RAVL1 Activates Brassinosteroids and Ethylene Signaling to Modulate Response to Sheath Blight Disease in Rice

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ABSTRACT

Rhizoctonia solani causes sheath blight disease in rice; however, the defense mechanism of rice plants against *R. solani* remains elusive. To analyze the roles of brassinosteroid (BR) and ethylene signaling on rice defense to *R. solani*, wild-type (WT) rice and several mutants and overexpressing (OX) lines were inoculated with *R. solani*. Mutants *d61-1* and *d2* were less susceptible to sheath blight disease, *bri1-D* was more susceptible, and *rav11* and *d61-1/EIL1 Ri5* were similarly susceptible compared with WT. The double mutant *rav11/d61-1* was phenotypically similar to the *rav11* mutant. Transcriptome analysis, chromatin immunoprecipitation assay, electrophoretic mobility shift assay, and transient

assays indicated that RAVL1 might directly activate *Ethylene insensitive 3-like 1 (EIL1)*, a master regulator of ethylene signaling. Mutants *ers1* and *d61-1/RAVL1 OX* were resistant to sheath blight disease, whereas *EIL1* RNAi mutants and *RAVL1 OX* were more susceptible than WT. *BRI1* and *D2* expression in *EIL1 Ri5/RAVL1 OX* and *EIL1* expression in *d61-1/RAVL1 OX* indicated that RAVL1 activates *BRI1/D2* and *EIL1*, respectively, independent of BR and ethylene signaling. Our analyses provide information on how BR and ethylene signaling regulate sheath blight disease and on the regulatory function of RAVL1 in rice sheath blight disease.

Sheath blight is one of the three major diseases in rice (*Oryza sativa* L.) and is caused by the fungal pathogen *Rhizoctonia solani* (Savary et al. 1995). Sheath blight threatens rice throughout its growth cycle, from seedling to heading, and causes lesions on leaves, sheaths, and panicles. At the late stage of infection, the whole plant withers and lodges (Savary et al. 1995). Sheath blight can reduce rice yield by 8 to 50%, depending on disease severity, crop stage at which the infection occurs, and environmental conditions (Savary et al. 2000). Currently, chemicals are extensively used as the main strategy for disease control (Savary et al. 2000), albeit increasing health risks, production cost, and environmental pollution. Thus, plant resistance based on host–pathogen interaction is considered a hot spot of research. Previous studies have shown that overexpression of chitinase, β -1,3-glucanase, and polygalacturonase inhibiting protein (OsPGIP1) enhances rice resistance against *R. solani* (Mao et al. 2014; Shah et al. 2009; Wang et al. 2015).

Brassinosteroids (BRs) are a major category of phytohormones. In *Arabidopsis thaliana*, their signaling starts with BRASSINOSTEROID INSENSITIVE 1 (BRI1) receptor, which leads to the dissociation of BRI1 kinase inhibitor 1 (BKI1) and association of BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1). The activation of BRI1-BAK1 ultimately results in the dephosphorylation and activation of BRASSINAZOLE-RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 (BES1), two transcription factors that regulate target gene expression in response to BRs (Kim and Wang 2010; Li and Chory 1997; Li et al. 2002; Nam and Li 2002;

Yang et al. 2011). The stability of BES1 and BZR1 is strongly enhanced in *bes1-D* and *bzr1-D* gain-of-function mutants, respectively, and rescues the BR-insensitive dwarf phenotype of the *bri1* BR-receptor mutant (Wang et al. 2002; Yin et al. 2002). In rice, a B3 DNA binding domain protein related to ABI3/VP1-Like 1 (RAVL1) has been identified as an upstream regulator in BR homeostasis, as it binds to the E-box in the promoter sequences of BR-receptor and BR biosynthetic genes (Je et al. 2010). A previous study showed that a mutation at the BR receptor *BRI1* enhances disease resistance in *Brachypodium distachyon* and barley (*Hordeum vulgare*) against necrotrophic and hemibiotrophic pathogens showing short asymptomatic phase (Goddard et al. 2014). Additionally, BR application causes 86% reduction in the severity of Fusarium head blight (FHB) in wheat due to *Fusarium culmorum*, and 33% reduction in FHB-associated yield losses in barley (Ali et al. 2013), suggesting that the BR signaling mechanism regulating the response to different pathogens is complex.

