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Developmental Cell

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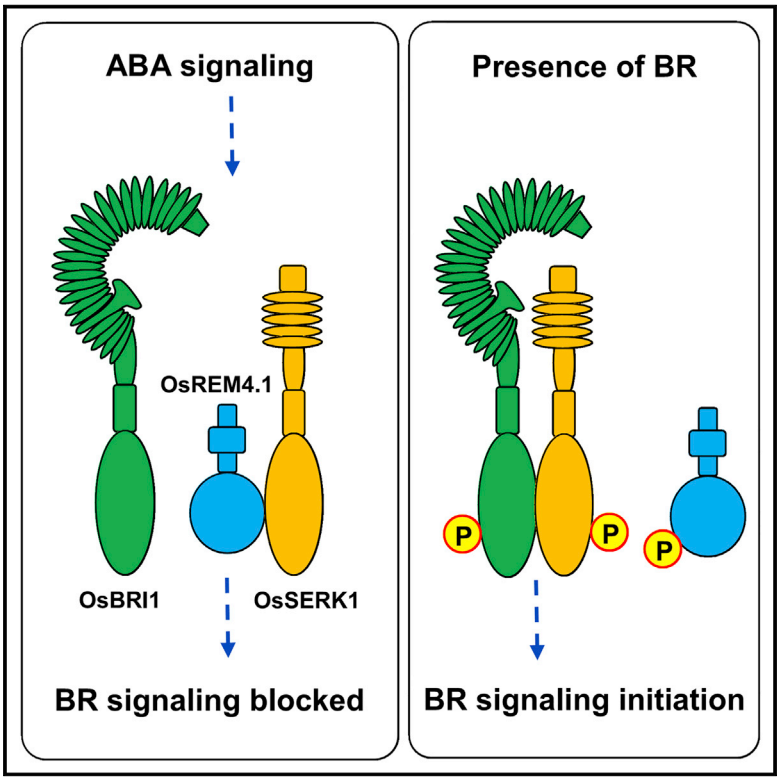
Graphical Abstract



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In Brief

The hormones abscisic acid (ABA) and brassinosteroid (BR) exhibit antagonistic interactions during plant development. Gui et al. now identify rice OsREM4.1 as a link between the ABA and BR signaling pathways. OsREM4.1, which is transcriptionally upregulated by ABA, inhibits the formation and activation of a BR receptor kinase (OsBRI1-OsSERK1) complex.

Highlights

- OsREM4.1 expression is upregulated by ABA through OsbZIP23
- OsREM4.1 negatively regulates BR signaling output
- OsREM4.1 interacts with OsSERK1 and inhibits OsBRI1-OsSERK1 complex formation
- OsREM4.1 can be phosphorylated by active OsBRI1 to dissociate from OsSERK1

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OsREM4.1 Interacts with OsSERK1 to Coordinate the Interlinking between Abscisic Acid and Brassinosteroid Signaling in Rice

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SUMMARY

Crosstalk among phytohormones is crucial for balancing plant growth and adjustment to various environments. Abscisic acid (ABA) and brassinosteroids (BRs) exhibit antagonistic interactions during many plant development processes, but little is known about the molecular mechanism mediating those interactions. Here, we identified a rice (*Oryza sativa*) remorin gene, *OsREM4.1*, whose expression is upregulated by ABA through the transcriptional activator OsbZIP23. OsREM4.1, in return, negatively regulates BR signaling output. We discovered that OsREM4.1 interacts with OsSERK1 to inhibit its interaction with rice BR receptor OsBRI1. Moreover, OsBRI1 could phosphorylate OsREM4.1 to reduce the binding affinity of OsREM4.1 to OsSERK1. These results demonstrate that OsREM4.1 is transcriptionally regulated by ABA and functions as an OsBRI1 substrate and OsSERK1-interacting protein to inhibit the formation and subsequent activation of the OsBRI1-OsSERK1 receptor complex. Our findings provide insight into the mechanism by which the antagonistic interactions between ABA and BRs are coordinated in rice.

INTRODUCTION

Crosstalk among plant hormones plays important roles in regulating plant development and adjustment to their environmental conditions (Vert and Chory, 2011). Abscisic acid (ABA) and brassinosteroids (BRs) play antagonistic roles in a variety of plant development processes. Both hormones share hundreds of transcriptional targets likely to be affected in the opposite direction (Nemhauser et al., 2006). In *Arabidopsis*, exogenous ABA treatment can inhibit BR signal transduction through unknown components downstream of Brassinosteroid-Insensitive 1 (BRI1), a leucine-rich-repeat receptor-like kinase functioning as the BR-binding receptor, but upstream of Brassinosteroid-Insensitive 2 (BIN2, one of the ten *Arabidopsis* GSK3-like kinases) in the BR signaling pathway (Zhang et al., 2009b). Although many studies have shown crosstalk between the two hormones, a critical unanswered question is which component coordinates the antagonistic interaction between BR and ABA signaling.

Studies in *Arabidopsis* have identified a group of key components in the BR signaling pathway from receptor kinases to transcription factors (Kim and Wang, 2010). It is believed that BRI1 and its co-receptor BRI1-Associated receptor Kinase 1 (BAK1) form a complex to mediate BR signaling (Li et al., 2002; Nam and Li, 2002). Structural characterization confirmed that the extracellular domains of BRI1 and BAK1 interact with each other in a BL-dependent manner (Sun et al., 2013). A process of reciprocal and sequential phosphorylation in the BRI1 and BAK1 receptor kinase complex is a key step toward activating the BR signaling cascade (Kim and Wang, 2010; Wang et al., 2005, 2008). The activity of the BRI1 and BAK1 receptor kinase complex can be also regulated by other components. For example, the association or dissociation BRI1 Kinase Inhibitor 1 (BKI1) from the kinase domain of BRI1 regulates the activity of the BRI1 and BAK1 receptor kinase complex (Wang and Chory, 2006). In rice functional characterization of *OsBRI1*, *OsSERK1* and *OsBZR1* suggest a conserved BR signaling pathway in the two species (Bai et al., 2007; Li et al., 2009; Yamamuro et al., 2000; Zuo et al., 2014).

The ABA receptor and other downstream transcription factors are among the core components that constitute the ABA signaling transduction pathway (Fujii et al., 2009; Ma et al., 2009; Park et al., 2009; Yoshida et al., 2010). ABA receptor proteins PYR1/PYL/RCAR bind to ABA and interact with regulator proteins to relay the ABA signal to downstream basic leucine zipper (bZIP) transcription factors, ABRE (ABA-responsive element) binding factors/binding proteins (ABFs/AREBs) (Fujii et al., 2009; Yoshida et al., 2010). The activated ABFs/AREBs function to regulate the expression of the ABA-responsive genes in various environments (Yoshida et al., 2010).

Remorins are plant-specific proteins that display a variety of functions, including impairing virus X movement in potato (Perraki et al., 2014; Raffaele et al., 2009), regulating bacterial infections by interacting with symbiotic receptors (Lefebvre et al., 2010), coping with abiotic stress (Checker and Khurana, 2013; Yue et al., 2014), playing a role in susceptibility to beet curly top virus in *Arabidopsis* (Son et al., 2014), and regulating

