New insights into \textit{bsr-d1}-mediated broad-spectrum resistance to rice blast

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Abstract

\textit{bsr-d1}, an allele encoding a transcription factor identified from the rice cultivar Digu, confers durable, broad-spectrum resistance to infections by strains of \textit{Magnaporthe oryzae}. \textit{bsr-d1} was predicted to inhibit \textit{M. oryzae}-induced expression of \textit{Bsr-d1} RNA and degradation of hydrogen peroxide to achieve resistance to \textit{M. oryzae}. However, the global effect of biological process and molecular function on blast resistance mediated by \textit{Bsr-d1} remains unknown. In this study, we compared transcriptomic profiling between \textit{Bsr-d1} knockout (\textit{Bsr-d1KO}) lines and the wild type, TP309. Our study revealed that \textit{bsr-d1} mainly regulates the redox state of plant cells, but also affects amino acid and unsaturated fatty acid metabolism. We further found that BSR-D1 indirectly regulates salicylic acid biosynthesis, metabolism, and signal transduction downstream of the activation of H$_2$O$_2$ signalling in the \textit{bsr-d1}-mediated immune response. Furthermore, we identified a novel peroxidase-encoding gene, \textit{Perox3}, as a new \textit{BSR-D1} target gene that reduces resistance to \textit{M. oryzae} when overexpressed in TP309. These results provide new insights into the \textit{bsr-d1}-mediated blast resistance.

Keywords

hydrogen peroxide (H$_2$O$_2$), \textit{Magnaporthe oryzae}, peroxidase, resistance, rice blast, salicylic acid, transcriptome analysis
1 | INTRODUCTION

Plants employ two main layers of immune responses, pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI), to defend against pathogens. PTI is mediated by pattern recognition receptors (PRRs) localized on cell membranes whereas ETI is triggered by nucleotide-binding oligomerization domain-like receptors (NLRs) localized in cytoplasm (Jones and Dangl, 2006). These immune responses trigger many molecular events, including activation of mitogen-activated protein kinases (MAPKs), induction of defence gene expression, production of defence signal molecules, reactive oxygen species (ROS) (Kaku et al., 2006), and phytohormones (De Vleeschauwer et al., 2013), and production of antimicrobial chemicals like phytoalexins and phytoanticipins (Dixon, 2001). To date, many important defence-related genes have been extensively studied in plants, such as Arabidopsis RPM1 (Xiao et al., 2001), rice Xa21 (Song et al., 1995) and bsr-d1 (Li et al., 2017), wheat Yr36 (Fu et al., 2009), and barley mlo (Büschges et al., 1997). MicroRNAs also play important roles in plant defence, such as Arabidopsis miR393 (Navarro et al., 2006), and tomato miR482 and miR2118 (Shivaprasad et al., 2012).

In response to the rice blast fungus, members of 10 miRNA families positively or negatively regulate rice defence against Magnaporthe oryzae, such as miR160, miR164, miR166, miR167, miR169, miR319, miR396, miR398, miR444, and miR7695 (Li et al., 2019b). Analyses of their signalling mechanisms and pathways have provided great insights into plant defence responses.

Rice blast, caused by M. oryzae, is a devastating disease and has been under extensive study. As a result, the rice–M. oryzae pathosystem has become a successful premier model for studying the molecular basis of plant–fungal interactions (Li et al., 2019a). Currently, some atypical resistance (R) genes, such as Pi21 (Fukuoka et al., 2009) and Ptr (Zhao et al., 2018), have attracted much attention. Pi21 encodes a proline-rich protein containing a metal-binding domain and a loss-of-function allele (pi21) confers nonrace-specific, durable resistance (Fukuoka et al., 2012; Li et al., 2016), and varieties that carry race-specific R genes such as Pi-k (Li et al., 2006), Pi33 (Vergne et al., 2007), Pi1, and Pi9 (Wei et al., 2013). These studies have identified several biological processes, including “response to oxidative stress”, “carbohydrate metabolic process”, “response to biotic stimulus (fungus)”, and “extracellular region” (Bagnaresi et al., 2012; Wang et al., 2014; Li et al., 2016), that are involved in immune response against blast.