Ethylene is a gaseous molecule that plays numerous roles in plant growth, development, and response to environmental stresses (Morgan and Drew 1997; van Loon et al. 2006). In *A. thaliana*, five endoplasmic reticulum (ER)-localized ethylene receptors, namely ETHYLENE RESPONSE 1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR 1 (ERS1), ERS2, and ETHYLENE INSENSITIVE 4 (EIN4), have been identified as negative regulator of ethylene signaling (Chang et al. 1993; Hua et al. 1995; Sakai et al. 1998). CONSTITUTIVE TRIPLE RESPONSE (CTR) 1 (Raf-like CTR1) is also a key negative regulator of ethylene signaling, which functions downstream of the receptors and upstream of EIN2 (Kieber et al. 1993). In the absence of ethylene, CTR1 directly phosphorylates the C-terminal domain of EIN2 to prevent EIN2-signal transferring from the ER to the nucleus (Ju et al. 2012). Furthermore, the signal is transduced from the ER to the nucleus via the EIN2-EIN3/ETHYLENE INSENSITIVE 3-LIKE 1 (EIL1) pathway (Ju et al. 2012; Qiao et al. 2012; Wen et al. 2012), in which EIL1 activates downstream signals, including ethylene response factors (ERFs) (Yang et al. 2015a). Exogenous application of ethylene induces the expression of pathogenesis-related (PR) genes, such as *PR1*, *PR5*, and *PR10*, in rice (Agrawal et al. 2001). Overexpression of an ERF in the roots of *Medicago truncatula* (*MtERF1-1*) enhanced its resistance to *R. solani* anastomosis group (AG)-8 (Anderson et al. 2010), whereas

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overexpression of a gene encoding an ethylene synthesis enzyme in rice (*OsACS2*) enhanced its resistance to multiple diseases, including sheath blight and blast (Helliwell et al. 2013). However, more research is needed to elucidate the role of BR and ethylene signaling in the regulation of sheath blight disease in rice.

Here, we studied BR action against sheath blight disease using *br11*, *d2*, and *rav11* mutants. The latter was the only susceptible mutant, and thus was further analyzed using transcriptomic, biochemical, genetic, and molecular methods. Results showed that *RAVL1* directly activates *EIL1*, an ethylene signaling gene that positively regulates the defense mechanism against sheath blight disease. Taken together, our results provide information on the role of BR and ethylene signaling in the regulation of sheath blight disease as well as the linkage between BR and ethylene signaling in rice.

MATERIALS AND METHODS

Plant growth and inoculation. Wild-type (WT) rice (*Oryza sativa* Japonica, cultivars Dongjin and Nipponbare) and *rav11*, *d61-1*, *d2*, *br11-D*, *ers1*, and *EIL1* RNAi mutants, as well as *RAVL1*, *d61-1/RAVL1*, and *EIL1* *Ri5/RAVL1* overexpressing (OX) lines (Je et al. 2010; Xuan et al. 2017; Yang et al. 2015b) were used. All plants were grown under greenhouse conditions (temperature, 23 to 30°C; relative humidity 80%; and 12 h light: 12 h dark) at Shenyang Agricultural University, China, and propagated by selfing. One-month-old plants were used for inoculation with *R. solani* AG-1 (Prasad and Eizenga 2008). In brief, a 10-cm-long piece was cut from the second youngest leaf of the main tiller and placed on moistened filter paper in a Petri dish (diameter, 36 cm; height, 2.5 cm). Each replicate comprised six leaves, and four replicates per line were used, in a completely randomized design. Colonized potato dextrose agar (PDA) blocks (diameter, 7 mm) were excised using a circular cutter and placed on the abaxial surface of each leaf piece (Supplementary Fig. S1). Leaves were incubated in a chamber with continuous light at 25°C for 72 h. The filter paper was kept moist with sterile water. After 72 h, the length and width of the lesions within each leaf piece were measured using Image J Fiji software and the approximate percentage of leaf covered with lesions was calculated (Eizenga et al. 2002; Prasad and Eizenga 2008; Savary and Mew 1996).