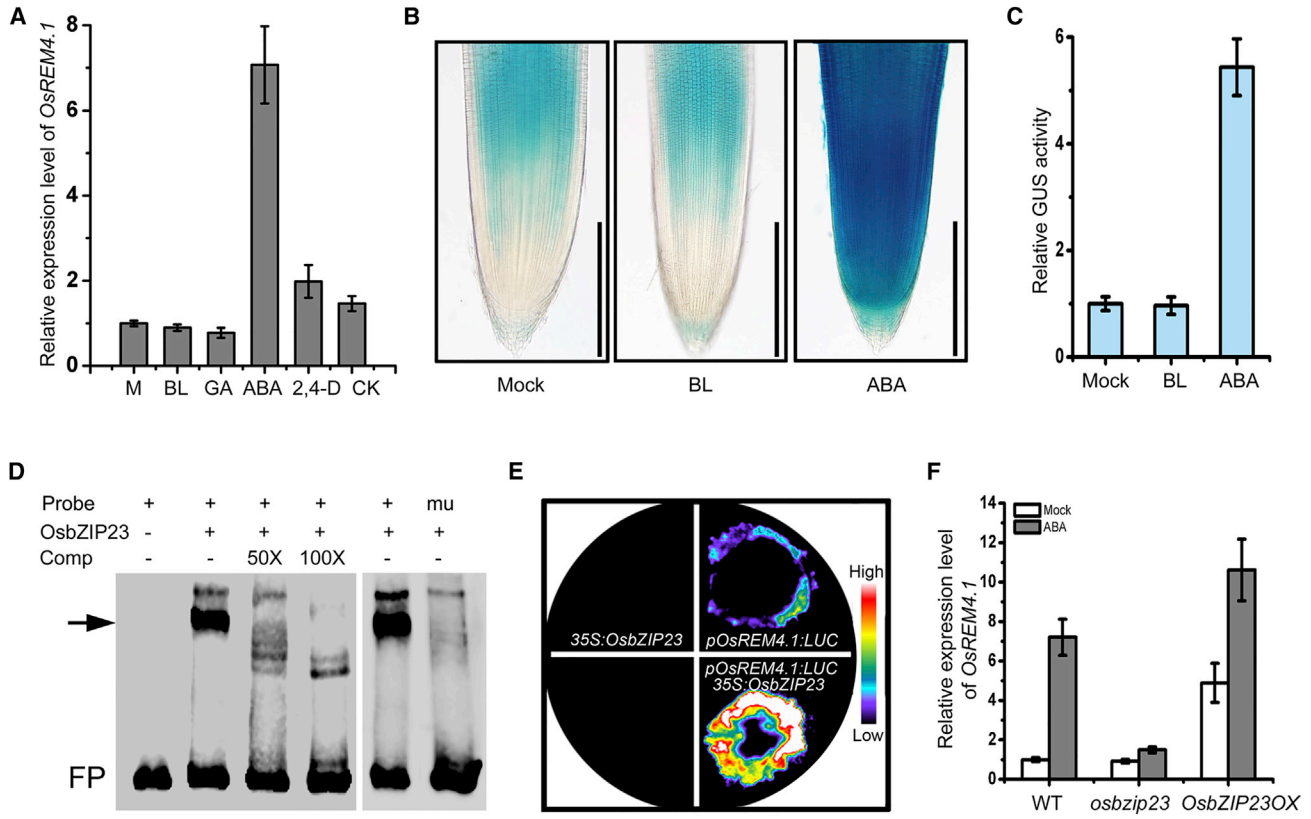

Figure 1. Phytohormone ABA Regulates *OsREM4.1* Expression

(A) *OsREM4.1* transcript level in rice under various phytohormone treatment for 6 hr. M, mock; BL, brassinolide (the most active BR type compound); GA, gibberellin; ABA, abscisic acid; 2,4-D, 2-(2,4-dichlorophenoxy) acetic acid; CK, cytokinin. The results are means ± SE of three biological repeats.

(B and C) *pOsREM4.1-GUS* staining (B) and quantitative *GUS* activity (C) under ABA or BL treatment for 6 hr. The results are means ± SE of three biological repeats. Scale bar, 500 μm.

(D) EMSA analysis of the OsbZIP23 binding to OsREM4.1 promoter. The arrow indicates the upshifted bands. mu, mutated promoter; FP, free probe.

(E) OsbZIP23 enhances *OsREM4.1* expression in tobacco leaves.

(F) *OsREM4.1* transcript level in 1-week-old seedlings of the wild-type (WT), *OsbZIP23* mutant (*osbzip23*), and *OsbZIP23* overexpression (*OsbZIP23OX*). The results are means ± SE of three biological repeats.

plasmodesmatal conductance through interacting with actin (Gui et al., 2014, 2015).

In this study, we identified a rice remorin gene, *OsREM4.1*, whose expression is upregulated by ABA through the transcriptional activator OsbZIP23. The OsREM4.1 protein directly interacts with OsSERK1 to repress BR signaling output by inhibiting the formation and/or activation of the OsBRI1-OsSERK1 receptor complex. In addition, OsREM4.1, which we show is a substrate of OsBRI1, can be phosphorylated by active OsBRI1. The phosphorylated OsREM4.1 is released from OsSERK1, thus allowing the formation of OsBRI1-OsSERK1 complex and subsequent activation of the BR signaling pathway. Our finding reveals a mechanism for coordinating the antagonistic interaction between ABA and BR signaling in rice.

RESULTS

OsREM4.1 Expression Is Specifically Regulated by Phytohormone ABA

Our recent study indicated that rice remorin proteins are involved in a variety of developmental processes (Gui et al., 2014). *OsREM4.1* gene was expressed in various tissues throughout the entire rice development process (Figure S1A). When rice plants were treated with various hormones, *OsREM4.1* expression was specifically induced by ABA but was not affected by BR, gibberellin, auxin, or cytokinin (Figure 1A). These results are consistent with the gene-expression data recorded in public microarray databases (Figure S1B). For verification of the ABA effect on *OsREM4.1* expression, a 1.3-kb fragment of the *OsREM4.1* promoter was fused to a β-glucuronidase (GUS) gene (*pOsREM4.1-GUS*) and transformed into rice. The resulting transgenic rice plants were treated with ABA or brassinolide (BL, the most active BR) and found that ABA, but not BL, could increase GUS activity (Figures 1B and 1C), further confirming the ABA effects on *OsREM4.1* expression.

ABA-Induced *OsREM4.1* Expression Is Mediated through OsbZIP23

ABA signaling is executed usually through a group of bZIP transcription factors, or ABFs/AREBs (Yoshida et al., 2010). Analyzing the *OsREM4.1* promoter sequence identified three ABREs while a search of a rice gene co-expression database

(www.ricearray.org) discovered an ABRE-binding factor *OsbZIP23* (*OsABF4*) that was co-expressed with *OsREM4.1*, with a high correlation coefficient. These two findings prompted us to test whether the ABA-induced *OsREM4.1* expression is mediated through the ABA-responsive transcription factor. We first performed an electrophoretic mobility shift assay (EMSA) to determine whether OsbZIP23 directly binds to the *OsREM4.1* promoter using an *Escherichia coli*-expressed recombinant OsbZIP23 protein fused with His tag (*OsbZIP23-His*) (Figure S2) and a 390-bp ABRE-containing fragment of the *OsREM4.1* promoter. As shown in Figure 1D, OsbZIP23 was able to bind to the wild-type (WT) *OsREM4.1* promoter fragment, and the in vitro protein-DNA binding was eliminated when the ABRE elements were mutated. We also tested whether the detected binding of OsbZIP23 to the ABRE-containing DNA fragment activates the *OsREM4.1* promoter. We fused a 1.3-kb *OsREM4.1* promoter fragment with a firefly luciferase (LUC) reporter gene (*pOsREM4.1-LUC*) and transformed the resulting transgene into tobacco-leaf epidermal cells via an agro-infiltration protocol (Sparkes et al., 2006). A low level of LUC activity was detected, indicating a basal expression level of the *pOsREM4.1-LUC* transgene in tobacco leaf (Figure 1E). When the *pOsREM4.1-LUC* construct was co-transformed with an *OsbZIP23* transgene driven by a constitutively active *35S* promoter, strong luminescence signals were detected in the infiltrated tobacco leaf (Figure 1E), confirming that OsbZIP23 could activate the *pOsREM4.1* promoter. Finally, we analyzed the *OsREM4.1* transcript levels in an *OsbZIP23* mutant (*osbzip23*) and *OsbZIP23*-overexpressing transgenic rice lines (*OsbZIP23OX*) (Xiang et al., 2008). As shown in Figure 1F, the *OsREM4.1* transcript level was very low in the *osbzip23* mutant but high in the *OsbZIP23OX* lines under normal conditions. When treated with ABA, *OsREM4.1* expression was significantly increased in the *OsbZIP23OX* and WT plants, but was not affected in the *osbzip23* mutant (Figure 1F). Taken together, these results demonstrated that OsbZIP23, which is an ABA-responsive transcription factor, binds to the *OsREM4.1* promoter to regulate the *OsREM4.1* expression.

OsREM4.1, in Association with the BR Signaling Pathway, Functions to Regulate Rice Growth

To investigate the physiological functions of OsREM4.1, we generated transgenic rice plants that overexpressed *OsREM4.1* or silenced *OsREM4.1* expression via RNAi (*RNAi*) (there is no available *OsREM4.1* mutant in public collections of rice mutants), and obtained a total of 18 independent *OsREM4.1* overexpression (*OsREM4.1OX*) transgenic lines and 23 independent *OsREM4.1* suppression (*OsREM4.1RNAi*) transgenic lines. Interestingly, the *OsREM4.1OX* lines displayed various degrees of dwarfism (Figures 2A, S3A, and S3B; Table S1). The observed growth phenotypes included dark-green, shorter, and wider flat leaf blades (Figures 2A, S3A, S3F, and S3G), shorter panicles, spikelets, and grains (Figures 2C, 2D, and S3D), narrower bending angle of the lamina joint (Figure 2E), and shorter leaf epidermis cells (Figure S3E). The severity of the dwarf phenotype was highly correlated with the transcript levels of the *OsREM4.1* gene (Figure 2K). In contrast, all of the *OsREM4.1RNAi* plants showed phenotypes of loose plant architecture with normal height, longer and inward rolling leaf blades (Figures 2A, S3A, S3F, and S3G), longer spikelets and grains (Figures 2C and 2D), wider lamina joint bending angle (Figure 2E), and longer leaf epidermis cells (Figure S3E). *OsREM4.1OX-13*, one of the 18 independent *OsREM4.1* overexpression lines of the T3 generation, and *OsREM4.1RNAi-2*, one of the 23 independent *OsREM4.1* suppression lines of the T3 generation (Table S1), were used in subsequent analyses.

Microscopic observation revealed that the cell length of the second internode was significantly shorter in *OsREM4.1OX*, but slightly longer in *OsREM4.1RNAi* plants, compared with WT (Figures 2B and 2F). Statistical analysis suggested that the reduction of cell length but not cell number may lead to shorter second internodes in *OsREM4.1OX* plants (Figures 2H and 2I). The vascular bundles in the leaf sheaths were also examined. The xylem vessels were obviously smaller in *OsREM4.1OX* but slightly larger in *OsREM4.1RNAi* plants compared with WT (Figures 2G and 2J). However, the number of sieve tubes and companion cells was higher in *OsREM4.1OX* plants (Figure 2G). Our phenotypic analyses revealed similarities between *OsREM4.1OX* transgenic plants and previously reported BR-deficient (*d2*, *d11* and *osdwarf4-1*) or -insensitive mutants (*d61* and *dlt*), which show dwarf stature, erect leaves, and shortened internodes (Hong et al., 2003; Sakamoto et al., 2006; Tanabe et al., 2005; Tong et al., 2009; Yamamuro et al., 2000). Moreover, the effect of *OsREM4.1* overexpression on vascular development is also similar to what was observed in *Arabidopsis* mutants lacking BRI1 and its two homologs, and in rice *d61-4*, a severe OsBRI1 mutant (Cano-Delgado et al., 2004; Nakamura et al., 2006). Taken together, these observations suggest that OsREM4.1 is related to BR synthesis or signaling that regulates rice growth and development.

