The Dual Effect of the Brassinosteroid Pathway on Rice Black-Streaked Dwarf Virus Infection by Modulating the Peroxidase-Mediated Oxidative Burst and Plant Defense

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The phytohormone brassinosteroid (BR) not only plays key roles in regulating plant growth and development but is also involved in modulating the plant defense system in response to pathogens. We previously found that BR application made rice plants more susceptible to the devastating pathogen rice black-streaked dwarf virus (RBSDV), but the mechanism of BR-mediated susceptibility remains unclear. We now show that both BR-deficient and -insensitive mutants are resistant to RBSDV infection. High-throughput sequencing showed that the defense hormone salicylic acid and jasmonic acid pathways were activated in the RBSDV-infected BR mutant. Meanwhile, a number of class III peroxidases (OsPrx) were significantly changed and basal reactive oxygen species (ROS) accumulated in BR mutants. Treatment with exogenous hormones and other chemicals demonstrated that the BR pathway could suppress the levels of OsPrx and the ROS burst by directly binding the promoters of *OsPrx* genes. Together, our findings indicate that BR-mediated susceptibility is at least partly caused by inhibition of the action of defense hormones, preventing the accumulation of the peroxidase-mediated oxidative burst.

Plant hormones play pivotal roles in a well-developed system that plants use to protect themselves from pathogen attack. The phytohormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are considered to be the key players in this plant defense system (De Vleeschauwer et al. 2013; Pieterse et al. 2012) but increasing evidence suggests that many other

hormones, including abscisic acid, auxins, and brassinosteroids (BR), are also involved in regulating plant resistance by crosstalk with the SA-JA-ET cascades (Adie et al. 2007; de Torres Zabala et al. 2009; De Vleeschauwer et al. 2012; Nahar et al. 2012). The phytohormone BRs, a class of steroid hormones, play key roles in regulating plant growth and development throughout the plant kingdom, influencing cell elongation, vascular differentiation, and stress tolerance (Sun et al. 2010; Wang et al. 2014). Bioactive BRs bind directly to BR receptor protein (BRI1), thereby inducing BRI1 dissociation from the negative regulator BKI1, phosphorylation, and activation of the coreceptor BAK1, and activation of the protein phosphatase BSU1. BSU1 dephosphorylates and inactivates the GSK3-like kinase BRASSINOSTEROID INSENSITIVE2 (BIN2). The inactivation of BIN2 results in the accumulation of the unphosphorylated transcription factors BZR1 and BES1. Unphosphorylated BZR1 and BES1 accumulate in the nucleus and regulate BR responses (Clouse 2014; Schumacher and Chory 2000; Wang et al. 2006, 2014; Yang et al. 2011).

Besides their critical role in growth regulation, there has been increasing evidence that BRs are also involved in plant defense to pathogen invasion (De Bruyne et al. 2014; Lozano-Durán and Zipfel 2015; Nakashita et al. 2003). In the first such report, exogenously applied BRs increased resistance in tobacco to tobacco mosaic virus (TMV) and were also active in rice (Nakashita et al. 2003). BR application was also shown to protect barley from several *Fusarium* diseases and promote resistance to cucumber mosaic virus in *Arabidopsis thaliana* (Ali et al. 2014; Zhang et al. 2015). Recent research suggested that BRs enhanced tobacco resistance to TMV through MEK2-SIPK cascades and the RBOHB-dependent reactive oxygen species (ROS) burst, while the BR master regulator BZR1 negatively mediated BR-induced virus resistance by inhibiting RBOHB-dependent ROS production, indicating the complexity of BR signaling in plant immunity against virus infection (Deng et al. 2016). In rice, BRs have been shown to promote susceptibility to pathogens (De Vleeschauwer et al. 2012; Nahar et al. 2013). Application of BRs rendered plants hypersusceptible to root-knot nematode *Meloidogyne graminicola* and the root-infecting oomycete *Pythium graminicola*. Our recent research revealed that application of BRs made rice more susceptible to RBSDV infection (He et al. 2017). These findings indicate that the effect and mode of action of BR on plant immunity is complicated, depending on the type of pathogen and host.

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RBSDV infects many plant species (rice, maize, barley, and wheat), typically leading to dwarfism and darkening of leaves (Shikata and Kitagawa 1977; Wang et al. 2009) and causing severe economic losses in Asia (Bai et al. 2002; Mertens 2004). The virus is a member of genus *Fijivirus*, family *Reoviridae*, and is transmitted by the small brown planthopper (*Laodelphax striatellus* Fallén) (SBPH) in a persistent, circulative manner (Wei and Li 2016). The RBSDV genome comprises 10 segments of double-stranded RNA (Wang et al. 2003; Zhang et al. 2008). Most segments encode a single protein but three (S5, S7, and S9) have two open read frames. The functions of some of the viral proteins are known. Both P5-1 and P9-1 interacted with P6 proteins to constitute the viroplasm (Sun et al. 2013a). P7-1 forms the tubular structures and localizes to the plasmodesmata (Isogai et al. 1998; Sun et al. 2013b). S7-2 interacts with S-phase kinase-associated protein 1 (SKP1) to form the SCF (Skp, Cullin, F-box) complex (Tao et al. 2017). S8 encodes a core capsid protein and acts as a transcriptional repressor (Liu et al. 2007). S10 encodes an outer capsid protein and could trigger the endoplasmic reticulum stress and unfolded protein response in plants (Sun et al. 2013c). RBSDV causes plant growth abnormalities, symptoms that are associated with a change of hormone homeostasis, but none of the viral proteins has been reported to directly alter the hormone pathway.