Hydrogen peroxide (H$_2$O$_2$) and salicylic acid (SA) are important signalling molecules in plant defence systems (De Vleeschauwer et al., 2013; Cerny et al., 2018). H$_2$O$_2$, being relatively stable, is the predominant ROS involved in mediating the response to biotic stress conditions (Mhamdi and Van Breusegem, 2018) and has received much research attention (Cerny et al., 2018). SA is one of the key phytohormones involved in biotic stress adaptation (De Vleeschauwer et al., 2013). SA and H$_2$O$_2$ signalling may interplay. For example, ectopic expression of the SA-hydroxylase transgene in tobacco impairs the hypersensitive response (HR) to pathogens mediated by H$_2$O$_2$ (Mur et al., 1997); H$_2$O$_2$ production in cell organelles induces SA biosynthesis, and leads to protective mechanisms such as stomatal closure and cell death (Saxena et al., 2016). Elevated H$_2$O$_2$ levels induce SA accumulation, while salicylate increases H$_2$O$_2$ levels in plants (Chamonggol et al., 1998). However, which of SA and H$_2$O$_2$ is the primary contributing factor to enhanced resistance to pathogens sometimes remains a contentious issue.

Currently, more than 70 defence-related genes that contribute to or regulate blast resistance have been identified; their encoded proteins are dispersed in the whole cell and their signalling pathways are intertwined (Li et al., 2019a). Among these genes, Pi9 (Liu et al., 2002), Pigm (Deng et al., 2017), pi21 (Fukuoka et al., 2009), and bsr-d1 (Li et al., 2017) are of the greatest interest because they confer broad-spectrum blast resistance and bring little or no significant yield penalty. bsr-d1, a natural, recessive allele from the rice variety Digu, encodes a C2H2-type transcription factor that directly regulates the expression levels of two peroxidase genes to modulate the rice immune response to M. oryzae.

Here, we assessed the global effects of BSR-D1 by comparing the transcriptomic profiles of Bsr-d1 knockout (Bsr-d1KO) and the wild-type TP309, and found that bsr-d1 regulates the redox state of cells, amino acid metabolism, and unsaturated fatty acid metabolic processes. Meanwhile, we found that H$_2$O$_2$ signalling occurs prior to SA signalling in the blast disease resistance mediated by bsr-d1. In addition to the two previously identified peroxidase genes, we further identified a new BSR-D1 target gene, Perox3 (LOC_Os01g73170), which confers enhanced susceptibility to M. oryzae when overexpressed in TP309.
RESULTS

2.1 The global effects of BSR-D1 in the redox state of rice, and amino acid and unsaturated fatty acid metabolic processes

To assess the global effects of BSD-D1, we first compared the gene expression profiles of Bsr-d1KO lines, which mimic bsr-d1 action conferring enhanced resistance, and the wild type, TP309, and identified a total of 164 differentially expressed genes (DEGs) (Figure 1a). Fifty DEGs were up-regulated, whereas 114 DEGs were down-regulated in Bsr-d1KO, indicating a change in expression of a relatively limited number of genes on knockout of Bsr-d1.

To identify the molecular pathways that are specifically involved in Bsr-d1KO lines, we performed gene ontology (GO) analysis on all DEGs. We identified 22 enriched GO terms in Bsr-d1KO plants (Figure 1b). Among them, only three GO terms, namely peroxidase activity (GO: 0,004,601), oxidoreductase activity acting on peroxide as acceptor (GO: 0,016,684), and response to oxidative stress (GO: 0,006,979), were highly significantly enriched (p < .01). Interestingly, these three GO terms are all associated with reduction-oxidation reaction regulating the redox state of cells containing the same seven enriched DEGs (LOC_Os07g48010, LOC_Os01g73170, LOC_Os07g48020, LOC_Os07g48050, LOC_Os04g59150, LOC_Os04g59200, LOC_Os01g22249). These results suggest that Bsr-d1 closely regulates the redox state of the cell.

