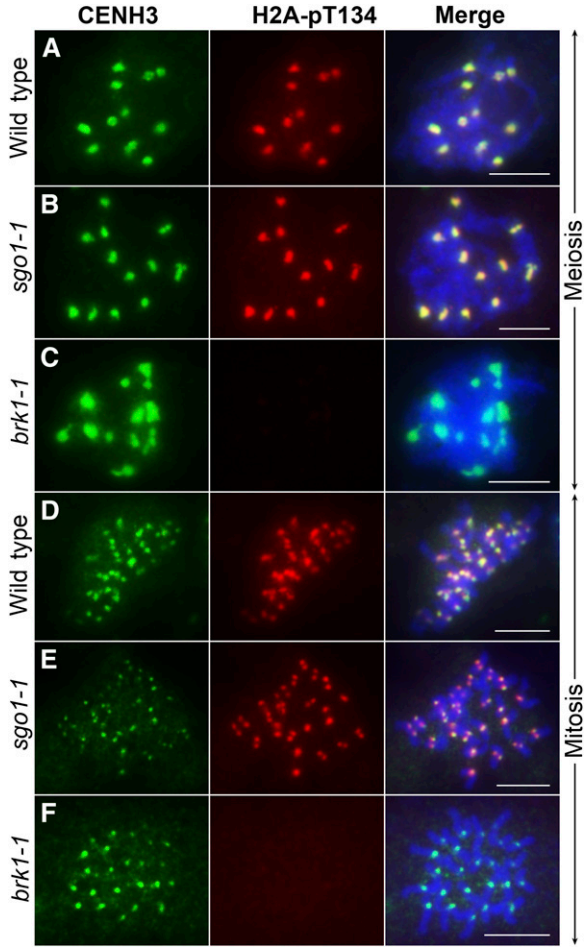

Figure 6. BRK1 Is Essential for Centromeric H2A-T134 Phosphorylation.

(A) to **(C)** Immunolocalization of H2A-pT134 and CENH3 at the pachytene stage of the wild type **(A)**, *sgo1-1* **(B)**, and *brk1-1* **(C)**.
(D) to **(F)** Immunolocalization of H2A-pT134 and CENH3 during prophase of the wild type **(D)**, *sgo1-1* **(E)**, and *brk1-1* **(F)**.
Chromosomes stained with DAPI (blue). Bars = 5 μm.