In a previous study, we found that the BR genes and pathway were inhibited in RBSDV-infected plants and that exogenous epibrassinolide (BL) application rendered rice susceptible to RBSDV (He et al. 2017). BRs regulate many aspects of normal plant growth and development, and disrupting BR biosynthesis results in dwarf phenotypes, dark-green and epinastic leaves, reduced apical dominance, delayed flowering, and senescence (Ye et al. 2011). Some of these effects resemble RBSDV symptoms and it therefore appears that the BR pathway plays important roles in RBSDV infection. However, the detailed mechanism of BR-mediated susceptibility during RBSDV infection remains unclear.

In this study, the role of the BR pathway in response to RBSDV was investigated using BR-deficient and -insensitive mutants and high-throughput sequencing. The results indicate that BR-mediated susceptibility is due to the suppression of the peroxidase-mediated oxidative burst and plant defense.

RESULTS

BR sensitivity is enhanced in RBSDV-infected plants.

Our previous results revealed that the BR pathway was suppressed in RBSDV-infected plants, and exogenous BL

treatment made plants more susceptible to RBSDV (He et al. 2017). Those results indicated that the BR pathway played a negative role in plant defense against RBSDV infection. To better understand the relationship between the BR pathway and RBSDV, we analyzed the sensitivity of mock- or RBSDV-infected plants to BL by lamina inclination assay. The lamina inclination, which is affected by BR treatment in rice, can be used as an indicator of BR sensitivity (Tong et al. 2009; Wada et al. 1981). The leaf angle of mock- and RBSDV-infected plants was similar without BL treatment (7 to 9 degrees) but greatly increased after treatment with 1 μ M exogenous BL at 37°C in the dark for 24 h (>30 degrees in mock plants and 62 degrees in those infected by RBSDV [Fig. 1]). These results suggested that RBSDV infection altered the sensitivity of rice to BL, making RBSDV-infected plants more sensitive to BL treatment.

A BR biosynthesis-deficient mutant was less susceptible to RBSDV.

BL-mediated susceptibility to RBSDV infection prompted us to clarify how the BR biosynthesis pathway influences viral infection. Previous studies indicated that *OsDWARF4* encodes a cytochrome P450 (*CYP90B2*) (Sakamoto et al. 2006) that plays important roles in C-22 hydroxylation during BR biosynthesis, thus affecting lamina joint bending (Sakamoto et al. 2006). The BR biosynthesis-deficient mutant *RI90B* was constructed by an RNA interference (RNAi) construct against the *CYP90B2* or *OsDWARF4* gene (Supplementary Fig. S1). The expression of *OsDWARF4* in the *RI90B* transgenic mutant was markedly reduced compared with wild-type (WT) plants. To test whether the mutants have any influence on insect SBPH mortality, mutant seedlings were infested with SBPH at five insects per seedling and insect mortality was evaluated 3 and 5 days later. The SBPH mortality in *RI90B* mutant plants was not significantly different from that in rice cultivar Nipponbare (NIP) controls (Supplementary Fig. S2). *RI90B* transgenic plants were then inoculated with RBSDV using viruliferous SBPH (or virus-free insects for the controls). After 30 days, symptoms in the *RI90B* mutant were milder than in NIP plants (Fig. 2A) and viral incidence (as determined by reverse transcription polymerase chain reaction [RT-PCR]) was significantly reduced (from >45 to <30%) (Fig. 2B). There were corresponding decreases in RBSDV RNA levels, as measured by RT-quantitative (q)PCR (Fig. 2C), and in the expression levels of viral P8, as shown by Western blot (Fig. 2D). The finding that the BR biosynthesis-deficient mutant *RI90B* was more resistant to

Fig. 1. Brassinosteroid sensitivity of rice black-streaked dwarf virus (RBSDV)-infected plants. **A**, Effects of epibrassinolide (BL) treatment of wild-type (Mock) and RBSDV-infected plants in the lamina inclination experiment. **B**, Statistical data for lamina joint-bending angle assays from one of three experiments that had very similar results. Each treatment contained at least 15 plants. *P* value at the top of columns indicates significant difference by Fisher's least significant difference test.

RBSDV attack is further evidence that the BR biosynthesis pathway is involved in rice susceptibility to virus infection.

A BR signaling-insensitive mutant was less susceptible to RBSDV.