To better understand the molecular pathways associated with the GO terms, we also analysed metabolic processes in Bsr-d1KO lines. We identified a total of 20 metabolic pathways that are affected by Bsr-d1 knockout (Figure 2). These pathways are mainly associated with amino acid (phenylalanine, cysteine, and methionine) and unsaturated fatty acid (α-linolenic acid and linoleic acid) metabolic processes (Figure 2). These results indicate that Bsr-d1 regulates amino acid and unsaturated fatty acid metabolism, which is associated with energy utilization and storage.
2.2 | The role of salicylic acid in bsr-d1 mediated blast resistance

Bsr-d1 regulates the cellular redox state, which often cross-talks with signalling of hormones such as SA, jasmonic acid (JA), and abscisic acid (ABA) when plants defend against pathogens (De Vleesschauwer et al., 2013; Cerny et al., 2018). Therefore, we analysed changes in hormone signalling pathways in Bsr-d1KO. We found that three DEGs in the "plant hormone signal transduction" pathway were affected by Bsr-d1 knockout. Two of the three DEGs are associated with SA signal transduction, whereas the third gene is associated with indoleacetic acid (IAA) signal transduction. Previous studies showed that M. oryzae inoculation activates the SA signal-transduction cascade (Shimono et al., 2007). These data suggest that SA signalling is probably involved in the bsr-d1-mediated immunity to M. oryzae. Therefore, we assessed the expression levels of those genes involved in SA biosynthesis and signal transduction (Figure 3a). We found that the SA biosynthesis gene OsICS was induced, whereas OsPAL remained unchanged in Bsr-d1KO plants; OsSSI2, whose product inhibits SA biosynthesis, remained unchanged. Meanwhile, OsSGT1, whose gene product catalyses the conversion of free SA into SA-O-β-glucoside (SAG), was induced in Bsr-d1KO plants. Three important genes in SA signal transduction, OsWRKY13, OsWRKY45, and OsNPR1, were all induced in Bsr-d1KO plants (Figure 5a). The results indicate that Bsr-d1 negatively regulates SA biosynthesis, metabolism, and signal transduction.

Both H₂O₂ and SA are involved in plant immune reactions (De Vleesschauwer et al., 2013; Li et al., 2017). However, there is still sometimes controversy concerning the hierarchy of H₂O₂ and SA in the signalling leading to disease resistance. To assess their relationship in blast disease resistance mediated by Bsr-d1 knockout, we asked whether BSR-D1 could bind to the promoters of the genes involved in SA biosynthesis, metabolism, or signal transduction, and activate or regulate these genes. We first determined whether BSR-D1 could bind to the promoter of each of the OsICS, OsSGT1, OsWRKY13, OsWRKY45, and OsNPR1 genes in the yeast one-hybrid assay. Our results show that the presence of BSR-D1 did not lead to activation of the HIS2 reporter when each promoter was fused to the HIS2 reporter gene (Figure 3b). This suggests that BSR-D1 in general does not directly bind to the promoters of these genes and thus indirectly regulates those genes involved in SA biosynthesis, metabolism, or signal transduction.

**FIGURE 2** Statistics of pathway enrichment comparing Bsr-d1 knockout (Bsr-d1KO) plants with the wild type TP309. Red font depicts up-regulated pathways, while blue font represents down-regulated pathways. Additionally, black font means both up- and down-regulated pathways.
2.3 | Identification of BSR-D1 binding target