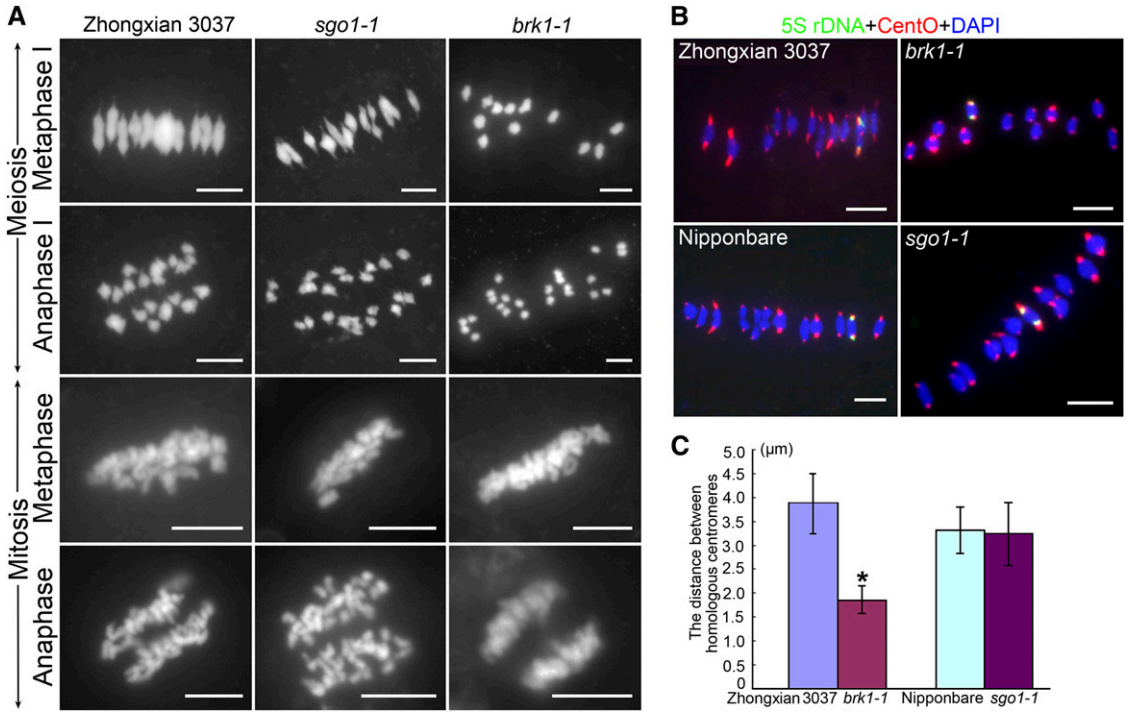

Figure 7. Deletion of BRK1 Reduces the Tension across Homologous Kinetochores at Metaphase I.

(A) Chromosome behavior during metaphase I and the onset of anaphase I, and mitotic metaphase and the onset of anaphase in Zhongxian 3037 (the wild type), *sgo1-1*, and *brk1-1*. During *brk1-1* metaphase I, the bivalents cannot line up on the equatorial plate and the centromeric chromatins are less stretched by microtubules than the wild type. Moreover, the separation of homologous chromosomes at the onset of anaphase I in *brk1-1* is not synchronous. Chromosomes stained with DAPI. Bars = 5 μm.

(B) Distance between homologous centromeres at metaphase I in the wild type, *brk1-1*, and *sgo1-1*. The homologous centromeres were probed with CentO (red) and 5S rDNA (green) through fluorescence in situ hybridization. Compared with the wild type, the average separation between homologous centromeres during metaphase I is reduced in *brk1-1*. Chromosomes stained with DAPI (blue). Bars = 5 μm.

(C) Quantitation of the distance between the center regions of CentO signals of chromosome 11 in Zhongxian 3037 (the wild type), *brk1-1*, Nipponbare (the wild type), and *sgo1-1*. Error bars represent SD (*$P < 0.01$).

the tension at metaphase (McEwen et al., 2001), we also employed this method to measure the tension across homologous kinetochores. Fluorescence in situ hybridization (FISH) was conducted with two cytological markers, CentO and 5S rDNA, on the metaphase I chromosomes of both Zhongxian 3037 (the wild type) and *brk1-1* plants (Figure 7B). CentO, a centromere-specific tandem repeat, is a molecular marker for all rice centromeres. The 5S rDNA is a tandemly repeated sequence located on the short arm close to the centromere of chromosome 11. We measured the distance between the center regions of CentO signals of chromosome 11 (indicated by 5S signals) at metaphase I in both *brk1-1* and the wild type. The average distance in *brk1-1* was 1.858 μm ($n = 40$), which is significantly shorter than the 3.872 μm ($n = 35$) measured in Zhongxian 3037 (Figure 7C) ($t = 18.472$, $P < 0.01$). To exclude the effect of the centromeric absence of SGO1 on the reduced tension in *brk1-1*, we also measured these distances in both Nipponbare (the wild type) and *sgo1-1* (Figure 7B). The average values were 3.236 μm ($n = 31$) and 3.307 μm ($n = 30$) in *sgo1-1* and Nipponbare, respectively, and the difference is not statistically significant (Figure 7C) ($t = 0.485$, $P > 0.05$). Therefore, we conclude that the absence of BRK1 dramatically reduces microtubule-induced tension across homologous kinetochores during metaphase I, and this defect is independent of the centromeric depletion of SGO1 in the *brk1-1* mutant.

BRK1 Is Required for Correcting the Merotelic Attachment of Paired Sister Kinetochores during Metaphase I

To find out what caused the reduced tension across homologous kinetochores, we detected the kinetochore-microtubule attachment during *brk1-1* metaphase I and compared it with that in the wild type. At the wild-type metaphase I, all 12 bivalents lined up on the metaphase plate with bundles of microtubule fibers connecting with kinetochores (indicated by CENH3) (Figure 8A). The kinetochore-attaching ends of the microtubules emitting from opposite poles were clear-cut. By contrast, in the *brk1-1* mutant, although all 12 bivalents could converge in the vicinity of the metaphase I plate, they were always out of alignment (Figure 8A), which is consistent with the observation for the metaphase I chromosomes stained with 4′,6-diamidino-2-phenylindole (DAPI) (Figure 7A). The kinetochore-attaching ends of microtubule bundles were not as distinguishable as those in the wild type because many microtubule fibers went through the spindle equator of