DWARF AND LOW-TILLERING (*DLT*) interacts with OsBZR1 to regulate BR responses (Tong et al. 2009, 2012). To investigate the role of BR signaling in plant immunity against viral infection, RBSDV was inoculated to a *dlt* mutant that had been shown to be insensitive to BL by the lamina-bending assay. Symptoms of RBSDV, including dwarfism and stunting, were less severe in the *dlt* mutant than in Zhonghua 11 (ZH11) plants (Fig. 3A). The disease incidence in ZH11 plants was close to 53%, while that in the *dlt* mutant was approximately 34% (Fig. 3B). The levels of RBSDV mRNAs and protein were also significantly lower in the mutant (Fig. 3C and D). SBPH mortality on the *dlt* mutant was not different from that on ZH11. These results demonstrate that the BR signaling mutant *dlt* was less susceptible to viral infection.

Transcriptomic analysis of a BR-insensitive mutant in response to RBSDV infection.

To shed light on BR-mediated susceptibility to viral infection, we next investigated the global transcriptome of the BR-insensitive mutant *dlt* and its WT, ZH11, treated with virus-free or viruliferous SBPH. A total of 611 and 713 differentially expressed genes (DEGs) were found in response to RBSDV infection in WT (ZH11-RB vs. ZH11-CK) and *dlt* plants (*dlt*-RB vs. *dlt*-CK), respectively, using the criteria of the absolute

value of $\log_2$ (fold change) ratio ≥ 1 and $P \leq 0.05$ (Fig. 4A; Supplementary Tables S1 and S2). Meanwhile, 691 DEGs were found to be involved in BR signaling (*dlt* vs. ZH11) (Supplementary Table S3). Consistent with our previous results (He et al. 2017), categories ranging from primary and secondary metabolism, ribosome to photosynthesis were over-represented in the ZH11-RB vs. ZH11-CK comparison by gene ontology (GO) analysis (Fig. 4B). Categories corresponding to phenylpropanoid biosynthesis, plant hormone signal transduction, starch and sucrose metabolism, nitrogen metabolism, and brassinosteroid biosynthesis were mainly overrepresented in the *dlt*-RB vs. *dlt*-CK comparison (Fig. 4B). Notably, a large number of genes were activated in the *dlt* in response to RBSDV infection based on hierarchical cluster analysis (Fig. 4C).

Since *dlt* was more resistant than the ZH11 to viral infection, we speculated that RBSDV might trigger a defense response in *dlt*. To this aim, we extracted the transcriptome data, using the following criteria: $\log_2$ (fold change) of *dlt*-RB vs. *dlt*-CK ≥ 1 and $> \log_2$ (fold change) of ZH11-RB vs. ZH11-CK, and $P \leq 0.05$. A total of 447 genes in *dlt*-RB vs. *dlt*-CK were significantly up-regulated compared with ZH11-RB vs. ZH11-CK (Supplementary Table S4). When this expression pattern was analyzed by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, a large number of genes were enriched in the plant-pathogen interaction (K04626) and plant hormone signal transduction (K04075) categories. Interestingly, JA signaling genes, such as *OsJAZs* and *OsMYC2*, were significantly increased in *dlt*-RB vs. *dlt*-CK compared with ZH11-RB vs. ZH11-CK (Fig. 5A). SA pathway regulatory gene WRKYs and

Fig. 2. Rice black-streaked dwarf virus (RBSDV) symptoms and gene expression on brassinosteroid synthesis mutant *RI90B* compared with the wild-type 'Nipponbare' (NIP). **A**, Mock- or RBSDV-infected NIP and *RI90B* plants. **B**, Viral incidence in NIP and *RI90B* plants. **C**, Relative expression levels of RBSDV *S2*, *S4*, and *S6* in RBSDV-infected NIP and *RI90B* plants, assessed by quantitative reverse transcription-polymerase chain reaction. The average values from three biological replicates are shown. An asterisk (*) at the top of columns indicates significant difference at $P < 0.05$. **D**, The expression level of viral P8 protein by Western blot.

pathogenesis-related (PR) proteins were also overrepresented in this pattern. RT-qPCR confirmed that the *OsMYC2*, *OsJAZ12*, *OsWRKY9*, *OsWRKY108*, and *OsPR1b* genes were expressed significantly more in the *dlt* mutant than in ZH11 plants in response to RBSDV infection (Fig. 5B). Thus, the results of RT-qPCR were consistent with the RNA-seq data. Overall, the results confirm our speculation that RBSDV infection activates the plant defense system pathways in the *dlt* mutant.

Effects of BR signaling on class III peroxidase and the oxidative burst.

The transcriptome data show that a number of class III peroxidases (EC 1.11.1.7) were markedly changed in the *dlt* mutant in response to viral infection (Supplementary Fig. S3). Class III peroxidases are present as large multigene families in land plants and are secreted into the cell wall or the surrounding medium and the vacuole (Cosio and Dunand 2009; Minibayeva et al. 2015). These enzymes mainly regulate the oxidative burst by controlling ROS (H_2O_2 and $O_2^{\cdot-}$) (Bindschedler et al. 2006; Mei et al. 2009; O'Brien et al. 2012). A previous report showed that higher basal ROS levels play vital roles in rice against RBSDV infection (Wu et al. 2017). Hence, we speculate that the BR pathway might affect the levels of *OsPrx* and the ROS burst, which would, in turn, influence viral infection. To explore the relationships between the BR pathway, *OsPrx*, and ROS burst, peroxidase activity was monitored in BR mutants *dlt* and *RI90B*. As shown in Figure 6A, *OsPrx* activity was significantly increased in both BR mutants *dlt* and *RI90B* compared with their respective WT plants. Nitroblue tetrazolium

(NBT) staining then showed that the accumulation of $O_2^{\cdot-}$ substantially increased in *dlt* and *RI90B* compared with the controls (Fig. 6B). Thus, the activity of *OsPrx* and the ROS burst were activated in BR mutants.