To analyze *R. solani* AG-1 infection-mediated expression of *BRI1* and *D2*, 1-month-old WT and *rav11* plants were inoculated with *R. solani* AG-1, and their leaves were sampled after 0, 24, 48, and 72 h. Relative fungal biomass was calculated using DNA-based quantitative PCR (qPCR). DNA was extracted from 10-cm rice leaf pieces presenting lesions (one piece per leaf; six leaves from each line at each sampling time) using the cetyltrimethyl ammonium bromide protocol. Relative fungal biomass was then measured based on cycle threshold (CT) values of *R. solani* *Phosphoglucate dehydrogenase* (*RsPDG*) and rice genomic *ubiquitin* (*OsUBI*) genes, according to $2^{[CT(OsUBI)-CT(RsPDG)]}$. The primers used for qPCR are listed in Supplementary Table S1.

Generation of crossing mutants and identification of homozygotes in the offspring. The F8 generation of the cross mutants *rav11/d61-1* and *d61-1/RAVL1* OX were obtained as previously described (Je et al. 2010). The F3 generation of *EIL1* *Ri5/d61-1* and *EIL1* *Ri5/RAVL1* OX plants were used, and offspring homozygosity for each genetic combination was isolated by genotyping 30 individual plants from each line, using gene specific primers (*EIL1* R or *RAVL1* R) and the vector primer 35S F. If all 30 plants carried the plasmid, the lines were considered homozygous for *EIL1* *Ri5* and *RAVL1* OX. Because *d61-1* is a point mutation mutant of *BRI1*, its homozygotes were isolated via the PCR and genotyping methods previously published (Je et al. 2010).

RNA extraction and quantitative real-time (qRT)-PCR analysis. Total RNA was isolated from 1-month-old seedlings with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and TRIzol reagent (Takara, Dalian, China), and then treated with RQ-RNase

free DNase (Promega, Madison, WI) to remove genomic DNA contamination. Complementary DNA was then generated using the GoScript Reverse Transcription kit (Promega), according to the manufacturer's instructions. The qRT-PCR was performed using the BIO-RAD CFX96 Real-time PCR system (Bio-Rad, Hercules, CA) with SYBR-Green (Takara). The values were normalized against *Ubiquitin* that was used as a housekeeping gene.

RNA deep sequencing and gene ontology (GO) analysis. Total RNA was extracted from 1-month-old WT and *rav11-1* plants as described above, and used for RNA sequencing at LC Biotech Co. (Hangzhou, China). Three biological replicates from each line were analyzed; thus, data presented here correspond to mean values, followed by the standard error of the mean, of three independent RNA sequencing experiments. Differentially expressed genes (DEGs) associated with biological process GO terms (Ye et al. 2006) were determined as follows: Fisher's exact test was used

Fig. 1. Evaluation of *d2*, *d61-1*, and *br11-D* mutants in response to sheath blight disease compared with wild-type (WT) rice (*Oryza sativa* L.). **A**, Response of *d2*, *d61-1*, and *br11-D* to *Rhizoctonia solani* anastomosis group (AG)-1 compared with WT. **B**, Percentage of leaf area covered with lesions in *d2*, *d61-1*, and *br11-D* compared with WT. Data represent means $\pm$ standard error ($n > 10$). **C**, Relative fungal biomass obtained by quantitative PCR [$2^{[CT(OsUBI)-CT(RsPDG)]}$] using genomic DNA. The data are means $\pm$ standard error ($n = 10$). Different letters indicate significant differences at $P < 0.05$.

to classify the enrichment of the GO category, and the false discovery rate (FDR) was calculated to correct the P value; the smaller the FDR, the smaller the error in judging P (Dupuy et al. 2007). Enrichment of GO terms among probe sets was found using one-tailed Fisher's exact test based on 2×2 contingency tables, as it provides a measure of the significance of the enrichment as it increases; the corresponding function is more specific, revealing GOs with a concrete function description in the experiment (Dunnick et al. 2012).