To further investigate the relationship between OsREM4.1 and BR biosynthesis or signaling, we examined the effects of exogenously applied BL and brassinozole (BRZ, a BR biosynthesis inhibitor) on *OsREM4.1OX* and *OsREM4.1RNAi* plants using several widely used BR sensitivity assays that measure coleoptile elongation, root growth inhibition, and lamina joint inclination. We found that BL significantly increased the coleoptile length in *OsREM4.1RNAi* and WT plants, but not in *OsREM4.1OX* transgenic line (Figures 3A and 3B), indicating that *OsREM4.1* overexpression inhibits BR sensitivity. Consistently, BRZ decreased the coleoptile length in WT and *OsREM4.1OX* plants significantly, but foliage leaves could still grow out of the coleoptile in BRZ-treated *OsREM4.1RNAi* seedlings, revealing that silencing *OsREM4.1* could enhance BR signaling (Figures 3A and 3B). We also examined the effects of BL/BRZ treatment on root elongation. As shown in Figures 3C and 3D, treatment with both BL and BRZ inhibited root elongation in WT seedlings, with BL treatment also causing increased lateral root formation. We also found that the *OsREM4.1OX* seedlings were less sensitive to both treatments, whereas the *OsREM4.1RNAi* seedlings exhibited a similar BL sensitivity as the WT seedlings but were more resistant to BRZ-induced inhibition of root growth. Finally, we used the lamina inclination assay to quantify the BR sensitivity of the WT and transgenic seedlings. Figures 3E and 3F shows that BL significantly increased the lamina joint bending of *OsREM4.1RNAi* seedlings compared with the WT control but had little impact on *OsREM4.1OX* plants.

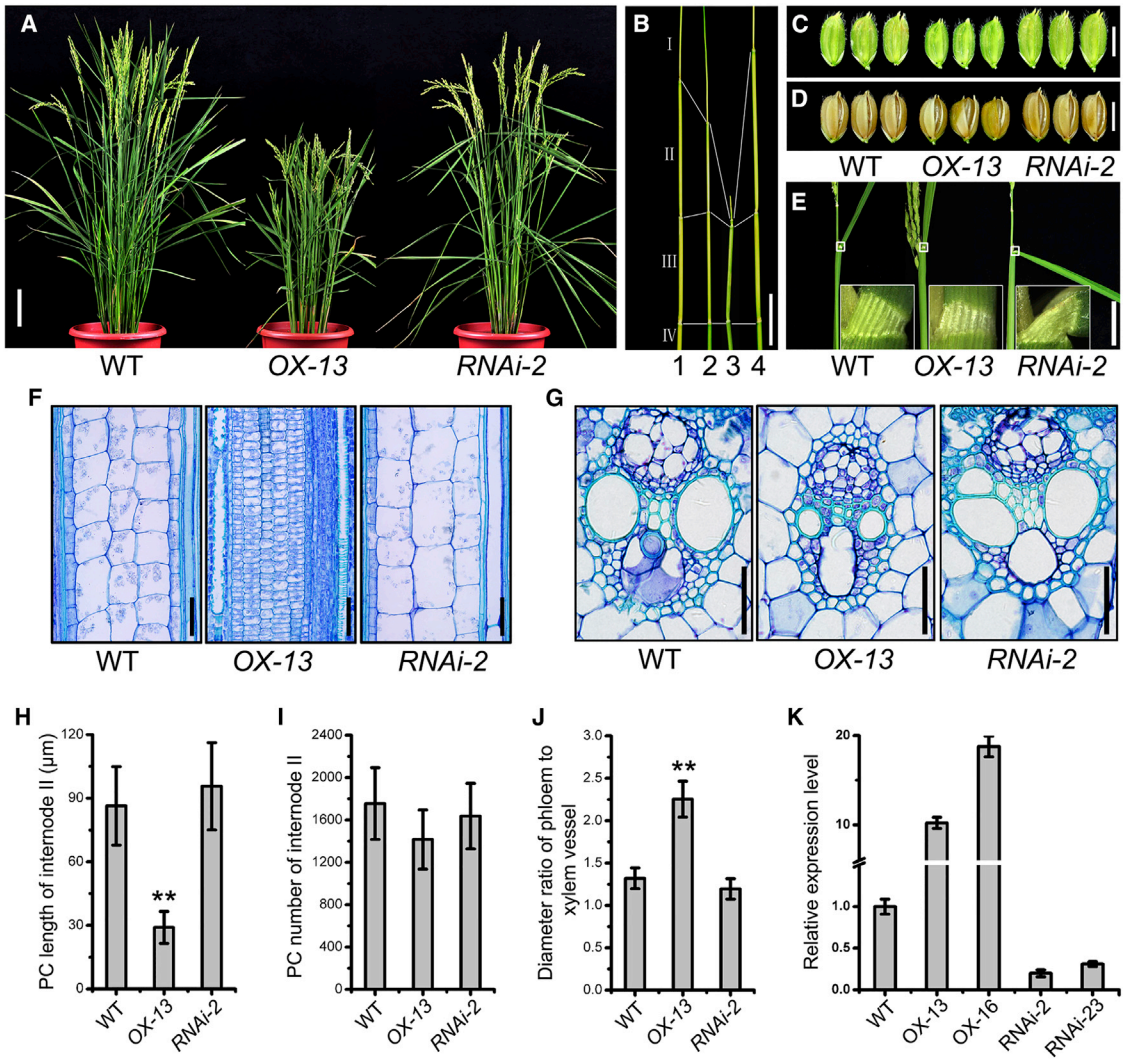

Figure 2. Phenotypes of the *OsREM4.1* Overexpression and *RNAi* Transgenic Plants

(A) The WT, *OsREM4.1* overexpression (*OX-13*), and *OsREM4.1 RNAi* (*RNAi-2*) plants at the grain-filling stage. Scale bar, 10 cm.

(B) Internodes of (1) WT, (2) *OX-4*, (3) *OX-13*, and (4) *RNAi-2* plants at the matured stage. Scale bar, 5 cm.

(C and D) Matured spikelets (C) and grains (D). Scale bars, 4 mm.

(E) Lamina joint bending angle of the flag leaf. Inset: zoomed-in image of the lamina joint of flag leaf. Scale bars, 3 cm.

(F) Longitudinal sections of the second internodes. Scale bars, 100 μm.

(G) Cross-sections of the leaf sheaths at the second internodes. Smaller xylem vessels in *OX-13* but larger in *RNAi-2* plants. Scale bars, 50 μm.

(H and I) Parenchyma cell (PC) length (H) and number in the second internodes (I). Data in (H) are means ± SE of 100 cells; those in (I) are means ± SE of the second internodes in ten plants. **$p < 0.01$, significant difference compared with WT (t test).

(J) Diameter ratio of phloem to xylem vessel. The results are means ± SE of 20 individual vascular bundles. **$p < 0.01$, significant difference compared with WT (t test).

(K) *OsREM4.1* expression in 3-week-old seedlings. Data are means ± SE of three individual plants.

We measured the transcript levels of *CPD* and *BR6OX*, two BR biosynthetic genes known to be feedback regulated, and *DREPP*, a known BR-responsive gene. As expected, BL treatment significantly suppressed the expression of both *CPD* and *BR6OX* but strongly increased the transcript levels of *DREPP* (Figure 3G). Consistent with the antagonism between ABA-BR, ABA treatment increased the transcript levels of *CPD* and *BR6OX* but decreased the abundance of *DREPP* transcript (Figure 3G). More importantly, the transcript levels of *CPD* and *BR6OX* were significantly lower while *DREPP* transcript abundance was markedly higher in *OsREM4.1RNAi* plants than in the WT control (Figure 3H). By contrast, the transcript abundance of *CPD* was greatly increased whereas the transcript level of *DREPP* was strongly reduced by overexpression of *OsREM4.1* (Figure 3H). The results of the physiological assays and gene-expression analyses strongly suggest that OsREM4.1 negatively regulates BR signaling in rice.