We next monitored the activity of *OsPrx* in rice seedlings treated with BL and brassinazole (BRZ) (an inhibitor of the BR biosynthesis pathway). The activity of *OsPrx* was decreased in the BL-treated seedlings but increased in the BRZ-treated seedlings compared with the controls (Fig. 6C). NBT staining similarly showed that BL- and BRZ-treated seedlings had decreased and increased accumulation of $O_2^{\cdot-}$, respectively (Fig. 6D). These results show that the BR pathway negatively modulated the activity of *OsPrx* and the ROS burst.

The BR pathway modulated ROS accumulation in a peroxidase-dependent manner.

The relationship between the BR pathway, peroxidase, and ROS levels was further examined by treating rice seedlings with salicylhydroxamic acid (SHAM), an inhibitor of peroxidase (Brouwer et al. 1986; Dunand et al. 2007; Khokon et al. 2011; Lv et al. 2018). The *RI90B*, *dlt*, and WT seedlings were treated with 10 mM SHAM, and the samples were collected 12 h later. As shown in Figure 7A, the activity of *OsPrx* was significantly decreased in SHAM-treated plants compared with the controls. The activity of *OsPrx* in BR mutants was markedly more than that in WT plants (consistent with earlier results (Fig. 6A), but there was no significant difference in activity of *OsPrx* between SHAM-treated BR mutants and SHAM-treated WT plants. The ROS levels in these plants were then measured by NBT staining. ROS levels were greater in BR mutants than

Fig. 3. Rice black-streaked dwarf virus (RBSDV) symptoms and gene expression on brassinosteroid signaling mutant *dlt* compared with the wild-type ZH11. **A**, Mock- or RBSDV-infected ZH11 and *dlt* plants. **B**, Viral incidence in ZH11 and *dlt* plants. **C**, Relative expression levels of RBSDV S2, S4, and S6 in RBSDV-infected ZH11 and *dlt* plants, assessed by quantitative reverse transcription-polymerase chain reaction. The average values from three biological replicates are shown. An asterisk (*) at the top of columns indicates significant difference at $P < 0.05$. **D**, The expression level of viral P8 protein by Western blot.

WT plants (Fig. 7B, left panels), again consistent with earlier results (Fig. 6B), but there were no such differences when plants had been treated with SHAM (Fig. 7B, right panels). Thus, the influence of the BR mutant on the activity of OsPrx and on ROS accumulation was inhibited by SHAM, indicating that BR-mediated ROS accumulation occurs by a peroxidase pathway.

To confirm the effect of peroxidase-mediated ROS accumulation on RBSDV infection, the SHAM-treated rice plants were inoculated with RBSDV and the levels of the genomic viral RNAs S2, S4 and S6 were determined by RT-qPCR at 30 days postinfection. The RBSDV RNA levels were much greater (five- to sixfold) in SHAM-treated ZH11 plants than in the untreated controls (Fig. 7C) showing that SHAM treatment increased rice susceptibility to viral infection. When the BR-insensitive mutant *dlt* was treated with SHAM and then inoculated with RBSDV, the RBSDV RNA levels were

significantly more than those in the untreated *dlt*. These results indicate that SHAM treatment undermines the antiviral property of the BR mutant and suggests that peroxidase-dependent ROS accumulation plays an important role in BR-mediated susceptibility to RBSDV infection.

The BR pathway directly modulated the expression of *OsPrx*.

The ability of the BR pathway to repress the activity of OsPrx prompted us to investigate whether the BR pathway directly affected the expression of *OsPrx* genes. The expression of *OsPrx* genes was significantly less in BL-treated seedlings than in mock plants 6 h after BL treatment (Fig. 8A). We therefore analyzed the promoters of these genes, using the software PlantCARE (Lescot et al. 2002). Interestingly, BR response elements [known as BRRE, CGTG (C/T)G] were found in these *OsPrx* promoters (Fig. 8B). In *Arabidopsis*, BR transcription

Fig. 4. RNA-seq analyses of ZH11 and *dlt* plants in response to rice black-streaked dwarf virus (RBSDV) infection. **A**, A Venn diagram shows the overlap between sets of genes differentially expressed in *dlt* vs. ZH11 under mock- and RBSDV-infected conditions at 30 days postinfection. Data were taken from three biological replicates, each containing a pool of eight to 10 plants. Genes were considered differentially expressed according to the following criteria: false discovery rate ≤ 0.05 and the absolute value of $\log_2$ (fold change) ratio ≥ 1 . **B**, Gene ontology analysis of differential gene expression in RBSDV vs. mock in ZH11 or *dlt* plants. **C**, Hierarchical cluster analysis of the differentially expressed genes in ZH11 or *dlt* under mock or RBSDV infection conditions.