Our transcriptomic profiling identified seven DEGs that can potentially regulate the cell redox state. Previously, two BSR-D1 target genes (LOC_Os05g04470 and LOC_Os10g39170) were identified that encode peroxidases (Li et al., 2017). However, we do not know whether these seven newly identified DEGs are target genes of BSR-D1 or not. In order to assess whether they are targets of the BSR-D1 protein, we tested binding of BSR-D1 to the promoter of each of the above seven genes in the yeast one-hybrid assay in which BSR-D1 was fused to GAL4 AD and each promoter fused to the HIS2 reporter gene. Our results showed that BSR-D1-GAL4 AD only bound to the promoter of LOC_Os01g73170 (hereby named Perox3) because specific activation of the HIS2 reporter only occurred to Perox3 in the presence of BSR-D1; several other candidates showed autonomous activation of the HIS2 reporter in the absence of BSR-D1 (Figure 4a). Meanwhile, the ChIP-seq results were also validated by carrying out real-time PCRs to quantify the presence of these promoters. These promoters were pulled down approximately 2-fold more frequently compared to the control, which had no antibodies added (Figure 4b). The results suggest that the BSR-D1 protein binds to the Perox3 promoter and activates Perox3 expression. This result suggests that BSR-D1 may indirectly regulate the other six DEGs.

To evaluate whether or not Bsr-d1 directly regulates Perox3 expression in planta, we assessed the RNA expression level of Perox3 in the Bsr-d1KO lines by reverse transcription quantitative PCR (RT-qPCR). The results showed that the Perox3 RNA level was reduced 2- to 4-fold compared to wild type (Figure 4c), suggesting that BSR-D1 directly binds to the Perox3 promoter and activates Perox3 expression in rice.

2.4 | Validation of the role of Perox3 in blast disease resistance

To assess the involvement of the Perox3 gene in blast disease resistance, we generated transgenic rice plants that had the Perox3 gene either overexpressed or knocked out. It is hard to accurately assay degree resistance in a resistant or hypersusceptible background. Therefore, we obtained three Perox3 overexpression (Perox3-ox) lines in the TP309 background, which is moderately susceptible (not resistant or hypersusceptible) to M. oryzae isolate ZB15, and confirmed their elevated Perox3 RNA levels (Figure S1a). Meanwhile, we used CRISPR/Cas9 technology to knock out the endogenous Perox3 gene (Perox3-KO) in TP309. We selected a 23-nt sequence in the Perox3 gene as the target site for Cas9 cleavage (Figure S1b), generated multiple putative transgenic lines, and verified its knockout by sequencing. We found two lines (named Perox3-KO#1 and #2) containing the mutation in the target site; Perox3-KO#1 and #2 each carry a one-base insertion in the target site (Figure S1b), truncating the Perox3 open reading frame.

Perox3-ox and Perox3-KO lines were challenged with the ZB15 blast isolate by punch-inoculation using detached leaves. These three Perox3-ox lines developed blast lesions approximately 30%-90% longer than TP309 (Figure 5a,b), indicating higher susceptibility. To confirm these lesion length results, we measured the amount
Figure 5  Role of Perox3 on blast resistance. (a) Punch inoculation of Perox3 overexpression (Perox3-ox) plants. Two leaves each of Perox3-ox #1, #2, and #3, Perox3-KO #1 and #2, and the wild type TP309 are shown. Detached leaves of 3-week-old plants were punch-inoculated. (b) Quantification of lesion length of each sample in (a). (c) Determination of blast fungal biomass. Fungal growth was determined on inoculated leaves at 6 days post-inoculation. Fungal biomass, measured as MoPot2 by quantitative PCR, in the inoculated leaves was normalized to OsUbq DNA. The blast isolate ZB15 was used for inoculations. Error bars represent SD from three replications. Asterisks represent significant differences (*p < .01)

3  |  DISCUSSION

We have previously reported the identification of the bsd1 allele that confers broad-spectrum blast resistance and the discovery of its underlying mechanism (Li et al., 2017). Here, we have further conducted a transcriptomic analysis to assess the global effect of knocking out the Bsr-d1 gene, which mimics the effect of bsd1, and found that redox regulatory genes, including a novel peroxidase gene Perox3, are the primary targets of BSR-D1. We empirically confirmed the binding of BSR-D1 to the Perox3 promoter. We also determined that genes involved in SA biosynthesis, metabolism, and signalling are indirectly regulated by BSR-D1, downstream of the regulation of H2O2 levels and H2O2 signal transduction. Thus, our findings significantly advance our understanding of the bsd1-mediated broad-spectrum resistance to M. oryzae and have several important implications.