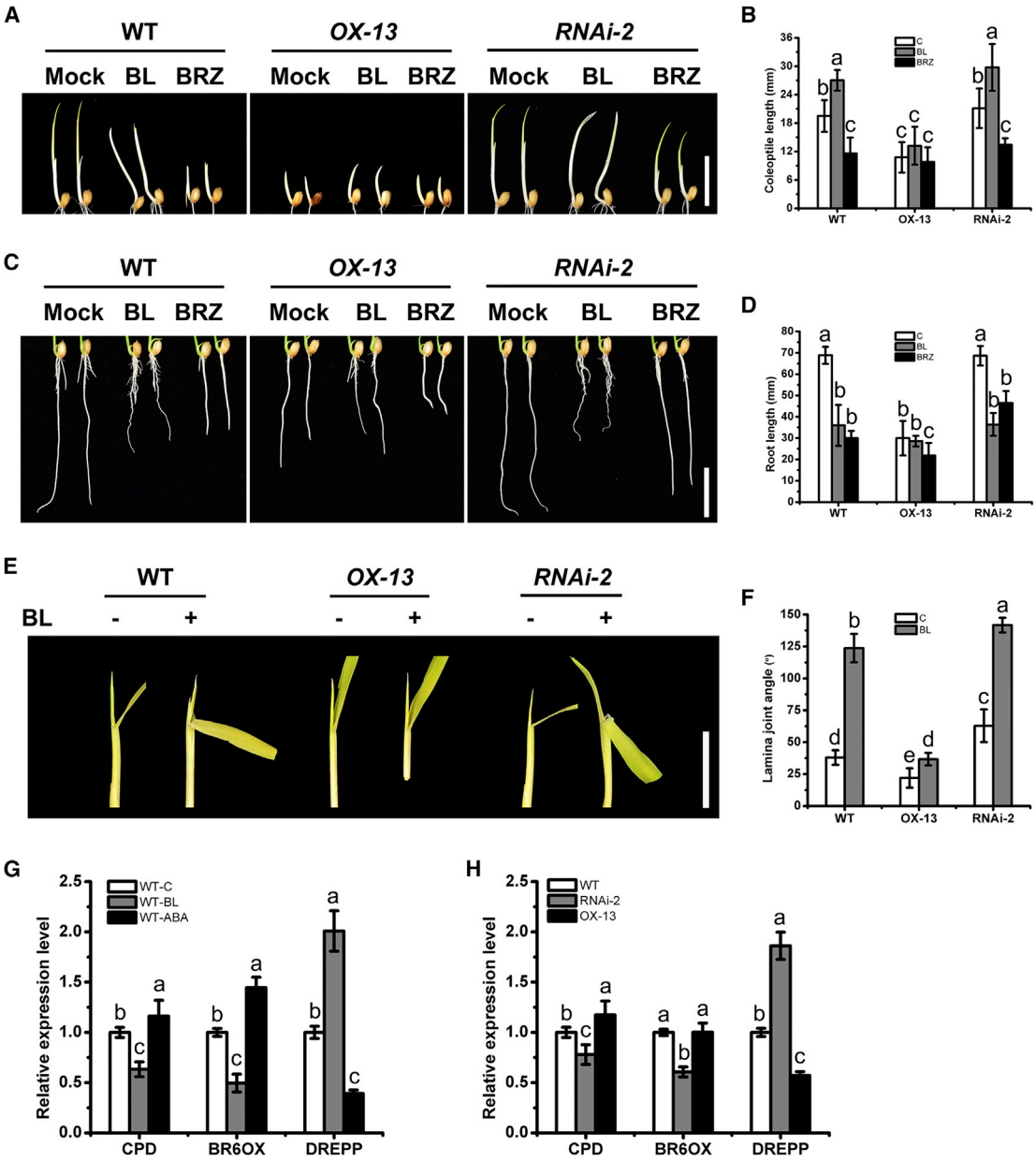

Figure 3. Responses to BR Treatment in *OsREM4.1*-Modified Plants

(A) Coleoptile elongation response to 2 μM BL or 5 μM BRZ in the dark. In WT plants, BL treatment promotes coleoptile elongation, while BRZ inhibits it. WT and *RNAi-2* plants coleoptile are highly sensitive to BL or BRZ treatments, while *OX-13* coleoptile reduced sensitivity to BL or BRZ. Scale bar, 2 cm.

(B) Statistical analysis of the coleoptile length under treatment with 2 μM BL or 5 μM BRZ. Values are means ± SE (n = 15).

(C) Root elongation response to 2 μM BL or 5 μM BRZ under light. Scale bar, 2 cm.

(D) Statistical analysis of the root length under treatment with 2 μM BL or 5 μM BRZ. Values are means ± SE (n = 15).

(E) Lamina joint inclination response to 10 nM BL. WT and *RNAi-2* plants lamina joint are highly sensitive to BL treatment, while *OX-13* lamina joint reduced sensitivity to BL. Scale bar, 2 cm.

(F) Statistical analysis of the lamina joint angle under treatment with 10 nM BL. Values are means ± SE (n = 10).

(G) Transcript level of the BR-responsive genes *CPD*, *BR6OX*, and *DREPP* in WT under treatment with 10 μM BL or 100 μM ABA.

(H) Transcript level of the BR-responsive genes *CPD*, *BR6OX*, and *DREPP* in WT, *RNAi-2*, and *OX-13* plants.

Lowercase letters in (B), (D), and (F)–(H) indicate significant differences at $p < 0.01$ by ANOVA.

OsREM4.1 Is a Plasma Membrane Protein and Interacts with OsSERK1 by Directly Binding to Its Kinase Domain

To find out where OsREM4.1 is localized subcellularly, we generated two transgenic constructs: *OsREM4.1-GFP* and *GFP-OsREM4.1*. Confocal microscopic analyses of the transformed protoplasts revealed strong green fluorescence signals on the plasma membrane (PM) (Figure 4A). The PM localization of OsREM4.1 was also confirmed by confocal microscopic

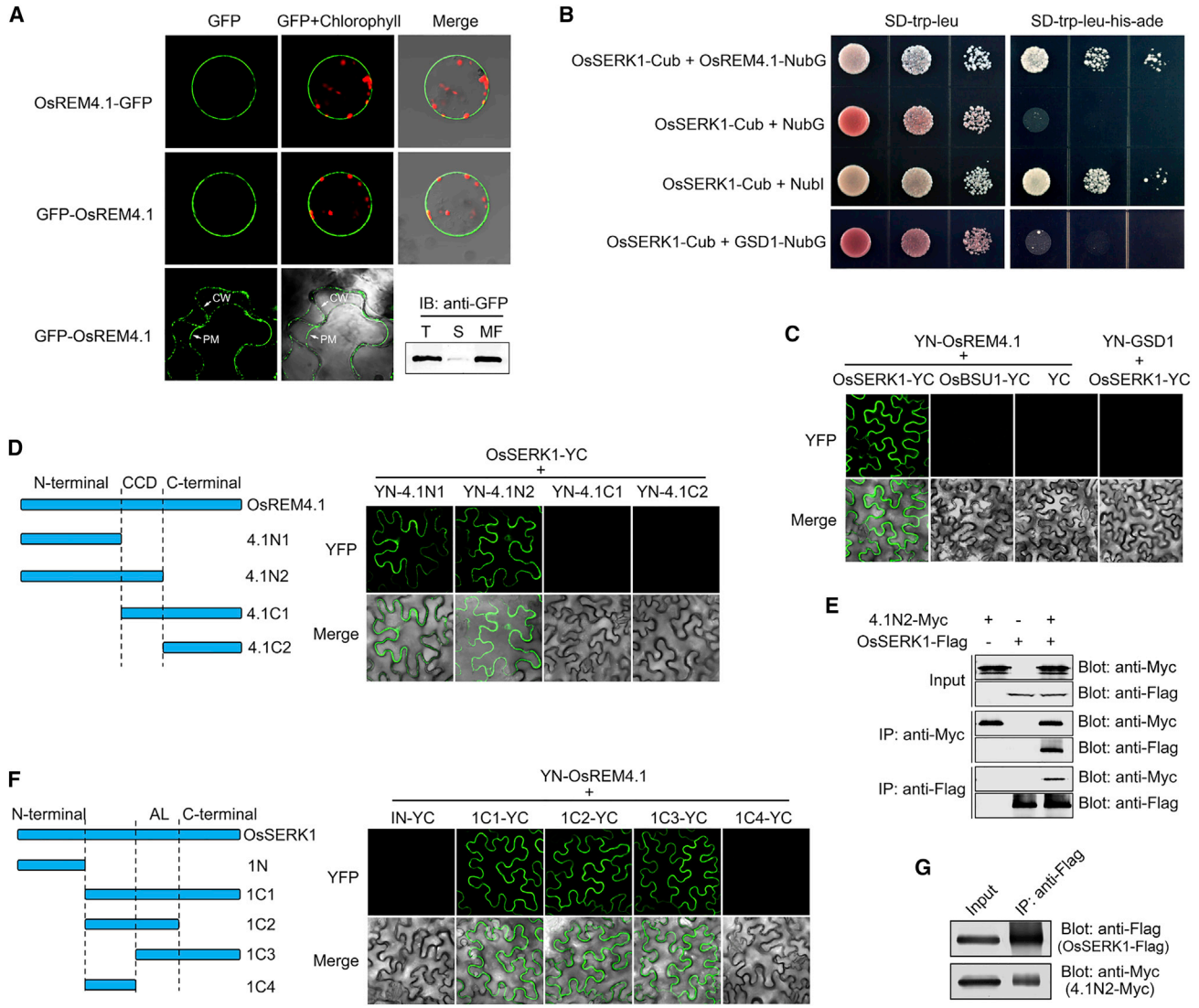

Figure 4. OsREM4.1 Is Localized on the PM and Interacts with OsSERK1 by Binding to Its Kinase Domain

(A) *OsREM4.1-GFP* and *GFP-OsREM4.1* were expressed in rice protoplasts or tobacco-leaf cells. Western blot analysis of the GFP-OsREM4.1 from rice protoplasts. CW, cell wall; PM, plasma membrane; T, total proteins; S, soluble proteins; MF, microsomal fraction.