Chromatin immunoprecipitation (ChIP) assay. Rice callus samples (8 g) collected from transgenic plants expressing 35S:RAVL1-green fluorescence protein (GFP) and 35S:GFP were used

for ChIP assay. Preabsorption with a preimmune serum was performed before immunoprecipitation with an anti-GFP monoclonal antibody (Clontech, Mountain View, CA). Immunoprecipitates were analyzed by qRT-PCR. Each input DNA level was used as a control to normalize the contents of immune-precipitated DNA in the qRT-PCR assay (Je et al. 2010).

Electrophoretic mobility shift assay (EMSA). To produce RAVL1 recombinant proteins, the open reading frame sequence of RAVL1 was subcloned into the pET28a expression vector, and the resulting pET28a-RAVL1 plasmid was used for the transformation of *Escherichia coli* strain BL21 DE3. Recombinant proteins were harvested after a 3-h treatment with 0.4 mM isopropyl

Fig. 2. Evaluation of *ravl1* mutants and RAVL1 overexpression (OX) plants in response to sheath blight disease compared with wild-type (WT) rice (*Oryza sativa* L.). **A**, Response of *ravl1-1*, *ravl1-2*, RAVL1 OX, and RAVL1-GFP OX to *Rhizoctonia solani* anastomosis group (AG)-1 compared with WT. **B**, Percentage of leaf area covered with lesions in *ravl1-1*, *ravl1-2*, RAVL1 OX, and RAVL1-GFP OX compared with WT. Data represent means $\pm$ standard error ($n > 10$). **C**, Relative fungal biomass obtained by quantitative PCR [$2^{\text{CT}(\text{OsUBI})-\text{CT}(\text{RsPGD})}$] using genomic DNA. The data are means $\pm$ standard error ($n = 10$). Different letters indicate significant differences at $P < 0.05$.

Fig. 3. Evaluation of *ravl1/d61-1* double mutant in response to sheath blight disease compared with wild-type (WT) rice (*Oryza sativa* L.) and parental lines. **A**, Response of *ravl1/d61-1* (F8) to *Rhizoctonia solani* anastomosis group (AG)-1 compared with WT and parental lines. **B**, Percentage of leaf area covered with lesions in *ravl1/d61-1* compared with WT and parental lines. Data represent means $\pm$ standard error ($n > 10$). **C**, Relative fungal biomass obtained by quantitative PCR [$2^{\text{CT}(\text{OsUBI})-\text{CT}(\text{RsPGD})}$] using genomic DNA. The data are means $\pm$ standard error ($n = 10$). Different letters indicate significant differences at $P < 0.05$.

β -D-1-thiogalactopyranoside at 27°C. For EMSA, a standard binding reaction was performed in a total volume of 20 μ l by incubating 1 μ g of purified protein, 40,000 cpm 32P-labeled DNA probe, and 1 μ g of poly dI-dC, which blocks any nonspecific binding between protein and probe DNA, in a reaction buffer (25 mM HEPES-KOH, pH 7.5; 100 mM KCl; 0.1 mM ethylenediaminetetraacetic acid [EDTA], 10% [vol/vol] glycerol, and 1 mM dithiothreitol) at 25°C for 30 min. The products were resolved in 8% polyacrylamide gel electrophoresis in 0.5 $\times$ Tris/Borate/EDTA buffer (Je et al. 2010). For probe labeling, 20-nucleotide DNA fragments were synthesized and end-labeled with [γ -32P]-labeled ATP using T4 polynucleotide kinase (NEB, Ipswich, MA). For the competition assay, the recombinant RAVL1 protein was incubated with 50-fold excess unlabeled P2 and mP2 probes for 30 min at 25°C, before adding labeled probes.