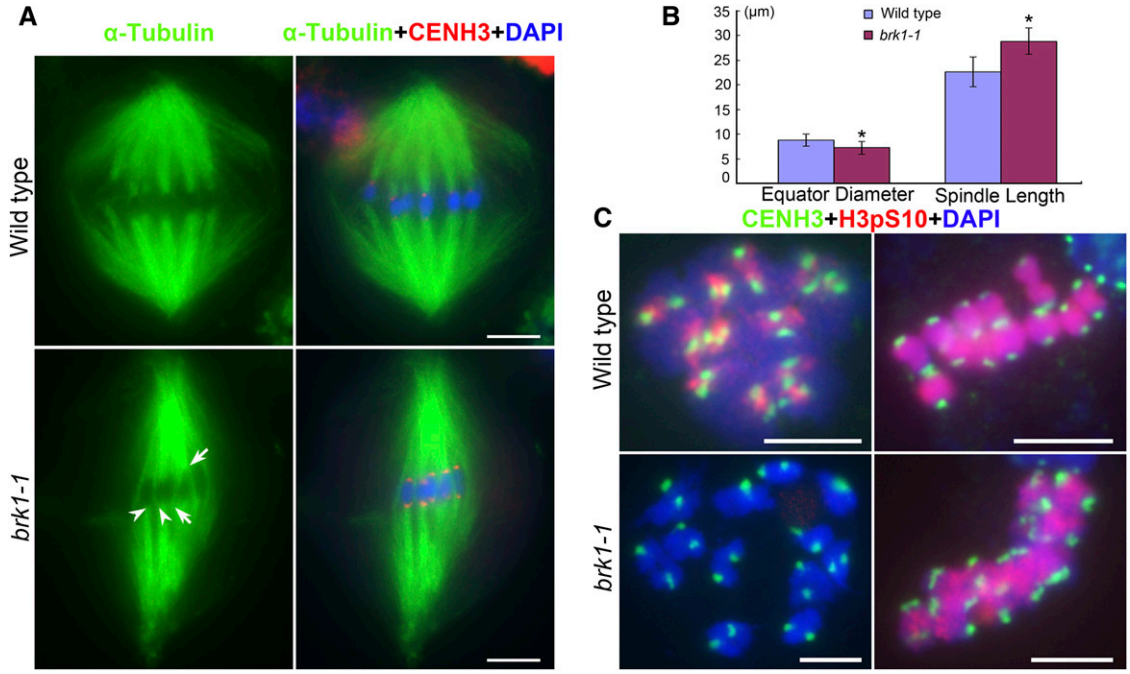

Figure 8. Defective BRK1 Causes Uncorrected Merotelic Attachment of Paired Sister Kinetochores at Metaphase I.

(A) Kinetochore (red)–microtubule (green) attachment at wild-type and *brk1-1* metaphase I. The merotelic attachment of paired sister kinetochores during *brk1-1* metaphase I is indicated by arrows, which results in the spindle elongation. Furthermore, one bundle of microtubule fibers could merotelically attach to the two adjacent kinetochores during *brk1-1* metaphase I (indicated by arrowheads), which prevents the bivalents from dispersing on the spindle equator. Chromosomes stained with DAPI (blue). Bars = 5 μm.

(B) Quantitation of the diameter of metaphase I spindle equator and the spindle length in both the wild type and *brk1-1* mutant. Error bars represent SD (*$P < 0.01$).

(C) The distribution of H3-pS10 (red) during diakinesis and metaphase I in both the wild type and *brk1-1* mutant. The inner centromeric recruitment of H3-pS10 at *brk1-1* diakinesis is abolished, while its entire chromosome distribution during metaphase I is not affected. Kinetochores indicated by CENH3 (green) and chromosomes stained with DAPI (blue). Bars = 5 μm.

brk1-1 metaphase I. Some of these fibers attached to the kinetochores facing the opposite pole, which resulted in one pair of sister kinetochores being connected by microtubules emitting from the different poles at the same time (Figure 8A; see Supplemental Figure 7 online). This kind of microtubule attachment belongs to the merotelic attachment of paired sister kinetochores that compromise tensions across homologous kinetochore pairs.