(B) Interaction of OsREM4.1 with OsSERK1 was verified by a DUAL membrane yeast two-hybrid system.

(C) Interaction of OsREM4.1 with OsSERK1 was detected by BiFC analysis.

(D) Schematic representation of a series of truncation mutations of OsREM4.1. The truncated *OsREM4.1* fragments were fuse with *YN* and co-expressed with *OsSERK1-YC*, respectively. N, N-terminal sequence; C, C-terminal sequence; CCD, coiled-coil domain.

(E) Interaction between OsREM4.1 and OsSERK1 was detected by coIP in tobacco. Input, extracted proteins; IP, immunoprecipitated proteins; anti-Myc, Myc antibodies; anti-Flag, FLAG antibodies.

(F) Schematic representation of a series of truncation mutations of OsSERK1. The truncated OsSERK1 fragments were fused with YC and co-expressed with *YN-OsREM4.1*, respectively. AL, activation loop.

(G) Interaction between OsREM4.1 and OsSERK1 was detected by coIP in rice.

examination of tobacco epidermal cells transformed with the *GFP-OsREM4.1* transgene (Figure 4A). Consistently, the presence of GFP-tagged OsREM4.1 was also detected in the membrane fraction but not in the soluble fraction (Figure 4A) after subcellular fractionation and subsequent immunoblot analysis of GFP-OsREM4.1-expressing rice protoplasts. Based on these results, we concluded that OsREM4.l is a PM-localized protein.

Next, *OsREM4.1-3Flag* transgenic rice plants were generated to identify potential OsREM4.1-interacting proteins. Through immunoprecipitation with FLAG agarose beads followed by liquid chromatography-tandem mass spectrometry analysis, a list of putative OsREM4.1-interacting proteins were identified (Table S2) including OsSERK1 (Os08g0174700 in the Rice Annotation Project Database/Os08g077600 in the Rice Genome Annotation Project), a rice homolog of *Arabidopsis* SERK1,

which is similar to BAK1 (Santiago et al., 2013). To test whether OsREM4.1 directly interacts with OsSERK1, we used a Split-Ubiquitin-based membrane yeast two-hybrid system (Dualsystems Biotech) with OsSERK1 fused to the C-terminal half of the yeast ubiquitin (Cub) as the bait and OsREM4.1 and GSD1 (Grain Setting Defect 1; another remorin protein as a control) fused to the N-terminal half of the yeast ubiquitin (NubI or NubG carrying an isoleucine to glycine mutation) as the prey. As shown in Figure 4B, yeast cells co-expressing OsREM4.1-NubG/NubI and OsSERK1-Cub grew well on a histidine (His)-free medium, while cells co-expressing NubG/GSD1-NubG and OsSERK1-Cub could not grow on the His-free medium (Figure 4B), demonstrating a direct interaction between OsREM4.1 and OsSERK1 in yeast cells. OsREM4.1-OsSERK1 interaction was further confirmed by a bimolecular fluorescence complementation (BiFC) assay performed in a tobacco expression system. When the complementation fusion proteins were correctly expressed (Figure S4), a strong fluorescent signal, as shown in Figure 4C, was clearly detected at the cell surface of tobacco-leaf epidermal cells that co-expressed OsREM4.1 tagged with the N-terminal half of YFP (YN-OsREM4.1) and OsSERK1 fused with the C-terminal half of YFP (OsSERK1-YC). In contrast, no fluorescence signal was detected in the tobacco-leaf cells co-expressing OsSERK1-YC and YN-GSD1 or in the tobacco-leaf cells co-expressing YN-OsREM4.1 and YC-OsBSU1, a YC-tagged rice homolog of *Arabidopsis* BSU1 known to play a key role in BR signaling.

The BiFC assay was used to further study the interaction between OsREM4.1 and OsSERK1. We generated a series of truncated *OsREM4.1* fragments (N-terminal region of *OsREM4.1*, *4.1N1* and *4.1N2*; C-terminal region of *OsREM4.1*, *4.1C1* and *4.1C2*) fused with the YN coding sequence and individually co-transformed these constructs with *OsSERK1-YC* in tobacco leaves. Correct expression of the fusion proteins was examined by western blots (Figure S4). Confocal microscopic analyses of the transformed tobacco leaves revealed strong fluorescent signals in leaf cells co-expressing *OsSERK1-YC* with *YN-4.1N1* or *YN-4.1N2* but no fluorescent signal in leaf cells co-expressing *OsSERK1-YC* and *YN-4.1C1* or *YN-4.1C2* (Figure 4D), indicating that OsSERK1 interacts with the N-terminal region of OsREM4.1. The interaction was independently verified by co-immunoprecipitation (coIP) with tobacco leaves or transgenic rice plants. When 4.1N2-Myc and OsSERK1-FLAG were co-expressed, both proteins were co-immunoprecipitated together (Figures 4E and 4G). We also generated a series of truncated *OsSERK1* fragments (N-terminal region of *OsSERK1*, *1N*; C-terminal region of *OsSERK1*, *1C1*, *1C2*, *1C3*, and *1C4*) fused with the YC coding sequence and transformed them individually with *YN-OsREM4.1* into tobacco leaves. Confocal microscopic analyses of the co-transformed tobacco leaves revealed that OsREM4.1 interacted with OsSERK1 at the activation-loop region of the OsSERK1 kinase domain (Figure 4F).

OsREM4.1 Binds to OsSERK1 to Inhibit the Formation and Activation of the OsBRI1-OsSERK1 Receptor Complex

Our finding that OsREM4.1 directly binds to the kinase domain of OsSERK1 prompted us to test whether the OsREM4.1-OsSERK1 interaction might interfere with the formation and/or activation of the OsBRI1-OsSERK1 receptor complex, which is essential to initiate BR signaling. We co-transformed *OsBRI1-YN* and *OsSERK1-YC* with or without *mCherry-OsREM4.1* into tobacco leaves and used confocal microscopy to analyze the OsBRI1-OsSERK1 interaction. As shown in Figure 5A, the fluorescent signal was detected in leaf cells co-expressing *OsBRI1-YN* and *OsSERK1-YC*, or in leaf cells co-expressing *OsBRI1-YN*, *OsSERK1-YC*, and *mCherry-GSD1*, validating that OsBRI1-YN and OsSERK1-YC interact in the system and that mCherry-GSD has no effect on the interaction. However, the fluorescent signal was not detected in the leaf cells co-expressing *OsBRI1-YN*, *OsSERK1-YC*, and *mCherry-OsREM4.1*, suggesting that mCherry-OsREM4.1 inhibits OsBRI1-YN/OsSERK1-YC interaction. Interestingly, BL treatment resulted in recovery of the fluorescent signal in the leaf cells co-expressing *OsBRI1-YN*, *OsSERK1-YC*, and *mCherry-OsREM4.1*. In addition, fluorescent signal enhancement was observed in the BL-treated cells co-expressing *OsBRI1-YN* and *OsSERK1-YC* or *OsBRI1-YN*, *OsSERK1-YC*, and *mCherry-GSD1*. This suggests that BL treatment could not only enhance the OsBRI1-YN/OsSERK1-YC interaction but also nullify the inhibitory effect of mCherry-OsREM4.1 on the OsBRI1-YN/OsSERK1-YC interaction. The inhibitory effect of OsREM4.1 on the interaction between OsBRI1/OsSERK1 was also confirmed by coIP analysis (Figure 5B). OsBRI1-FLAG and OsSERK1-Myc were produced along with mCherry-OsREM4.1 in tobacco leaves. The co-expressed proteins were isolated and precipitated using anti-FLAG antibody-coupled agarose beads. Western blot examination indicates that co-precipitation of OsSERK1-Myc with OsBRI1-FLAG was significantly reduced by mCherry-OsREM4.1. These results suggest that OsREM4.1 inhibited the interaction between OsBRI1 and OsSERK1, while BL treatment alleviated this inhibition.

To further analyze the impact of BL treatment on the OsBRI1-OsREM4.1-OsSERK1 interaction in rice cells, we expressed *YN-OsREM4.1*, *OsSERK1-YC*, and *OsBRI1-mCherry* in rice protoplast. When *YN-OsREM4.1* and *OsSERK1-YC* were co-expressed, fluorescent signal was detected in protoplasts treated with or without BL (Figure 5C). However, When *YN-OsREM4.1*, *OsSERK1-YC*, and *OsBRI1-mCherry* were co-expressed, fluorescent signal was significantly decreased under BL treatment compared with the mock-treated control (Figure 5C), suggesting that the OsREM4.1-OsSERK1 interaction was inhibited when OsBRI1 was activated by BL treatment. Such an inhibitory effect of BL-activated BRI1 on the OsREM4.1-OsSERK1 binding was also confirmed by coIP analysis. When *YN-OsREM4.1*, *OsSERK1-YC-Myc*, and *OsBRI1-mCherry* were co-expressed in tobacco leaves, as shown in Figure 5D, binding of YN-OsREM4.1 to OsSERK1-YC-Myc resulted in their co-immunoprecipitation. Such binding was significantly reduced under BL treatment. Together, our results strongly suggested that the OsREM4.1-OsSERK1 interaction was inhibited when OsBRI1 was activated by BL treatment.