factor BZR1 can recognize the BRRE motif to suppress expression of the BR biosynthesis genes (He et al. 2005; Sun et al. 2010). To demonstrate the direct binding of OsBZR1 to the promoter of *OsPrx*, electromobility shift assays (EMSA) were performed, using a recombinant His-OsBZR1 fusion protein and digoxigenin (DIG)-labeled probes derived from the *OsPrx* promoters that included at least one of the BRREs (Supplementary Table S5). OsBZR1 was constructed into pCold-TF-His vector to form the TF-His-OsBZR1 fusion protein. As shown in Figure 8B, TF-His-OsBZR1 fusion protein specifically bound the DIG-labeled probes (indicated by an arrow) and that this binding could compete with unlabeled probes. To determine whether OsBZR1 directly regulates *OsPrx* expression,

we performed chromatin immunoprecipitation (ChIP) qPCR using a transgenic line *OsBZR1-D* (a gain-of-function mutation of OsBZR1) expressing the fully functional FLAG OsBZR1 (Qiao et al. 2017). Consistent with BR-mediated susceptibility to RBSDV infection, the BR-sensitive mutant *OsBZR1-D* was more susceptible to RBSDV attack (Supplementary Fig. S4). ChIP-qPCR assays showed that FLAG OsBZR1 bound specifically to the BRRE motif in the *OsPrx* promoters but not to other regions in the promoters (Fig. 8C). These results indicate that OsBZR1 modulates the expression of *OsPrx* genes by directly binding their promoters. Overall, the negative effect of the BR pathway on *OsPrx* activity and the ROS burst contributed to BR-mediated susceptibility to RBSDV.

Fig. 5. The effect of brassinosteroid signaling on the jasmonic and salicylic acids (JA/SA) pathway in response to rice black-streaked dwarf virus (RBSDV) infection. **A**, Genes involved in the JA/SA signaling pathway in *dlt* vs. ZH11 in response to RBSDV infection. Genes whose names are shaded are up-regulated in the RNA-seq data, and **B**, reverse transcription-quantitative polymerase chain reaction verification of the expression of the JA/SA genes. Mock- or RBSDV-infected plants were used at 30 days postinfection. Each sample had three biological replicates with five technical replicates, and each biological sample consisted of seven to 10 pooled plants. An asterisk (*) at the top of columns indicates significant difference at $P < 0.05$.

DISCUSSION

RBSDV-infected plants usually have dark green leaves, severe stunting, and growth abnormalities. These symptoms are often associated with a change of hormone homeostasis. The direct association between viral symptoms and hormone change was well exemplified by another rice virus, rice dwarf virus (RDV) (Zhu et al. 2005). Rice plants infected with RDV exhibited a dwarf phenotype and had reduced gibberellic acid (GA) levels. RDV P2 outer capsid protein interacted with *ent*-kaurene oxidase, a key factor in the biosynthesis of gibberellins, and disturbed GA biosynthesis, resulting in viral symptoms. In addition, RDV P2 could bind OsIAA10 and reprogram auxin signaling to enhance viral infection (Jin et al. 2016), while RDV Pns11 interacted with *S*-adenosyl-L-methionine synthetase to manipulate ethylene biosynthesis and also benefited viral infection (Zhao et al. 2017). In our previous report, the BR pathway was down-regulated in RBSDV-infected plants (He et al. 2017). Disrupting the BR pathway often results in dwarf phenotypes and darkened leaves in plants. Hence, the downregulated BR pathway in RBSDV-infected plants may contribute to the viral symptoms. In the present study, the BR sensitivity assay showed that RBSDV-infected plants were hypersensitive to exogenous BL treatment, indicating that RBSDV infection changed BR sensitivity in rice and that the BR pathway plays important roles in the rice-RBSDV interaction.

BRs are a unique class of growth-promoting plant hormones that affect various physiological process, including germination, etiolation, vegetative growth, flowering, and fertility.

Increased evidence indicates that BRs act as vital modulators of plant immunity (Belkhadir et al. 2012; Lozano-Durán and Zipfel 2015). A recent study showed that BR can suppress basal immunity to the root pathogen *P. graminicola*. The BR pathway is exploited by *P. graminicola* as a virulence factor to promote disease by suppressing SA and GA-mediated defenses (De Vleeschauwer et al. 2012). In the rice-*M. graminicola* interaction, the BR pathway was shown to promote infection by inhibiting the JA-mediated defense (Nahar et al. 2013). These findings indicate that the roles of BR in plant immunity are varied and the potential mechanisms of BR-modulated immunity complex, depending on the type of pathogen and host. Our recent reports showed that application of exogenous BL could increase rice susceptibility to RBSDV (He et al. 2017). Here, we reveal the potential mechanism of BR-mediated susceptibility to viral infection using BR-deficient and -insensitive mutants of rice and high-throughput RNA sequencing. Consistent with the previously revealed negative role of BR in plant defense, the mutants had enhanced resistance to RBSDV (Figs. 2 and 3). RNA sequencing revealed that important modulators of the JA and SA pathways, including JAZs, MYC2, WRKYs, and PR proteins, were activated in the BR-insensitive mutant when infected by RBSDV. This implies that activation of JA- and SA-mediated defenses contributed to the resistance of the BR mutants. We have previously shown that JA inhibits BR-mediated susceptibility to RBSDV infection (He et al. 2017) and it therefore appears that BR-mediated susceptibility is antagonized by JA-mediated defenses during RBSDV infection of rice. An antagonistic relationship between BR and JA is also involved in plant growth and development. For example, BR is