We also observed an abnormal spindle structure at metaphase I in *brk1-1* meiocytes. For example, the bivalents on the spindle equator in the *brk1-1* mutant were not as dispersive as in the wild type, while the spindle length in *brk1-1* was elongated a little bit as compared with the wild type (Figure 8A). To quantify these changes, we measured both the diameter and length of the spindle at metaphase I in *brk1-1* and the wild type. We found the spindle diameter to be shorter in *brk1-1* (7.22 μm, $n = 54$) than in the wild type (8.81μm, $n = 30$; Figure 8B). Consistent with this observation in *brk1-1*, we found that one bundle of microtubule fibers could merotelically attach to the two adjacent kinetochores, which might inhibit the bivalents from dispersing on the metaphase I plate (Figure 8A). Furthermore, the spindle length at metaphase I is 22.65 μm in the wild type ($n = 34$), whereas this value is 28.88 μm in *brk1-1* ($n = 52$; Figure 8B). It has been suggested that the additional spindle elongation is a consequence of the difficulty in chromosome separation (Yang and Ma, 2001), so the elongated metaphase I spindle in the absence of BRK1 could have resulted from the uncorrected merotelic attachment.

Because Aurora B kinase is essential for sensing the centromere tension and correcting the erroneous kinetochore-microtubule attachment (Lampson and Cheeseman, 2011), we speculated that the deletion of BRK1 might affect the localization of Aurora kinase. There are two Aurora kinases in rice, belonging to plant Aurora α and Aurora β families, respectively (Demidov et al., 2005); however, their functions have not been studied so far. As the phosphorylation of histone H3 at Ser-10 by Aurora B is conserved from animals to plants (Demidov et al., 2005; Kawabe et al., 2005), we also detected the distribution of H3-pS10 in both wild-type and *brk1-1* meiocytes. We found that H3-pS10 starts to localize to the inner centromeric regions at diakinesis and then spreads to the whole chromosome at metaphase I in the wild type (Figure 8C). By contrast, in the *brk1-1* mutant, although H3-pS10 could still localize to the entire chromosome during metaphase I, its inner centromeric recruitment at diakinesis was abolished (Figure 8C). Thus, our data implied that BRK1 is required for the phosphorylation of inner centromeric histone H3 during diakinesis. The merotelic attachments frequently occur

during prometaphase, but most will be corrected at metaphase to guarantee the fidelity of chromosome separation (Gregan et al., 2011). Therefore, our results suggested that BRK1 is also required for correcting merotelic attachments of paired sister kinetochores during the early stage of metaphase I to ensure that proper tension is generated across homologous kinetochores.

DISCUSSION

Temporal Localization of BRK1 on the Kinetochore

The centromeric localization of Bub1 is widely conserved in eukaryotes from yeast to humans, although its temporal distribution differs somewhat. During mammalian mitosis, Bub1 localizes to the kinetochore early in prophase, and it has been demonstrated that the kinetochore localization of Bub1 is more stable than the other Bub proteins (Howell et al., 2004; Johnson et al., 2004; Musacchio and Salmon, 2007). Bub1 kinetochore levels in the human mitotic cell cycle are downregulated upon the kinetochore-microtubule attachment (Taylor et al., 2001), and a similar temporal distribution of Bub1 is also detected in mouse fibroblasts (Taylor and McKeon, 1997). Parallel to the situation in mammalian mitosis, it is difficult to detect frog Bub1 on the kinetochore at anaphase (Sharp-Baker and Chen, 2001).

In fission yeast, the timing of Bub1 dissociation from kinetochores is markedly delayed compared with that reported so far for other eukaryotes (Bernard et al., 1998). When fission yeast cells enter mitosis, Bub1 is recruited to the centromeres from the nuclear periphery and remains there until telophase. It has been proposed that the anaphase localization of Bub1 at kinetochores may be because of its nonessential, structural function within the kinetochore (Bernard et al., 1998). Furthermore, fission yeast Bub1 is associated with the kinetochore throughout the entire meiotic process, from the end of the horsetail stage to anaphase II (Bernard et al., 2001).

Our results have shown that BRK1 is recruited to kinetochores from premeiotic interphase to early microspore formation during meiosis and from interphase to telophase during mitosis, and its kinetochore levels are less affected by the kinetochore-microtubule attachment. The only stages during which BRK1 dissociates from the kinetochore are early premeiotic and mitotic interphase. BRK1 can thus be regarded as one stable component of the kinetochore-associated proteins during both meiosis and mitosis. The temporal distribution of BRK1 resembles that of fission yeast Bub1 to some extent.