Because OsREM4.1 interacts with OsSERK1, we were interested to know whether OsREM4.1 could also inhibit the kinase activity of OsSERK1. We therefore generated a recombinant OsBRI1 kinase domain with a His_6 tag (amino acids 747–1,121, named OsBRI1-CD) and its kinase-dead variant carrying the K^{835}-E mutation (named OsBRI1m-CD), the His_6-tagged

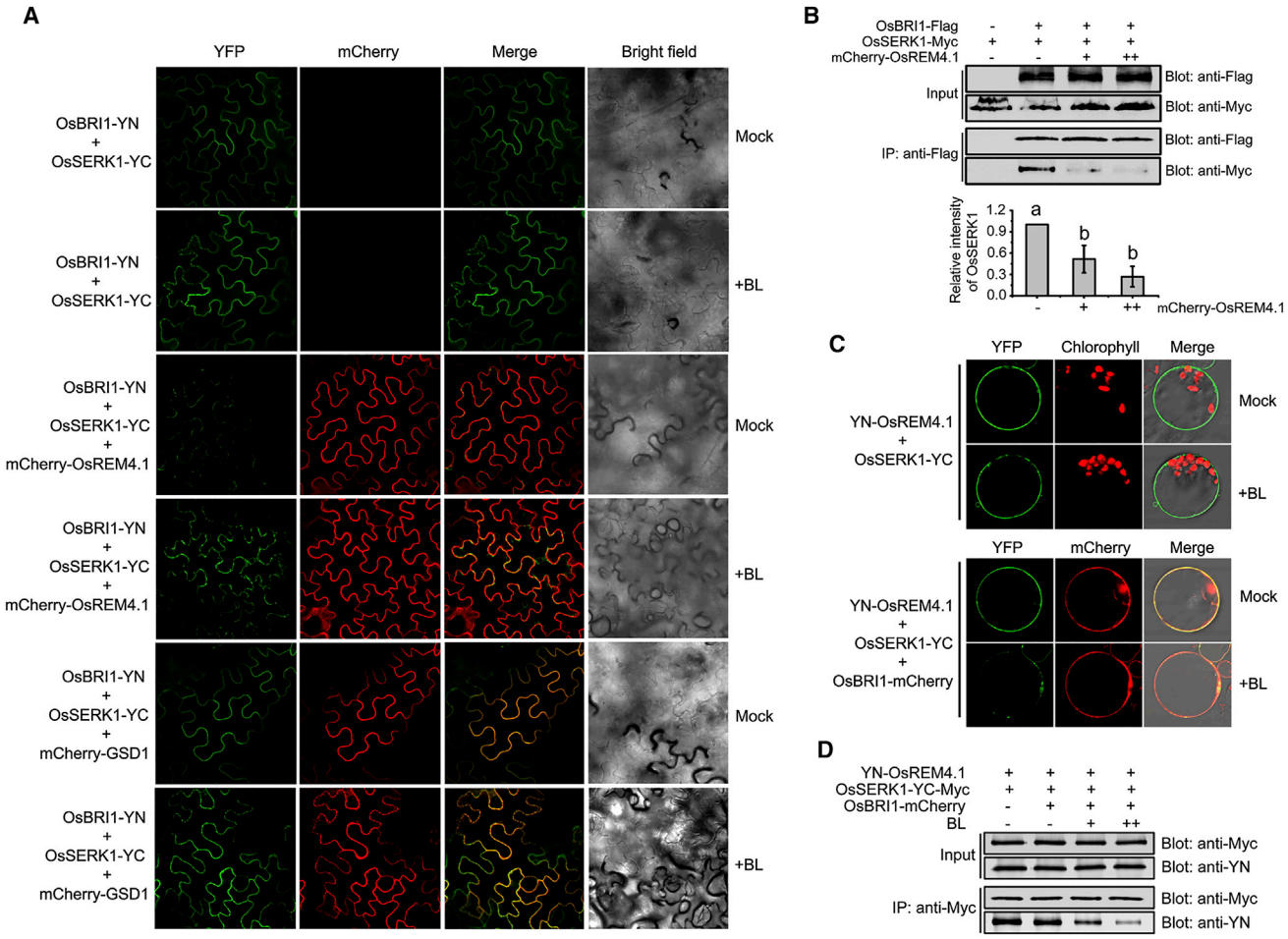

Figure 5. OsREM4.1 Interferes with the Interaction between OsBRI1 and OsSERK1

(A) *OsBRI1-YN* and *OsSERK1-YC* were co-expressed with or without *mCherry-OsREM4.1* and *mCherry-GSD1* in tobacco leaves. YFP fluorescent signal was detected when *OsBRI1-YN* and *OsSERK1-YC* was co-expressed without the presence of *mCherry-OsREM4.1* or in the presence of *mCherry-GSD1*, but not detected when *OsBRI1-YN* and *OsSERK1-YC* was co-expressed in the presence of *mCherry-OsREM4.1*. BL treatment recovered YFP fluorescent signal in co-expression of *OsBRI1-YN*, *OsSERK1-YC*, and *mCherry-OsREM4.1* and enhanced the YFP fluorescence in other co-expressions. YFP fluorescent channel in green and mCherry fluorescent channel in red.

(B) *OsSERK1-Myc* and *OsBRI1-FLAG* with different quantities of *mCherry-OsREM4.1* (one and three times) were expressed in tobacco leaves. Protein extract was immunoprecipitated with anti-FLAG antibodies. The signal density of the immunoprecipitated OsSERK1 was quantified based on the three biological repeats. Lowercase letters indicate significant difference at $p < 0.01$ by ANOVA.

(C) *YN-OsREM4.1* and *OsSERK1-YC* were co-expressed with or without *OsBRI1-mCherry* in rice protoplasts. YFP fluorescent signal was significantly reduced when *YN-OsREM4.1* and *OsSERK1-YC* was co-expressed with *OsBRI1-mCherry* under BL treatment compared with mock treatment for 3 hr. Chloroplast auto-fluorescence in red.

(D) *OsSERK1-YC-Myc* and *YN-OsREM4.1* were expressed or co-expressed with *OsBRI1-mCherry* in tobacco leaves for 48 hr and then treated with different quantities of BL (+, 2 μM; ++, 5 μM) for 3 hr.

N-terminal region of OsREM4.1 (amino acids 1–205, named OsREM4.1-ND), the His_6-tagged N-terminal region of GSD1 (amino acids 1–414, named GSD1-ND), and a glutathione S-transferase (GST)-tagged recombinant OsSERK1 kinase domain (amino acids 263–624, named OsSERK1-CD) and its kinase-dead counterpart with the K^{329}-E mutation (named OsSERK1m-CD) (Figure S5). The K^{835}-E mutation in OsBRI1m-CD and the K^{329}-E mutation in OsSERK1m-CD correspond to the K^{911}-E mutation of *Arabidopsis* BRI1 (Oh et al., 2000) and the K^{317}-E mutation of *Arabidopsis* BAK1 (Wang et al., 2008), which were previously shown to inactivate the kinase activity of BRI1 and BAK1, respectively. The purified recombinant proteins were used for in vitro kinase assays. As shown in Figure 6A, the WT recombinant OsSERK1 and OsBRI1 kinases autophosphorylated and transphosphorylated each other, whereas the kinase-inactive mutant proteins failed to exhibit any detectable autophosphorylation activity. Incubation of OsSERK1-CD with increasing concentrations of OsREM4.1 had little effect on the OsSERK1 autophosphorylation (Figure 6B). Interestingly, while no change in the transphosphorylation of OsBRI1m-CD by OsSERK1-CD was detected when the two recombinant kinases were preincubated before adding OsREM4.1-ND to the reaction mixture, the OsSERK1-CD-catalyzed transphosphorylation of OsBRI1m-CD was significantly reduced when OsSERK1-CD