Fig. 6. The effect of the brassinosteroid pathway on peroxidases and reactive oxygen species levels. **A**, Peroxidase activity in ZH11, *dlt*, NIP, and *RI90B* seedlings. Each result was derived from not less than three biological repeats; an asterisk (*) at the top of columns indicates significant difference at $P < 0.05$; **B**, Nitroblue tetrazolium (NBT) staining in ZH11, *dlt*, NIP, and *RI90B* seedlings. **C**, Peroxidase activity in rice seedlings treated with epibrassinolide (BL) or brassinazole (Brz) or an untreated mock. Three biological replicates were performed. An asterisk (*) at the top of columns indicates significant difference at $P < 0.05$. **D**, NBT staining of rice seedling treated with BL or Brz or an untreated mock.

involved in negatively modulating both JA signaling and JA-inhibitory root growth in *Arabidopsis*. In rice, application of methyl-jasmonate impairs the increase of BR-induced lamina joint inclination. BR has been shown to suppress SA defenses against *P. graminicola* invasion of rice and this suppression occurs downstream of SA biosynthesis but upstream of the master SA regulators *OsNPR1* and *OsWRKY45*. A recent study showed that preapplication of SA largely decreased the occurrence of RBSDV in rice (Huang et al. 2018).

Class III peroxidases, a group of heme-containing glycosylated proteins, are involved in a broad range of physiological processes throughout the plant life cycle (Cosio and Dunand 2009). Recently there have been several demonstrations that peroxidases have a role in the oxidative burst. Peroxidases generate the oxidative burst by producing ROS (O_2^- and $\cdot OH$) through the hydroxylic catalytic cycles (Bindschedler et al. 2006; Daudi et al. 2012). The peroxidase-dependent oxidative burst plays an important role in plant host-pathogen interactions. In cotton, superoxide anions were massively generated by peroxidases in response to *Xanthomonas campestris* attack (Mei et al. 2009). Transgenic *Arabidopsis* plants expressing an antisense heterologous French bean peroxidase cDNA exhibited an impaired oxidative burst and were susceptible to both fungal and bacterial pathogens (Bindschedler et al. 2006). In *Arabidopsis*, T-DNA insertion mutants with attenuated

expression of *PRX33* and *PRX34* were demonstrated to decrease ROS levels in response to microbe-associated molecular patterns (Daudi et al. 2012). These results suggested that the peroxidase-dependent oxidative burst played vital roles in *Arabidopsis* basal resistance. Although peroxidases have a significant role during pathogen attacks, the interaction between peroxidases and plant hormones has rarely been reported. In the present study, several peroxidases were significantly changed in the BR mutant during RBSDV infection. Further research revealed that BR-deficient and -insensitive mutants exhibited an increased peroxidase activity and a higher ROS burst. Application of exogenous hormones and the peroxidase inhibitor SHAM suggested that the BR pathway negatively modulated the ROS burst by suppressing peroxidase activity. EMSA and ChIP-qPCR assays indicated that BR signaling transcription factor BZR1 directly interacted with the peroxidase promoters to repress their expression. Hence, this study reveals a significant mechanism in which the crosstalk between BRs and the peroxidase-mediated ROS burst was involved in determining the outcomes of the rice-RBSDV interaction.

In conclusion, our results offer a novel mechanism for BR-mediated susceptibility to RBSDV infection involving the inhibition of the JA- and SA-mediated plant defense response and the reduction of peroxidase-mediated ROS accumulation (Fig. 9).

Fig. 7. Brassinosteroids modulate reactive oxygen species accumulation via a peroxidase pathway. **A**, Peroxidase activity in ZH11, *dlt*, *NIP*, and *RI90B* seedlings under mock or salicylhydroxamic acid (SHAM) treatment. Each result was derived from not less than three biological repeats; an asterisk (*) at the top of columns indicates significant difference at $P < 0.05$. **B**, Nitroblue tetrazolium staining in ZH11, *dlt*, *NIP*, and *RI90B* seedlings treated with SHAM or mock. **C**, Relative expression levels of rice black-streaked dwarf virus (RBSDV) S2, S4, and S6 in RBSDV-infected ZH11 and *dlt* plants under SHAM treatment, assessed by quantitative reverse transcription-polymerase chain reaction. Each treatment used at least 30 seedlings, and three biological replicates were performed. Different letters at the top of columns indicate significant differences at $P < 0.05$ by Fisher's least significant difference tests.

MATERIALS AND METHODS

Plant materials and growth conditions.