The APC/C requires activators (Cdc20 or Cdh1) to specifically recognize the D-box and/or KEN box motifs on its substrates (Pfleger and Kirschner, 2000). It has been reported that two KEN box motifs in human Bub1 can promote its degradation by the APC/C with Cdh1 during mitotic exit (Qi and Yu, 2007). There are also two KEN boxes in mouse Bub1 and only one in frog and budding yeast Bub1. As stated above, in these organisms, Bub1 dissociates from kinetochores after metaphase and finally disappears at anaphase. By contrast, we did not find any KEN box in rice BRK1 or fission yeast Bub1; consistent with this, the kinetochore localization of Bub1 in these two organisms can persist to telophase. Therefore, we speculate that the persistence of BRK1 on the kinetochore might be related to its absence of a KEN box motif. In addition to the regulation of protein synthesis, the degradation of Bub1 might also be regulated by its phosphorylation state (Qi and Yu, 2007). It will be important to further reveal the regulatory mechanism of BRK1 disassociation from kinetochores at the mitotic exit.

The Role of BRK1 in Generating Proper Tension across Homologous Kinetochores at Metaphase I

Although the sterility of *brk1* mutants is caused by precocious separation of sister chromatids after metaphase I, which results from a lack of the cohesin-protecting function of SGO1 at centromeres, *brk1* mutants still present kinetochore defects unshared with *sgo1*. As our data show, BRK1 is required for correcting the merotelic kinetochore-microtubule attachment of paired sister kinetochores to ensure the generation of proper tension between homolog kinetochores at metaphase I. The other fission yeast shugoshin protein, Sgo2, can interact with Bir1/Survivin, which forms the CPC together with Aurora B, INCENP, and Borealin, and is required for the recruitment of CPC to centromeres (Kawashima et al., 2007; Vanoosthuyse et al., 2007). Because Bub1 is essential for the centromeric recruitment of Sgo2 through phosphorylation of H2A in fission yeast (Kawashima et al., 2010), Aurora B localization is also perturbed by the deletion of Bub1 (Hauf et al., 2007). In addition, the mono-orientation of sister kinetochores during metaphase I is affected by the defective Bub1 (Bernard et al., 2001). Thus, it has been argued that the deletion of Aurora B from centromeres caused by perturbed Sgo2 localization in the *S. pombe bub1* mutant results in the generation of merotelic attachment of paired sister kinetochores, which further leads to defects in monopolar attachment at metaphase I (Hauf et al., 2007).

In plants, it has been predicted there are also two members of the shugoshin family, while the function of plant Sgo2 is unknown (Watanabe, 2005; Wang et al., 2011). Thus, whether plant *Sgo2* is a functional gene and BRK1 is also required for Sgo2 localization are unclear. Three Aurora kinases have been identified in *Arabidopsis thaliana*, all of which could phosphorylate Ser-10 in histone H3 (Kawabe et al., 2005). Among them, Aurora3 mainly localizes to the centromeric region in mitosis, while the distribution of Aurora1 and Aurora2 is rather dynamic (Demidov et al., 2005). The function of plant Aurora kinase and their meiotic distribution pattern are still unknown. According to our assay, the phosphorylation of H3-S10 at the inner centromeric regions at diakinesis is dependent on BRK1. We thus speculate that BRK1 is required for the proper localization of one Aurora kinase family member during the diakinesis–metaphase I transition, which is essential for correcting the incorrect kinetochore-microtubule attachment at the early stage of metaphase I. However, the whole chromosomal recruitment of the other Aurora kinase, which is independent of BRK1, guarantees the final fidelity of homologous separation at the onset of anaphase I, even in the absence of BRK1.

Unlike fission yeast, the deletion of BRK1 in rice never perturbs the mono-orientation of sister kinetochores in meiosis I. However, compared with the integrated sister kinetochores in the wild type, 2.969% of bivalents per meiocyte (n = 32) in *brk1-1*

presented detached sister kinetochores at metaphase I (a little more severe than 2.750% in *sgo1-1*, $n = 16$). In this situation, the erroneous kinetochore-microtubule attachments are more prone to occur, so there must be an additional monitoring system to correct them, which might be the reason why the role of BRK1 in correcting merotelic attachments is prominent in meiosis I rather than in mitosis.