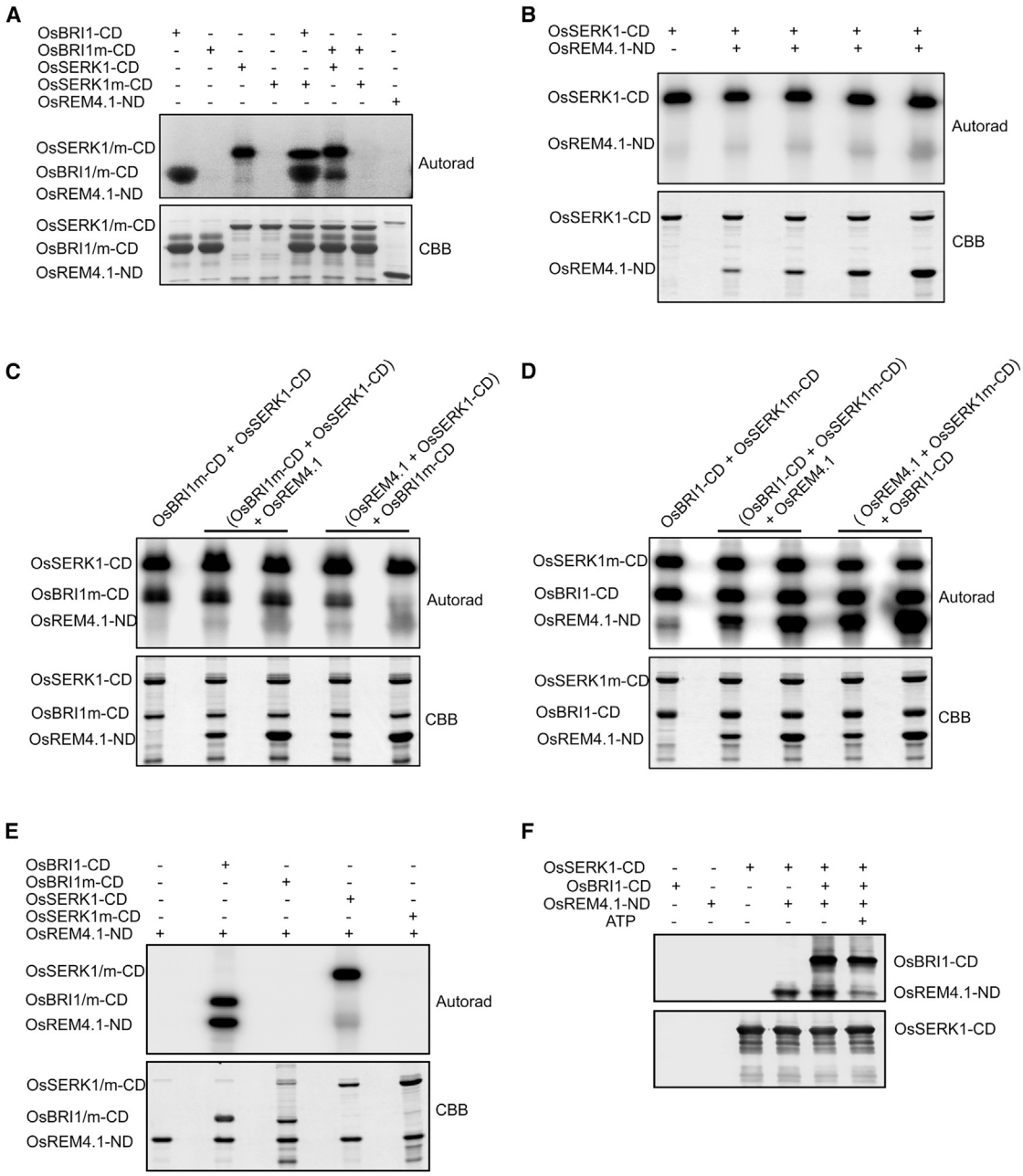

Figure 6. OsREM4.1 Represses OsSERK1-Catalyzed Transphosphorylation of OsBRI1

(A) The kinase domains (OsBRI1-CD and OsSERK1-CD), mutated kinase domains (OsBRI1m-CD and OsSERK1m-CD), and OsREM4.1 N-terminal part (OsREM4.1-ND) were generated in *E. coli* and purified. OsBRI1-CD and OsSERK1-CD were detected to have autophosphorylation and transphosphorylation activities. Bottom panel shows Coomassie brilliant blue R250 (CBB) stained protein loading for the autoradiograph.

(B) Different amounts of OsREM4.1-ND protein incubated with OsSERK1-CD did not affect OsSERK1 autophosphorylation.

(C) Preincubation of OsSERK1-CD with OsREM4.1-ND in the reaction mixture significantly inhibited the OsSERK1-CD-catalyzed transphosphorylate of OsBRI1m-CD.

(D) Preincubation of OsSERK1m-CD with OsREM4.1-ND has little effect on the OsBRI1-CD-catalyzed phosphorylation of OsSERK1m-CD.

(E) OsBRI1-CD strongly catalyzed OsREM4.1 phosphorylation, while OsSERK1-CD catalysis was scarce if at all.

(F) GST-OsSERK1-CD was coupled with glutathione agarose beads and used to interact with unphosphorylated or phosphorylated OsREM4.1. The phosphorylated OsREM4.1 showed much weaker binding to OsSERK1.

was preincubated with OsREM4.1 (Figure 6C). In addition, preincubation of OsSERK1m-CD with OsREM4.1 had little effect on the OsBRI1-CD-catalyzed transphosphorylation of OsSERK1m-CD (Figure 6D). By contrast, another rice remorin, GSD1, which could be phosphorylated by either OsSERK1-CD or OsBRI1-CD (Figure S6), had little effect on

the OsSERK1-CD-catalyzed transphosphorylation of OsBRI1m-CD. Taken together, our kinase assays suggested that the OsREM4.1-OsSERK1 interaction prevents the OsSERK1-catalyzed transphosphorylation of OsBRI1, which is thought to be crucial for initiation of BR signaling.

OsREM4.1 Is a Substrate of OsBRI1

During our in vitro kinase assays, we discovered that OsREM4.1 was strongly phosphorylated when mixed with OsBRI1-CD and OsSERK1m-CD (Figure 6D), suggesting that OsREM4.1 is likely to be phosphorylated by OsBRI1-CD, as OsSERK1m-CD was a dead kinase (Figure 6A). Therefore, the phosphorylation of OsREM4.1 was further examined. The results show that WT OsBRI1 was able to strongly phosphorylate OsREM4.1, while the WT OsSERK1 exhibited little phosphorylation activity against OsREM4.1 (Figure 6E). As expected, both kinase-inactive OsBRI1m-CD and OsSERK1m-CD were not able to phosphorylate OsREM4.1 (Figure 6E). We therefore concluded that OsREM4.1 is likely a substrate for OsBRI1.

OsREM4.1 Phosphorylation Affects Its Binding to OsSERK1

Our findings of OsREM4.1-OsSERK1 interaction and OsREM4.1 phosphorylation by OsBRI1 prompted us to examine whether OsBRI1-catalyzed phosphorylation of OsREM4.1 affects the binding of OsREM4.1 to OsSERK1. We performed an in vitro GST pull-down assay by incubating purified GST-OsSERK1-CD bound to glutathione agarose beads with OsREM4.1 and OsBRI1-CD in the presence or absence of ATP. As shown in Figure 6F, the presence of ATP in the incubation mixture significantly reduced the OsREM4.1-OsSERK1 binding. Such a result is consistent with our BL treatment experiments revealing that BL-activated OsBRI1 inhibited the OsREM4.1-OsSERK1 binding.

DISCUSSION

Studies have shown that plant hormones ABA and BR play antagonistic roles in regulating many plant developmental processes; however, it is unknown how these two hormone-mediated regulatory processes are interconnected. In this study, we demonstrate that OsREM4.1 coordinates the antagonistic interaction between ABA and BR signaling processes in rice. Our evidence reveals that ABA regulates *OsREM4.1* expression through transcription factor OsbZIP23. OsREM4.1, interacting with OsSERK1, acts as a regulator to modulate the association of OsBRI1 and OsSERK1 and the activation of the OsBRI1-OsSERK1 receptor complex.

OsREM4.1 Is Regulated by ABA Signal and Plays a Role in Modulating BR Signaling Output

OsREM4.1 expression is tightly regulated by the hormone ABA through elements in its promoter sequence. In a typical ABA signal transduction pathway, ABREs are recognized by transcription factors of bZIP-type AREBs or ABFs (Yoshida et al., 2010). OsbZIP23 has been shown to be a key transcription factor responsible for activating ABA-responsive molecular networks in rice (Xiang et al., 2008). Our results demonstrate that OsbZIP23 regulates *OsREM4.1* transcript levels by directly binding to the *OsREM4.1* promoter. OsbZIP23 is activated and *OsREM4.1* expression is upregulated in the presence of ABA signal, while *OsREM4.1* transcription is kept to a basal level in the absence of ABA signal.

OsREM4.1 suppression in rice led to characteristics such as wider bending angle at the lamina joint, typical phenotypes observed in BR synthesis, or signal-enhanced mutants (Tanaka et al., 2009; Tong et al., 2012; Zhang et al., 2009a). Meanwhile, *OsREM4.1* overexpression in rice displayed phenotypes such as dwarf stature, erect leaves, and shortened internodes, similar to both BR-deficient (*d2*, *d11* and *osdwarf4-1*) and BR-insensitive (*d61* and *dlt*) mutants (Hong et al., 2003; Sakamoto et al., 2006; Tanabe et al., 2005; Tong et al., 2009; Yamamuro et al., 2000). These results suggest that *OsREM4.1* acts in association with the BR biosynthesis or BR signaling process.

Moreover, *OsREM4.1OX* transgenics were insensitive to BL treatment while *OsREM4.1RNAi* transgenics were more sensitive, suggesting that *OsREM4.1* plays a role in negatively regulating BR response in rice. Response to BR signal typically involves the downregulation of BR biosynthesis genes, such as *CPD* and *BR6OX* in a feedback manner (Sun et al., 2010; Yu et al., 2011), and upregulation of BR signaling transduction genes such as *DREPP* (Sun et al., 2010). Measurement of these genes expression can be considered an indicator of BR signaling activity (Tong et al., 2009; Wang et al., 2002). In our study, the transcript levels of *CPD* and *BR6OX* were downregulated in *OsREM4.1*-suppressed plants but little changed in plants overexpressing *OsREM4.1*. In contrast, *DREPP* expression was enhanced in *OsREM4.1RNAi* plants but suppressed in *OsREM4.1OX* plants. These results suggest that *OsREM4.1* expression is negatively related to the level of BR signaling activity.