3.1  Degradation of H2O2 is the primary target for BSR-D1

H2O2 is a relatively stable nonfree-radical ROS that is involved in programmed cell death (PCD) of infected and surrounding cells under pathogen attack (Birch et al., 2018). We previously identified the Bsr-d1 gene that directly up-regulates peroxidase gene expression to suppress the accumulation of H2O2, impairing blast disease resistance (Li et al., 2017). Our results of transcriptomic profiling presented here further support our previous model because all three significant GO terms enriched in Bsr-d1KO lines are associated with redox state regulation. Among the three GO terms, response to oxidative stress (GO: 0,006,979) is often specifically enriched in resistant rice varieties (Bagnaresi et al., 2012; Wang et al., 2014; Li et al., 2016), which is consistent with our model.

H2O2 is a major redox metabolite and at high concentrations induces oxidative damage to biomolecules (Cerny et al., 2018). To avoid oxidative damage to cellular structures, plants mainly use enzymatic antioxidants, such as superoxide dismutases (SODs), catalases (CATs), and peroxidases (POXs), to scavenge H2O2 (Xie et al., 2019). Our previous results showed that two peroxidase genes induced by BSR-D1 are employed by M. oryzae to suppress blast disease resistance (Li et al., 2017). In this study, we identified a third peroxidase gene, Perox3, which also negatively regulates blast disease resistance (Figure 5). The multiple peroxidase genes involved in suppressing H2O2 accumulation may be a host cellular mechanism to safeguard the cell from oxidative damage. However, this mechanism was hijacked by M. oryzae through activation of the Bsr-d1 gene and used to counter the ROS burst induced during the rice immune response on M. oryzae infection (Li et al., 2017). To counter this M. oryzae strategy, the rice host Digu has developed a bsd1 allele that can no longer be activated by M. oryzae blocking the activation of bsd1 and its target peroxidases, leading to the accumulation of H2O2 needed for resistance to M. oryzae (Li et al., 2017).

3.2  The role of unsaturated fatty acids in the interface between rice and M. oryzae

Multiple molecular events occur in the rice–M. oryzae interaction (Bagnaresi et al., 2012; Wei et al., 2013; Wang et al., 2014; Li et al., 2016). Here, we identified three highly significantly enriched GO
terms associated with reduction–oxidation reactions regulating the redox state of cells in Bsr-d1KO, namely peroxidase activity (GO: 0,004,601), oxidoreductase activity acting on peroxide as acceptor (GO: 0,016,684), and response to oxidative stress (GO: 0,006,979). In particular, response to oxidative stress (GO: 0,006,979) is specifically involved in blast resistance, as reported previously (Bagnaresi et al., 2012; Wang et al., 2014; Li et al., 2016), which further supports the importance of ROS in plant immunity (Mittler et al., 2004). Our results show that amino acid (phenylalanine, cysteine, and methionine) metabolic processes regulated by Bsr-d1 might provide needed carbon and nitrogen sources for rice or M. oryzae are similar to the previously reported transcriptomic profiling results using IRBL18 (Wei et al., 2013). Additionally, Bsr-d1 affects the unsaturated fatty acid (α-linolenic acid and linoleic acid) metabolic processes. Previous studies have reported that there is a relationship between ROS and unsaturated fatty acids. For example, ROS, such as •O2-, directly oxidize unsaturated fatty acids (Wagner et al., 2004). A single •OH can result in peroxidation of many polyunsaturated fatty acids and peroxidation of unsaturated fatty acids may produce malondialdehyde (MDA), which is responsible for cell membrane damage (Sharma et al., 2012). This report indicates that unsaturated fatty acids are involved in the response of defending against blast fungus. Thus, our transcriptomic results are consistent with previous reports supporting the notion that unsaturated fatty acids may be another battlefront in the interaction between rice and M. oryzae.