Plant Bub1 May Not Function in the SAC

The *S. cerevisiae bub1* mutant strain grows well in the absence of spindle-inhibiting drugs (Hoyt et al., 1991). Therefore, it can be concluded that the SAC is a nonessential device in budding yeast, and it only becomes vital when the kinetochore-microtubule attachment is disturbed. Similarly, rice BRK1 is recruited to centromeres during mitosis, but no defective phenotype of its mutants was observed during the vegetative growth. Thus, we ask, what are the mitotic roles of BRK1? Does it become essential to the fidelity of mitotic chromosome segregation only when microtubules are destabilized? Two aspects make it difficult to address these questions. One is the lack of optimal cell lines in rice with which the mitotic steps can be timed. The other is the extreme weakness of SAC in rice. When we treated the root tip meristems of wild-type rice with 1 μM oryzalin, a microtubule-destabilizing agent, for only 2 h, it was found that some extremely condensed sister chromatids had already separated from each other in the mitotic cells. In addition, some multimicronuclei cells, formed by decondensation of the dispersive chromosomes, could also be observed. As these two phenomena are characteristics of SAC silencing, we suggest that rice mitotic cells can bypass the arrest at metaphase caused by the disturbed kinetochore-microtubule attachment much more easily than metazoans and yeast do. This notion is consistent with the statement that most plant cells can quickly escape the conditions inhibiting spindle assembly and enter the next cell cycle with a 4N restitution nucleus (Rieder and Maiato, 2004). Under the same treatment, we could not detect any significant differences between *brk1-1* and the wild-type root meristem cells.

The lack of a Gle2 binding sequence domain in BRK1 is surprising because it is very important for the spindle checkpoint activation and rather few Bub1/BubR1s lack the Bub3 binding domain (Brady and Hardwick, 2000; Klebig et al., 2009; Bolanos-Garcia and Blundell, 2011). Even the yeast Mad3, the homolog of vertebrate BubR1 lacking the kinase domain, still reserves the domain for binding Bub3 (Hardwick et al., 2000). In contrast with Bub1, plant Bub3 is relatively conserved. For example, *Arabidopsis* BUB3.1 is 52% identical to human Bub3 over its entire length (Caillaud et al., 2009). The absence of a KEN box in BRK1 further excludes the possibility that BRK1 can activate the SAC through direct interaction with APC/C^{Cdc20} (Sczaniecka et al., 2008). Furthermore, neither the Bub3 binding domain nor the KEN box is present in the *Arabidopsis* Bub1 candidate (NP_179656.4) (Karpov et al., 2010). Therefore, we speculate that Bub1 lost its classical functions in SAC during evolution of the plant kingdom.

According to reports, it is possible that other conserved SAC proteins cooperate in plants to delay the onset of anaphase until all the kinetochores are correctly attached by microtubules. During mitosis in maize and wheat (*Triticum aestivum*), MAD2 localizes to kinetochores that lack attached microtubules (Yu et al., 1999; Kimbara et al., 2004). In addition, when treated with a microtubule-destabilizing agent, *Arabidopsis* BUBR1, BUB3.1, and MAD2 can localize to the unattached kinetochores, where they interact with each other and may conduct the evolutionarily conserved functions of SAC proteins (Caillaud et al., 2009). *Arabidopsis* BUBR1 contains an N-terminal TPR motif and two KEN boxes; however, like yeast Mad3, it also lacks the kinase domain. Therefore, as far as these are concerned, it seems that among the core spindle checkpoint kinases, the Aurora family and Mps1 may be the best candidates to transduce the SAC signals in plants (Demidov et al., 2005). Consistent with this conjecture, it was reported that in human Bub1-deficient cells, the mitotic arrest depends on Aurora kinase activity (Morrow et al., 2005). Although it has been predicted that the Mps1 homolog exists in plants (Winey and Huneycutt, 2002), its biological function has not been characterized yet.

METHODS

Plant Materials

The *blk1-1* mutant was isolated from the *indica* rice (*Oryza sativa*) variety Zhongxian 3037 following $^{60}Co \sim \gamma$-ray radiation. Seeds of the *Tos17* insertion line NE7019, which we called *blk1-2*, were kindly provided by the Rice Genome Resource Center of the National Institute of Agrobiological Sciences. The other meiotic mutant, *sgo1-1*, was isolated in our lab previously (Wang et al., 2011). All plant material was cultivated in paddy fields.

Molecular Cloning of *BRK1* and *Tos17* Insertion Site Mapping

To fine map *BRK1*, STS markers (P1 to P5) were developed based on sequence differences between *indica* variety 9311 and *japonica* variety Zhonghua 11 according to data published on the National Center for Biotechnology Information website. All primer sequences are listed in Supplemental Table 1 online. Primer pair TosP (5′-ATTGTTAGGTTG-CAAGTTAGTTAAGA-3′) and 7019P (5′-TGTATCGCCAGCTTGATACG-3′) was used to amplify the *Tos17*-inserted regions of *brk1-2*. The PCR products were cloned into the pMD18-T vector (TaKaRa) and sequenced.