The WT rice NIP cultivar (*Oryza sativa* L. *japonica*), ZH11, Huaidao No. 5, and Wuyujing No. 3 were used for this study. The BR signaling-insensitive mutant *dlt* was provided by C. Chu. *OsBZR1-D* (a gain-of-function mutation of *OsBZR1*) was provided by X. Wang. The brassinosteroid-deficient mutant *RI90B* was constructed in this study. RBSDV-infected rice plants were initially collected from fields in Shandong Province, China, and were kept as a virus source in our laboratory. All rice plants were grown in a greenhouse at 28 to 30°C with a light and dark cycle of, respectively, 14 and 10 h.

Construction of vector and rice transformation.

To obtain the construct for RNAi of the *CYP90B2* or *OsDWARF4* gene, we transformed rice plants by introducing an RNAi structure. The RNAi-trigger regions were amplified by PCR. The PCR product from the *CYP90B2* or *OsDWARF4* gene was digested with *KpnI* and *BamHI* or *SpeI* and *SacI*, respectively, and was then cloned in two orientations into

pTCK303. The RNAi regions were designed under the control of the maize ubiquitin promoter gene. The recombinant plasmids were introduced into *Agrobacterium tumefaciens* (strain EHA105) using electroporation and were transformed into rice cultivar NIP.

Insect vector and RBSDV inoculation.

Virus-free SBPH (*Laodelphax striatellus*) were reared on Wuyujing No. 3 seedlings in our laboratory at 25°C under artificial light. Virus inoculation was essentially as described previously (He et al. 2017). Briefly, to acquire the virus, first- or second-instar SBPH were fed on RBSDV-infected plants for 3 to 5 days. The nymphs were transferred onto healthy Wuyujing No. 3 seedlings for 10 to 12 days (passing through the circulative period). Simultaneously, rice seedlings for viral inoculation were grown in a glass beaker (about 35 seeds per beaker) in a greenhouse at 28°C under artificial light for 10 days. RBSDV-carrying and virus-free planthoppers (mock) were used for inoculation. The inoculated rice seedlings were then grown in the field to develop viral symptoms (about 30 days). Plants infected with RBSDV exhibited stunting and

Fig. 8. Brassinosteroid (BR) directly modulates the expression of *OsPrx*. **A**, Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) verification of *OsPrx* gene expression in epibrassinolide (BL)-treated rice seedlings. Ten-day old rice seedlings were treated with 1 μ M BL. Each sample contained a pool of eight to 10 plants collected at 0 or 6 h after spraying. Three biological replicates were performed. An asterisk (*) at the top of columns indicates significant difference at $P < 0.05$. **B**, TF-His-OsBZR1 fusion proteins bind specifically to the digoxigenin-labeled BR response element (BRRE) probes of *OsPrx* in the electromobility shift assay. The shifted bands shown by the arrows indicate that OsBZR1 binds to the promoters of *OsPrx*. The competitor (unlabeled BRRE probe) was added in 10-fold excess amounts in the competitive assay. The negative control was TF-His protein (Mock). The fold enrichment represents binding efficiency ratio of antibody to no antibody. Black circles show BRRE motifs. P1 and P2 represent different regions in the *OsPrx* promoters: the P1 region contains BRRE motifs, and the P2 region contains BRRE motifs. **C**, Chromatin immunoprecipitation quantitative polymerase chain reaction assays on binding of OsBZR1 to the *OsPrx* promoters. Relative fold enrichments of FLAG:OsBZR1 in each examined region compared with wild type (WT) are shown. At least three biological replicates were performed. An asterisk (*) indicates significant differences ($P < 0.05$).

darkening of leaves. RT-PCR was used to assess the disease status of each plant and to thus determine the viral incidence. The inoculation experiment had at least three biological repeats. To evaluate the effect of rice plants on insect (SBPH) mortality, the rice seedlings were infested with SBPH at five insects per seedling and insect survival was assessed 3 and 5 days later.

Phytohormone treatment and BR-sensitivity tests.

For the phytohormone treatment assay, leaves of 10-day-old rice seedlings were sprayed with 1 μ M BL (Sigma) and 5 μ M BRZ (Tokyo Chemical Industry). To determine gene expression by RT-qPCR, samples were collected 0 and 6 h after spraying. For NBT staining and peroxidase activity measurement, the samples were collected 24 h after spraying. BL and BRZ were first dissolved in 100% ethanol to suitable concentrations as stock solutions (1:10) and were then diluted with sterile distilled water containing 0.1% Triton X-100. A water and 0.1% Triton X-100 solution was used as the mock control. The lamina joint assay followed published procedures with a slight modification (Tong et al. 2012). Briefly, the mock or RBSDV-infected rice seedling materials were cut into 2-cm-long segments and were immersed into 1 μ M BL for 24 h in darkness. Images were taken after 24 h of incubation, and the angles of leaf inclination were measured using a protractor. The experiments were repeated three times with similar results.

Peroxidase activity measurement.