Transient expression assay. Constructs, harboring an effector (35S:RAVL1), reporter (*pEIL1*), mutated promoter (*mpEIL1*), and internal control (35S:LUC), were used for the cotransformation of *A. thaliana* protoplasts (Yamaguchi et al. 2010). To generate *p35S:RAVL1*, the GAL4BD region of the p35S:GAL4BD vector was replaced by *RAVL1* cDNA (Xuan et al. 2017). To obtain the reporter

vectors, a 1.5-kb *EIL1* promoter sequence was constructed. In the mutated promoter, E-box element sequences (CANNTG) were substituted by the TTTT sequence. Native and mutated promoters of *EIL1* were cloned into the p35S:TATA:GUS vector by replacing the 35S:TATA region with *EIL1* promoters. Plasmids 35S:RAVL1 and *pEIL1*-GUS or 35S:RAVL1 and *mpEIL1*-GUS (2 μ g) were transformed into *A. thaliana* protoplasts via polyethylene glycol (PEG)-mediated transformation method. After 12 and 24 h of transformation, protoplast cells were harvested and proteins were extracted. β -Glucuronidase (GUS) activity was tested using the GUS assay kit (Promega), and its expression was normalized against luciferase (LUC) expression (Xuan et al. 2013). Luciferase was assayed using the Luciferase assay kit (Promega). Both PEG-mediated transformation and activity assays were performed as previously described (Yoo et al. 2007).

Statistical analyses. Statistical analyses were performed using Prism 5 (GraphPad, San Diego, CA). All data are expressed as means $\pm$ standard error (SE). One-way analysis of variance (ANOVA) was performed, followed by Bonferroni's multiple comparison tests. Differences among samples were considered significant at $P < 0.05$.

Fig. 4. Transcriptome profile and expression of ethylene-related genes in *ravl1* mutants and *RAVL1* overexpressing (OX) lines compared with wild-type (WT) rice (*Oryza sativa* L.). **A**, Heat map of differentially expressed genes (DEGs) in WT and *ravl1-1*. Green, red, and black are used to denote expression strength in the heat map. Only DEGs with $P < 0.05$ were selected for generating the heat map. **B**, Relative expression of *EIL1*, *ACO2*, *Os07g06300*, *Os07g06190*, *Os09g20350*, and *RAVL1* in *ravl1* mutants and *RAVL1* overexpression plants compared with WT. The mRNA levels of samples were normalized to that of *Ubiquitin* mRNA. Data represent means $\pm$ standard error ($n = 3$). The expressions of *EIL1*, *ACO2*, *Os07g06300*, *Os07g06190*, *Os09g20350*, and *RAVL1* in WT were defined as 1. Different letters indicate significant differences at $P < 0.05$.

RESULTS

BR signaling negatively regulates rice resistance to sheath blight disease. To investigate the BR function in rice resistance against *R. solani* AG-1, WT and *brl1* mutant *d61-1* (Yamamuro et al. 2000), BR biosynthesis deficient mutant *d2* (Hong et al. 2003), and *BR11* OX mutant *brl1-D* (Jeong et al. 2002) were inoculated with the fungus, and the percentage of leaf area covered with lesions was estimated. Results showed that the *d61-1* and *d2* were more resistant to *R. solani* AG-1 than WT plants, whereas *brl1-D* showed more severe symptoms than WT plants (Fig. 1A). The percentage of leaf area covered with lesions was 40% in WT, 15% in *d61-1*, 13% in *d2*, and 56% in *brl1-D* (Fig. 1B). Relative fungal biomass 72 h postinoculation was highest in *brl1-D*, while *d61-1* and *d2* showed lower fungal biomass than WT plants (Fig. 1C), indicating that BR signaling negatively regulates rice resistance to sheath blight disease.