Cloning the Full-Length *BRK1* cDNA

Total RNA was extracted from rice panicle using TRIzol reagent (Invitrogen), and 3 μg RNA was reverse transcribed with the oligo-dT(18) primer using MMLV-RT (Invitrogen). Gene-specific primers forward (5′-ATGGTTCTGCTCGAACGCAC-3′) and reverse (5′-GATGCGTATATGT-CTCGGT-3′) were designed to amplify the predicted coding region of *BRK1*. PCR products were cloned into the pMD18-T vector (TaKaRa) and sequenced. 3′- and 5′-RACE was performed according to the kit protocols (3′-Full RACE Core Set and 5′-Full RACE Core Set; TaKaRa). For 3′-RACE, the first and second PCRs were performed using 3′RACE-F1 (5′-CTGCTCAACGCGACCTCCAA-3′) and the 3′ adaptor (5′-AAGCAG-TGGTATCAACGCAGA-3′), and 3′RACE-F2 (5′-AGCCTCCGGATGTCC-TTCCA-3′) and the 3′ adaptor, respectively. For 5′-RACE, total RNA was reverse transcribed using the 5′ phosphorylated primer 5′RACE-RT (5′-CCTTACTGAAGAGAAACAGT-3′). First and second PCRs were performed using 5′RACE-S1 (5′-CTCAGGTCTATCAACAAGGCT-3′) and 5′RACE-R1 (5′-TGCTGCCTCGGGCACCACCAA-3′), and 5′RACE-S2 (5′-GATCGGCTCAAGATTTGTGT-3′) and 5′RACE-R2 (5′-CGTGCGTT-CGAGCAGAACCA-3′), respectively. The products of 3′-RACE-PCR and 5′-RACE-PCR were cloned and sequenced.

Real-Time PCR Expression Assay

Real-time PCR assay was conducted as described (Hong et al., 2010). Total RNA was extracted from the leaf, root, and panicle of Zhongxian 3037 and panicles of *brk1* mutants. Real-time PCR analysis was performed using the Bio-Rad CFX96 real-time PCR instrument and EvaGreen (BIOTIUM). Real-Time PCR was performed using primers *BRK*RT-F (5′-CTGCTCAACGCGACCTCCAA-3′) and *BRK*RT-R (5′-GCTACGAAGTTACGGAAGGT-3′) for *BRK1* and UBQF (5′-CAAGATGATCTGCCGCAAATGC-3′) and UBQR (5′-TTTAACCAGTCCATGAACCCG-3′) for *Ubiquitin*. The real-time PCR results were analyzed using Bio-Rad CFX Manager analysis software. Each experiment had three replicates.

Construction of *BRK1* RNA Interference Cassette and Rice Transformation

A 310-bp fragment of the *BRK1* cDNA sequence was amplified with the primer pair RNAiF, 5′-ATTCTCGAGGCCAGTGTCGATGGTAATACA-3′ (adding an *Xho*I site), and RNAiR, 5′-ACGAGATCTCAACGCTGTGCAGTGTTTCCA-3′ (adding a *Bgl*II site). The fragment was cloned into the *Bam*HI-*Sal*I and *Bgl*II-*Xho*I sites of the pUCRNAi vector. Subsequently, the stem-loop fragment was cloned into the pCAMBIA2300-Actin vector. Then the RNAi construct was transformed into an *Agrobacterium tumefaciens* strain, which was used for further rice transformation.

Antibody Production and Protein Gel Blot Analysis

Antibodies were produced as described (Wang et al., 2010). To generate the antibody against BRK1, a 330-bp fragment of the *BRK1* cDNA (amino acids 396 to 505) was amplified with primers BRKAbF, 5′-GTGAATTCCCGAAATCACAGACGAAACCTT-3′ (adding an *Eco*RI site), and BRKAbR, 5′-AGTCTCGAGTCCACAGCTCCACGTTCCAGT-3′ (adding an *Xho*I site). The fragment was cloned into the expression vector pGEX 4T-2. The BRKAb-GST peptides were purified using Glutathione Sepharose 4B (GE). Polyclonal antibodies against BRK1 were raised in rabbit and mouse.

Kinase Assays

The kinase domain of BRK1 (amino acids 208 to 560) and the mutant kinase domain (K287R) were expressed in *Escherichia coli* BL21 strain at 18°C using the pET28-Smt3 vector. The recombinant proteins were purified using Ni-chelating columns (Liu et al., 2010).