The leaves of 10-day rice seedlings were collected and were ground in liquid nitrogen, using phosphate buffer (pH = 7 to 7.4) to prepare a tissue homogenate. The homogenate was then centrifuged for 10 min in 3,000 $\times$ g at 4°C, and the supernatant

was used for the peroxidase activity assay, using the peroxidase assay kit (Jiancheng Bioengineering Institute) according to the manufacturer's instructions, with a 37°C reaction for 30 min, and measuring activity with a spectrophotometer (420 nm). Each experiment used not less than three biological repeats and each sample consisted of a pool of eight to 10 plants.

The NBT staining assay.

Histochemical staining of superoxide ($O_2^{\cdot-}$) was performed by NBT methods. Briefly, the second leaf of rice seedlings was cut into 1-cm pieces and was infiltrated into 6 mM NBT solution (2 ml) prepared in sodium citrate (pH 6.0). After vacuum infiltration for 10 min at 60 KPa of pressure and incubation at room temperature for 2 h, the chlorophyll was completely removed by bleaching in absolute ethanol. The samples were kept in 20% glycerol and were photographed under a stereomicroscope.

Total RNA extraction and qRT-PCR.

Total RNA was isolated from rice leaves with TRIzol reagent (Invitrogen), following the manufacturer's protocol. One to two micrograms of RNA was reverse-transcribed using the Tiangen fast quant RT kit (Tiangen). The qRT-PCR assay was conducted on an ABI7900HT sequence detection system (Applied Biosystems) with ChamQTM SYBR qPCR master mix (Low ROX Premixed). The reference sequence was *OsUBQ5* gene (AK061988) (Sun et al. 2015) and the relative expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ method. Each sample had three biological replicates with five technical replicates, and each biological sample consisted of seven to 10 pooled plants.

Protein analysis.

Protein extraction and Western blotting were done as described previously (Sun et al. 2013c). RBSDV-infected rice leaves were extracted with sodium dodecyl sulfate (SDS) lysis buffer (100 mM Tris-HCl, pH = 6.8, 10% SDS) and were boiled with 5 $\times$ protein loading buffer at 95°C for 10 min. To separate the proteins, 10 to 12% SDS-PAGE gels were used that were then transferred to polyvinylidene difluoride membranes by standard procedures. The membrane was blocked using 10% skimmed milk powder diluted in Tris-buffered saline with Tween 20 (TBST) (1 to 2 h) and, then, with dilute primary antibody (1:3,000) in blocking buffer (5% skimmed milk powder diluted in TBST) for 2 h. Then secondary antibody was added in blocking buffer (1:10,000) before incubation according to the manufacturer's instructions for 1.5 h. Anti-P8 polyclonal antibody (Xie et al. 2017) was used as the primary antibody. The actin antibody (Abbkine) was used to evaluate the reference protein. The proteins were visualized with the ECL kit (Pierce).

Gel mobility shift assay.

The full length of OsBZR1 was cloned into the pCold-TF vector and was then introduced into *Escherichia coli* DE3. TF-His-OsBZR1 fusion protein was expressed in *E. coli* BL21 (DE3) and was purified using EMSAs. The BRRE probes in the promoter of *OsPrx* were end-labeled using DIG as the hot probe. An unlabeled probe was used for the competition assays. The EMSA was performed using the LightShift Chemiluminescent EMSA kit (Thermo Scientific).

ChIP.

The 2-week-old WT and *OsBZR1-D* seedlings were collected and cross-linked in vivo for ChIP. After crosslinking, ChIP assays were performed as described using the EpiQuik plant ChIP kit (Epigentek). The anti-FLAG antibody (Sigma) was

Fig. 9. A proposed model to explain how brassinosteroid (BR) regulates plant susceptibility to rice black-streaked dwarf virus (RBSDV) infection in rice. Upon RBSDV infection, BR can inhibit jasmonic and salicylic acids-mediated plant defenses against virus infection. At the same time, the BR pathway prevents the reactive oxygen species (ROS) burst, which plays important roles in rice immunity. In this process, BR master regulator BZR1 can directly bind the promoters of *OsPrx* and inhibit the expression of *OsPrx*. The reduced activity of *OsPrx* leads to the suppression of ROS levels. Lines ending with arrows show activation. The lines ending with a perpendicular short line indicate suppression.

used for immunoprecipitation. The DNA was eluted from antibody-conjugated beads with elution buffer. The ChIP products were purified and used for qRT-PCR. The *OsUBQ5* gene (AK061988) was used to normalize qRT-PCR results.

RNA library construction and sequencing.

The methods of RNA library construction were described previously (He et al. 2017). For RNA sequencing, the RNA quantity and purity were determined by Z. Lianchuan (Hangzhou, China). The addition of adapters, size selection, and RNA-seq were done by Z. Lianchuan. The Illumina HiSeq 2000 platform was used for RNA sequencing. The rice genome (The MSU Rice Genome Annotation Project database version 7.0) was used for mapping of sequencing reads by Bowtie software. Blast2go software was used for the GO functional classes and KEGG pathways. A difference in gene expression was considered significant when the absolute value of $\log_2$ (fold change) ratio was ≥ 1 and $P \leq 0.05$.

Statistical analysis.

Differences were analyzed using one-way or two-way analysis of variance with Fisher's least significant difference tests. A P value ≤ 0.05 was considered statistically significant. All analyses were performed using ORIGIN 8.0 software.

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