Because *RAVL1* is a key transcription factor that directly activates the expression of *BR11*, a BR receptor gene, and BR synthetic genes (Je et al. 2010), *rav1l* mutants are insensitive to BR. Therefore, the two *rav1l* mutants (*rav1l-1* and *rav1l-2*) and *RAVL1* overexpression plants (*RAVL1* OX and *RAVL1-GFP* OX) (Je et al. 2010) were further utilized to examine response to sheath blight disease. The *rav1l* mutants showed responses to *R. solani* AG-1 similar to that of WT, whereas *RAVL1* OX lines were more

susceptible than WT (Fig. 2A). The percentage of leaf area covered with lesions was 40% in WT and *rav1l* mutants and 52% in *RAVL1* OX plants (Fig. 2B). In addition, 72 h postinoculation *RAVL1* OX plants showed higher fungal biomass than WT and *rav1l* mutants (Fig. 2C). The qRT-PCR performed to quantify the expression level of *BR11* (*Os01g0718300*) and *D2* (*Os01g0197100*) in the leaves of 1-month-old WT, *rav1l-1*, and *rav1l-2* plants at 0, 24, 48, and 72 h after *R. solani* AG-1 inoculation indicated that *BR11* and *D2* expressions were not altered by *R. solani* AG-1 infection, and were lower in *rav1l* mutants than in WT (Supplementary Fig. S2).

Mutation of *RAVL1* suppresses resistance to sheath blight disease in the *d61-1* mutant. Because *RAVL1* directly activates the expression of *BR11* and BR biosynthetic genes, including *D2* (Je et al. 2010), *d61-1* and *d2* mutants showed resistance to sheath blight disease. However, *rav1l* mutants showed a WT-like response to *R. solani* AG-1. Thus, we further examined the function of *RAVL1* and *BR11* by crossing *rav1l* (susceptible) and a *d61-1* (less susceptible than WT) mutants, and inoculating their F8 offspring with *R. solani* AG-1. The *rav1l/d61-1* plants were susceptible to *R. solani* AG-1, similar to that shown by *rav1l* (Fig. 3), indicating that *RAVL1* mutation suppressed *d61-1* resistance to *R. solani* AG-1.

Expression of ethylene-related genes differed between *rav1l* and WT plants. Although *RAVL1* is known as an activator of *BR11*, *rav1l* suppresses the phenotype of *d61-1*, suggesting that

Fig. 5. *RAVL1* directly binds to the *EIL1* promoter. **A**, Putative E-box elements within 1.5 kb of the *EIL1* promoter. P1, P2: probes used for chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift assay (EMSA). **B**, Relative ratios of immunoprecipitated DNA to input DNA in 35S:GFP and 35S:RAVL1:GFP. Ab: GFP antibody. **C**, Evaluation of *RAVL1* affinity to P2 and mutated P2 (mp2). Competition EMSAs were performed with 50-fold excess unlabeled probes (P2 and mp2). **D**, Transient expression assay by co-transfection with *p35S:RAVL1* and each of the vectors expressing beta-glucuronidase gene (*GUS*) under the control of P2 and mp2 *EIL1* promoters in *Arabidopsis thaliana* protoplast cells. The relative *GUS* activity with each combination was analyzed. Error bars are $\pm$ standard error of the mean ($n = 6$). *GUS* activity when expressing only *pEIL1* was defined as 1. Different letters indicate significant differences at $P < 0.05$.

RAVL1 may also regulate other genes involved in sheath blight disease resistance. The transcriptome of 1-month-old WT and *ravl1-1* mutant leaves was obtained to identify *RAVL1*-regulated genes. Genes in *ravl1-1* mutants showing significantly altered expression (transcript levels presenting more than twofold changes in relation to levels in WT) were either upregulated (123 genes) or downregulated (86 genes) compared with WT ($\log_2 \geq 1$; Fig. 4A). These DEGs were involved in diverse biological processes, including transcription, carbohydrate metabolic process, transport, defense response, and hormone-mediated signaling (Supplementary Fig. S3). Six DEGs were ethylene-related genes whereas no BS-related gene was identified (Supplementary Table S2). A