The kinase assay for BRK1 was performed as previously described with minor modifications (Tang and Innes, 2002). Briefly, ~500 ng of the BRK1 kinase domain fused with the SUMO tag and 12.5 μg MBP (Millipore) were mixed in 20 μL kinase buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, and 40 μM ATP), containing 5 μCi of [γ-^{32}P]ATP (5 μCi/μL). After incubation at room temperature for 30 min, the reactions were terminated by adding 5 μL of 5× loading buffer to each sample. The samples were then boiled for 3 min and separated on 12% SDS-polyacrylamide gels. After vacuum drying at 80°C for 1 h, the gels were exposed to Kodak x-ray film to detect the kinase activity.

Meiotic Chromosome Preparation, FISH, Immunofluorescence, and Cell Imaging

Young panicles of both the wild type and mutants were fixed in Carnoy's solution (ethanol:glacial acetic, 3:1). Microsporocytes undergoing meiosis were squashed in an acetocarmine solution. After freezing in liquid nitrogen for a few minutes, the cover slips were removed with a sharp razor, and then the slides were dehydrated through an ethanol series (70, 90, and 100%). Chromosomes were counterstained with DAPI in an antifade solution (Vector). FISH analysis was conducted as described (Zhang et al., 2005). The pTa794 clone containing the coding sequences for the 5S rRNA genes of wheat (*Triticum aestivum*) (Cuadrado and Jouve, 1994) and the pRCS2 clone containing the rice centromere-specific tandem repeat sequence CentO were used as the FISH probes.

For immunofluorescence, fresh young panicles were fixed in 4% (w/v) paraformaldehyde for 30 min at room temperature. Microsporocytes at various meiotic stages were squashed on a slide with PBS solution. After removing the cover slip and freezing in liquid nitrogen, the slide was dehydrated through an ethanol series (70, 90, and 100%). Then the slides were incubated with different antibody combinations as mentioned in Results (diluted 1:500 in TNB buffer [0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.5% blocking reagent]) in a moist chamber at 37°C for 4 h. After three rounds of washing in PBS, TRITC-conjugated goat anti-rabbit antibody and FITC-conjugated sheep anti-mouse antibody (1:1000, Southern Biotech) were added to the slides. The chromosomes were counterstained with DAPI. For microtubule immunofluorescence, the assay was conducted as previously described (Li et al., 2010). Briefly, the rice inflorescences were fixed in methanol:acetone (4:1, v/v) for 3 h and washed three times with PBS buffer. Pollen mother cells were then gently released onto poly-L-Lys–coated slides in PBS buffer. To avoid cell flattening, slides were dried for 10 min without covering cover slips. After that, a thin layer of agarose/gelatin (0.75% low melting agarose, 0.75% gelatin, and 0.3% Suc) was spread on the slides to maintain the shape of the male meiocytes. Then the slides were incubated with mouse monoclonal anti-α-tubulin (Sigma-Aldrich). Images were captured under the Zeiss A2 fluorescence microscope with a micro-charge-coupled device camera or taken by the confocal laser scanning microscope (Leica TCS SP5).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: BRK1, JQ990990; SGO1, HQ333477; CENH3, AY438639; and REC8, AY371049.

Supplemental Data

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

Supplemental Figure 1. Characterization of the *brk1-1* Mutant Phenotype.

Supplemental Figure 2. Expression Analysis of *BRK1*.

Supplemental Figure 3. Alignment of BRK1 with Bub1 from Human (Hs), Mouse (Mm), Frog (Xl), Budding Yeast (Sc), and Fission Yeast (Sp).

Supplemental Figure 4. Meiotic Chromosome Behavior in Both *brk1-2* and *BRK1^{RNAi}*.

Supplemental Figure 5. The Dissociation of BRK1 from Kinetochore Is Independent of Kinetochore-Microtubule Attachment.

Supplemental Figure 6. Immunolocalization of H2A-pT134 and SGO1 in *brk1-2* and Distribution of BRK1 in Both *brk1-2* and *BRK1^{RNAi}*.

Supplemental Figure 7. Confocal Micrographs of Metaphase I Spindle in the Wild Type and *brk1-1* Mutant.

Supplemental Table 1. Primers Designed for Map-Based Cloning of *BRK1*.

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AUTHOR CONTRIBUTIONS

Z.C. designed the project. M.W. and D.T. performed the study and analyzed the image data. M.W., Q.L., Y.J., Y.S., and K.W. performed the immunostaining experiment. M.W. and Z.C. wrote the article.

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