### 3.3 Signalling pathways in the bsr-d1-mediated defence response

Bsr-d1 regulates some of the genes associated with SA biosynthesis, metabolism, and signal transduction (Figure 3a). However, a previous report suggested that the levels of endogenous SA do not change significantly on pathogen attack in rice (Silverman et al., 1995), though exogenously applied SA can induce resistance to M. oryzae (Iwai et al., 2007). This indicates that bsr-d1 may regulate the SA signal transduction to achieve blast disease resistance.

H2O2 and SA are important signalling molecules in the plant immune response (De Vleesschauwer et al., 2013; Cerny et al., 2018). The two signal molecules can interplay (Saxena et al., 2016). For example, SA can increase H2O2 levels in plant tissues (Rao et al., 1997), while SA accumulation can also be induced by elevated H2O2 levels (Chamnongpol et al., 1998; Mhamdi et al., 2010). Arabidopsis GLUTATHIONE REDUCTASE1 plays an important role in increasing intracellular H2O2 production and SA accumulation in the Col-0 background under long-day conditions (Mhamdi et al., 2010). Similarly, Bsr-d1 appears to regulate both the genes that modulate H2O2 concentration and those that are involved in SA biosynthesis, metabolism, and signal transduction (Figures 3a and 4). H2O2 can be placed upstream or downstream of SA in their signalling cascades when plants respond to different environment stresses (Chen and Kessig, 1991; Cerny et al., 2018). In Bsr-d1-mediated signalling, BSR-D1 can directly bind to the promoter of peroxidase genes, but not to the promoters of the genes associated with SA biosynthesis, metabolism, and signal transduction, clearly suggesting that H2O2 signalling occurs prior to SA signalling in the blast disease resistance mediated by bsr-d1.

### 4 EXPERIMENTAL PROCEDURES

#### 4.1 Plant materials and blast infection procedures

Rice TP309 and transgenic lines including Bsr-d1KO, Perox3-ox, and Perox3-KO were grown in two tubs in a growth chamber at 28 °C in a 12-hr light/12-hr dark photoperiod with 75% humidity. Three-week-old rice plants were used for inoculation with M. oryzae isolate ZB15. M. oryzae spores were grown on complete agar medium for 2 weeks before producing spores. Spores were collected via flooding of the fungal agar cultures with sterile water, and the spore concentration in the suspension was adjusted to 5 × 10^6 conidia/ml before punch inoculation. Punch inoculation of detached rice leaves is modified based on Jia et al. (2003) with the following modification. First, 4 μl of spore suspension was placed at each of two spots on each leaf using a micropipette. Inoculated detached leaves were placed in 0.1% 6-benzylaminopurine (6-BA) in sterile water to keep moist. The lesion lengths of disease reactions were measured using a ruler 5 days post-inoculation. The relative fungal DNA amount was calculated using the threshold cycle value (Ct) of M. oryzae Pot2 DNA against the Ct of rice genomic ubiquitin DNA (Park et al., 2012).

#### 4.2 Transcriptome analysis

We used leaf samples at the three-leaf stage for RNA-Seq in this transcriptomic study. RNA quantification and qualification, library preparation for strand transcriptome sequencing, clustering and sequencing, and part data analysis were performed at Novogene Bioinformatics Technology Co., Ltd (Tianjin, China) following the manufacturer’s instructions. Differential expression analyses of two rice varieties were performed using the DESeq R package v. 1.18.0. DESeq provided statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting p values were adjusted using the Benjamini and Hochberg approach for controlling the false discovery rate. Genes with an adjusted p value <.05 found by DESeq were assigned as differentially expressed. GO enrichment analysis of DEGs was implemented by the GOSeq R package, in which gene length bias was corrected. GO terms with corrected p value <.05 were considered significantly enriched DEGs. The KOBAS software was used to test the statistical enrichment of DEGs in KEGG pathways.