previous study showed that ethylene contributes to rice resistance against *R. solani* (Helliwell et al. 2013). Therefore, *RAVL1* might positively regulate rice resistance against sheath blight disease through the ethylene signaling pathway. Based on this hypothesis, a qRT-PCR was performed to verify the expression levels of *EIL1* (*Os03g20790*), *ACO2* (*Os09g27750*), and three putative ethylene resistance genes, *Os07g06300* (ethylene-insensitive protein 2), *Os07g06190* (ethylene-insensitive protein 2), and *Os09g20350* (ethylene-responsive transcription factor) in WT, *ravl1-1* and *ravl1-2*, and *RAVL1* OX and *RAVL1-GFP* OX. The levels of *EIL1*, *Os07g06190*, and *Os09g20350* were lower in *ravl1* mutants and higher in *RAVL1* OX plants than in WT. However, the levels of

Fig. 6. Evaluation of *ers1* and *EIL1 Ri5*, and *d61-1/EIL1 Ri5* double mutants in response to sheath blight disease compared with wild-type (WT) rice (*Oryza sativa* L.). **A**, Response of *ers1* and *EIL1 Ri5* to *Rhizoctonia solani* anastomosis group (AG)-1 compared with WT. **B**, Percentage of leaf area covered with lesions in *ers1* and *EIL1 Ri5* compared with WT. Data represent means $\pm$ standard error ($n > 10$). **C**, Relative fungal biomass obtained by quantitative PCR [$2^{[CT(OsUBI)-CT(RsPGD)]}$] using genomic DNA. The data are means $\pm$ standard error ($n = 10$). **D**, Response of *d61-1/EIL1 Ri5* (F3) to *R. solani* AG-1 compared with WT and parental lines. **E**, Percentage of leaf area covered with lesions in *d61-1/EIL1 Ri5* compared with WT and parental lines. Data represent means $\pm$ standard error ($n > 10$). **F**, Relative fungal biomass obtained by quantitative PCR [$2^{[CT(OsUBI)-CT(RsPGD)]}$] using genomic DNA. The data are means $\pm$ standard error ($n = 10$). Different letters indicate significant differences at $P < 0.05$.

ACO2 and *Os07g06300* were higher in *ravl1* than in WT and *RAVL1* OX (Fig. 4B), similar to that obtained by RNA sequencing. In addition, *RAVL1* (*HM450152*) was highly expressed in *RAVL1* OX compared with WT, but this transcript was not detected in *ravl1* (Fig. 4B).

RAVL1 transcriptionally activates *EIL1* directly via promoter binding. *EIL1*, *Os07g06190*, and *Os09g20350* expressions were lower in *ravl1* mutants and higher in *RAVL1* OX's than in WT plants (Fig. 4B). The promoter sequences of *EIL1*, *Os07g06190*, and *Os09g20350* were analyzed to identify the presence of possible binding sites for RAVL1. We identified two putative E-box elements located within 1.5 kb of the *EIL1* promoter (Fig. 5A), whereas no putative E-box elements were observed within 1.5 kb of

Os07g06190 and *Os09g20350* promoters (data not shown). The ChIP assay using *35S::GFP* and *35S::RAVL1::GFP* transgenic plant calli showed that precipitation of RAVL1 enriched the P2 region, but not the P1 region, of the *EIL1* promoter (Fig. 5B). Additionally, EMSA results suggested that RAVL1 can bind to E-boxes in the P2 region, but not to E-box sequences mutated to TTTT (Fig. 5C). The competition EMSA performed to verify the binding affinity between RAVL1 and the P2 region revealed that this binding was greatly reduced in the presence of 50-fold excess unlabeled P2 but unaffected by unlabeled mP2 (Fig. 5C). To verify if these *cis*-elements were responsible for the transcriptional activation of the *EIL1* promoter via RAVL1 in vivo, we performed a transactivation assay by transient expression in *A. thaliana* protoplasts. Results