OsREM4.1 Modulates BR Signaling Output by Interfering with OsBRI1-OsSERK1 Complex Formation

OsSERK1 was identified as a co-precipitated protein in the OsREM4.1 complex. The direct interaction between OsREM4.1 and OsSERK1 was verified through yeast two-hybrid, BiFC, and coIP assays. Our protein-protein interaction assays demonstrated that OsREM4.1 binding to the OsSERK1 may block OsSERK1 interaction with OsBRI1. Our kinase activity analyses confirmed that OsSERK1-mediated transphosphorylation of OsBRI1 is inhibited by OsREM4.1. Thus, the interaction of OsREM4.1 with OsSERK1 interferes with OsBRI1-OsSERK1 complex formation and activation of the receptor kinase complex, to impede BR signaling output. A recent study showed that OsBKI1 interacts with OsBRI1 to inhibit the OsBRI1 interaction with OsBAK1/OsSERK1 and negatively regulates the kinase activity of BRI1-BAK1 receptor complex (Jiang et al., 2015). This study identifies OsREM4.1 as a regulatory protein that interacts with OsSERK1 to inhibit the OsBRI1-OsSERK1 interaction. BR signaling is involved in many developmental processes. The BR signaling may be tuned by different regulators in plants to keep it functioning properly. OsREM4.1, which is induced by ABA, functions in coordination with BR signaling and ABA signaling in rice development. It is quite possible that OsREM4.1 and OsBKI1 work independently to inhibit the activation of the rice BR receptor OsBRI1. Further investigation is required to fully understand how these two negative regulators are coordinated during the rice life cycle.

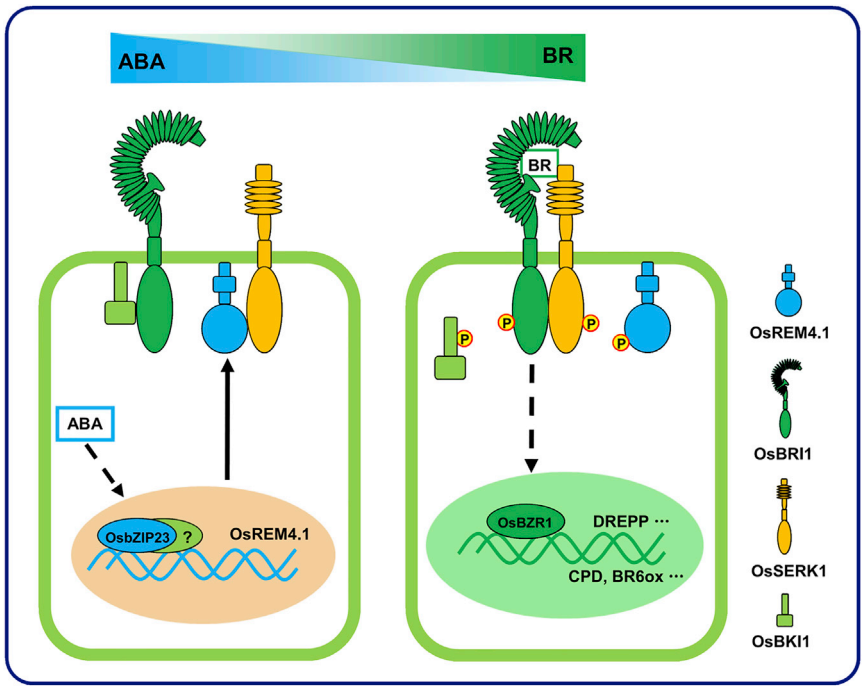

Figure 7. A Proposed Model for OsREM4.1's Role in Coordinating the Interlinking of ABA and BR Signaling

OsREM4.1 protein is localized on PM in association with OsBRI1-OsSERK1 complex formation. With increase of ABA presence, the ABA-responsive transcription factor OsbZIP23 gene is activated and upregulates *OsREM4.1* expression. OsREM4.1 protein interacts with OsSERK1 and interferes with OsBRI1-OsSERK1 complex formation, repressing BR signaling initiation. With increase of BR presence, BR binds to the extracellular domain of OsBRI1, which activates the OsBRI1 kinase for phosphorylation of OsREM4.1. The phosphorylated OsREM4.1, which has lower binding affinity to OsSERK1, causes disassociation of OsREM4.1 with OsSERK1. Thus, OsSERK1 is able to interact with OsBRI1 to form OsBRI1-OsSERK1 receptor kinase complex, and the BR signaling cascade is then activated.

OsREM4.1 Phosphorylation by OsBRI1 Undermines OsREM4.1 Repression of OsBRI1-OsSERK1 Complex Formation

OsREM4.1, as a substrate of OsBRI1, can be phosphorylated by activated OsBRI1. Unphosphorylated OsREM4.1 has a higher affinity for binding to OsSERK1 to form OsREM4.1-OsSERK1 complex and inhibit formation the OsBRI1-OsSERK1 receptor complex. When BL binds to OsBRI1, which in turn catalyzes phosphorylation of OsREM4.1, the phosphorylated OsREM4.1 has a lower affinity for binding to OsSERK1 and causes dissociation of the OsREM4.1-OsSERK1 complex. Thus, OsSERK1 is able to interact with OsBRI1 to form the OsBRI1-OsSERK1 receptor complex. OsREM4.1, whose phosphorylation status is regulated by BL, acts as a modulator to regulate the BR signaling output.

The dynamics of various plant hormones is crucial for plant growth and development (Vert and Chory, 2011). ABA and BR are dynamically present in plants and play antagonizing roles in a variety of plant development processes, which requires certain molecular mechanisms to coordinate such antagonizing hormone signaling in plants. As proposed in Figure 7, the evidence of this study suggests that OsREM4.1, through interaction with OsSERK1 to inhibit OsBRI1-OsSERK1 receptor complex formation, negatively regulates BR signaling output, while OsBKI1 interacts with OsBRI1 to regulate the OsBRI1-OsSERK complex activity (Jiang et al., 2015). When both ABA and BR signals are low, OsREM4.1 is present at a basal level and its binding to OsSERK1 obstructs the interaction between OsBRI1 and OsSERK1 to prevent the activation of BR signaling. When ABA signal is high, the increase of OsREM4.1 enhances its binding to OsSERK1. The binding interferes with the interaction between OsBRI1 and OsSERK1, hindering the formation of active OsBRI1-OsSERK1 receptor complex and resulting in the suppression of BR signaling. When BR signal is high, BR binding to BRI1 activates BRI1, which in turn phosphorylates OsREM4.1 and results in its dissociation from OsSERK1. The unbound OsSERK1 is then associated with OsBRI1 to fully activate the OsBRI1-OsSERK1 receptor complex. This model provides insights into how ABA signaling antagonizes BR signaling. ABA plays a brake-like role in restraining plant growth, especially under stress conditions, while BR functions as an accelerator to promote plant growth. *OsREM4.1*, which helps to coordinate the brake and accelerator, plays a pivotal role in the balance between plant growth and adjustment to ever-changing growth environments.

EXPERIMENTAL PROCEDURES

Plant Growth, Transgenic Lines, and Gene-Expression Analysis

WT rice plants used in this study were *Oryza japonica* cv. zhonghua 11 (ZH11). The WT and transgenic rice plants (*OsREM4.1OX*, *OsREM4.1RNAi*, and *OsREM4.1-3Flag*) were grown in the field at the Institute Experimental Farm or in a phytotron under conditions of 60% relative humidity, 12 hr photoperiod, 28°C constant temperature regime and, photon flux density at 200–250 μM m^{-2} s^{-1}. Gene expression and promoter GUS activity were examined as described by Gui et al. (2011) and quantitatively determined as described by Blazquez (2007). The luciferase reporter gene transient expression assay was performed in tobacco (*Nicotiana benthamiana*) leaves as described by Li et al. (2013).

Electrophoretic Mobility Shift Assay

OsbZIP23-His protein was expressed in *E. coli* and purified using Ni-nitrilotriacetic acid resin (Qiagen). Biotin-labeled OsREM4.1 promoter fragment and mutated OsREM4.1 promoter fragment were obtained by PCR amplification using 5′-biotin-labeled primers. EMSA was performed using a LightShift Chemiluminescent EMSA Kit (Pierce) according to the instructions.

BR Sensitivity Tests

Coleoptile lengths were measured after 1 week of growth in darkness. Root lengths were measured after 1 week of growth under normal light/dark cycles. The lamina joint inclination assay and excised leaf segment assay were performed as described previously (Wada et al., 1981). Detailed BR sensitivity tests are provided in Supplemental Experimental Procedures.

Protein-Protein Interaction Assays

A DUAL membrane yeast two-hybrid (Dualsystems Biotech), BiFC, coIP, and GST pulldown assays were used to measure the interaction between OsREM4.1 and OsSERK1. Detailed procedures are provided in Supplemental Experimental Procedures.

Protein Phosphorylation Assay

His6-tagged OsBRI1-CD, OsBRI1m-CD, OsREM4.1-ND and GSD1-ND proteins, and GST-tagged OsSERK1-CD and OsSERK1m-CD proteins were expressed in *E. coli* and purified using His and glutathione-Sepharose beads, respectively (Qiagen). In vitro phosphorylation assays were performed as previously described (Li et al., 2002). Detailed procedures are provided in Supplemental Experimental Procedures.

ACCESSION NUMBERS

The accession number for the remorin gene OsREM4.1 reported in this paper is GenBank: XM_015791500 or the Rice Genome Annotation Project database: LOC_Os07g38170.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2016.06.011.

AUTHOR CONTRIBUTIONS

J.G. performed most of the experiments. Z.S., C.L., and J.S. performed some of the experiments. J.L. analyzed the data and wrote the paper. J.G. and L.L. conceived the project, analyzed the data, and wrote the paper.

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