#### 4.3 RNA isolation and RT-qPCR

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) following the manufacturer’s protocols. cDNA was
synthesized using an RNA reverse transcription kit (Invitrogen Life Technologies). RT-qPCR was conducted using a Bio-Rad CFX96 Real-Time System coupled to a C1000 thermal cycler (Bio-Rad). The reference gene Ubiquitin 5 (Ubq5) was used for the normalization of all RT-qPCR data (Li et al., 2017). The sequences of the primers are listed in Table S1. The 2^{-ΔΔCt} method was used to calculate the relative expression levels with three technical repeats (Livak and Schmittgen, 2001).

4.4 | One-hybrid assays in yeast

The full-length cDNA sequence of transcription factor gene Bsr-d1 was amplified and fused in frame with the GAL4 activation domain in vector pGADT7-Rec2 (Clontech) forming construct pGADT7- Bsr-d1. Then, the fusion construct was cotransformed with the reporter construct (pHIS2-fused to each promoter of LOC_Os04g59150, LOC_Os04g59200, LOC_Os01g73170, LOC_Os07g48100, LOC_Os01g22249, LOC_Os07g48050, LOC_Os07g48020, OsICS, OsSGT1, OsWRKY13, OsWRKY45, and OsNPR1) into Y187 yeast cells (Clontech). The sequences of the primers are listed in Table S1. The locations of each promoter for Y1H are shown in Table S2. The empty vector pGADT7-Rec2 and the pHIS2- promoter were cotransformed as the negative control for mating experiments. DNA–protein interactions were determined by the growth of the transformants on the nutrient-deficient medium with 0 and 15 mM 3-amino-1,2,4-triazole (Clontech). The sequences of the primers are listed in Table S1. The expression levels were determined by the growth of the transformants on the nutrient-deficient medium with 0 and 15 mM 3-amino-1,2,4-triazole (Clontech). The full-length cDNA sequence of transcription factor gene OsWRKY13 was cloned into pCAMBIA2300 to generate the overexpression construct, pCAMBIA2300-OsWRKY13. The pCAMBIA2300-OsWRKY13 construct was introduced into TP309 through Agrobacterium-mediated transformation as described previously (Li et al., 2017). The regenerated transgenic plants carrying Perox3-ox were selected with G418. PCR-based genotyping was performed to verify the presence of the transgene as previously described (Li et al., 2016). Overexpression of Perox3 in the transgenic lines was confirmed by RT-qPCR.

For CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 construction, the 23 bp targeting sequence (including PAM) of Perox3 was confirmed using a BLAST search against the rice genome (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Hsu et al., 2013). The designed targeting sequence was synthesized and annealed to form the oligo adaptors. Vector pBGK032 was digested by Bsal and purified using a DNA purification kit (Tiangen). A ligation reaction (10 µl) containing 10 ng of the digested pBGK032 vector and 0.05 mM oligo adaptor was carried out and directly transformed to Escherichia coli competent cells to produce CRISPR/Cas9 plasmid. The CRISPR/Cas9 plasmids were introduced into Agrobacterium tumefaciens EHA105. Transformation of rice was performed as described above. Genomic DNA was extracted from these transformants and primer pairs flanking the desired target site were used for PCR amplification (Table S1). The PCR products (300–500 bp) were sequenced.

4.5 | Semi-in vivo chromatin immunoprecipitation (ChIP) and ChIP-qPCR

Total DNA of TP309 and purified GST-BSR-D1 were used for ChIP assays. The protocol has been published before (Li et al., 2017). Three-week-old seedlings were used for total DNA extraction. The total DNA was sheared into 100–500 bp fragments using an ultrasonic crusher. The glutathione-S-transferase (GST) fusion protein was affinity-purified on glutathione-agarose beads (BD Biosciences). DNA fragments enriched by GST-BSR-D1 were obtained using the procedure of semi-in vivo ChIP. The prepared DNA in ChIP was applied for qPCR using respective primer pairs (the amplified fragment with predicted conserved motif from −164 bp to −43 bp) of the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Zhu Z, Yin J, Chern M, et al. New insights into bsr-d1-mediated broad-spectrum resistance to rice blast. *Molecular Plant Pathology*. 2020;21:951–960. https://doi.org/10.1111/mpp.12941