Fig. 7. Genetic and phenotypic evaluation of *RAVL1* action on gene expressions and *d61-1/RAVL1* overexpressing (OX) double mutant in response to sheath blight disease compared with wild-type (WT) rice (*Oryza sativa* L.) and parental lines. **A**, Relative expression of *EIL1* in *d61-1/RAVL1* OX (F8) compared with WT and parental lines. **B**, Relative expression of *BRI1* and *D2* in *EIL1* Ri5/*RAVL1* OX (F3) compared with WT and parental lines. The mRNA levels of samples were normalized to that of *Ubiquitin* mRNA. Data represent means $\pm$ standard error ($n = 3$). The expressions of *EIL1*, *BRI1* and *D2* in WT were defined as 1. **C**, Response of *d61-1/RAVL1* OX to *Rhizoctonia solani* anastomosis group (AG)-1 compared with WT and parental lines. **D**, Percentage of leaf area covered with lesions in *d61-1/RAVL1* OX compared with WT and parental lines. Data represent means $\pm$ standard error ($n > 10$). **E**, Relative fungal biomass obtained by quantitative PCR [$2^{CT(UBI)-CT(RsPGD)}$] using genomic DNA. The data are means $\pm$ standard error ($n = 10$). Different letters indicate significant differences at $P < 0.05$.

suggested that RAVL1 can transactivate *pEIL1*, but not the E-box mutated promoter *mpEIL1* (Fig. 5D), indicating that RAVL1 might activate *EIL1* expression via promoter binding.

Ethylene signaling positively regulates rice resistance to sheath blight disease. To test the function of *EIL1* in response to *R. solani* AG-1, *EIL1* RNAi transgenic plants were first analyzed by qRT-PCR for the detection of *EIL1* expression; the results showed that *EIL1* was suppressed by 50 to 80% (Supplementary Fig. S4A). Phenotypic evaluation of *EIL1* RNAi in response to sheath blight disease revealed that transgenic plants were susceptible to *R. solani* AG-1. To confirm the role of ethylene signaling in response to sheath blight disease, we further analyzed the ethylene receptor ERS1 mutant *ers1* and *EIL1* RNAi 5 (*EIL1* Ri5). The *ers1* mutant showed a strong ethylene response phenotype to exogenous ethylene, indicating that *ERS1* is a negative regulator of ethylene signaling (Ma et al. 2014). The results showed that *ers1* was more resistant to *R. solani* AG-1 than WT, whereas *EIL1* RNAi 5 (*EIL1* Ri5) was more susceptible than WT (Fig. 6A, B, and C), indicating that ethylene signaling positively regulates rice resistance to sheath blight disease.

***EIL1* inhibition suppresses resistance to sheath blight disease in the *d61-1* mutant.** Because RAVL1 apparently activates the expression of *EIL1* and *BRI1*, two genes that have opposite functions in response to *R. solani* AG-1, we further examined the function of *EIL1* and *BRI1* by crossing *EIL1* Ri5 (more susceptible than WT) and *d61-1* (more resistant than WT) mutants and inoculating their F3 offspring with *R. solani* AG-1. The results showed that *d61-1/EIL1* Ri5 was susceptible to *R. solani* AG-1, similar to WT (Fig. 6D, E, and F), indicating that *EIL1* suppression reduced *d61-1* resistance to sheath blight disease (Fig. 6D, E, and F).

RAVL1 coordinates BR and ethylene signaling. Because RAVL1 directly activates the expression of BR and ethylene-related

genes, we examined if the activation of *EIL1* by *RAVL1* required BR signaling and if the *RAVL1* activation of *BRI1* and *D2* required ethylene signaling. To this end, we monitored the levels of *EIL1* in *RAVL1* OX/*d61-1* and those of *BRI1* and *D2* in *RAVL1* OX/*EIL1* Ri5. The results showed that levels of *EIL1* were slightly lower in *d61-1*, but markedly higher in *RAVL1* OX and *d61-1/RAVL1* OX (F8), than in WT (Fig. 7A). Additionally, the levels of *BRI1* and *D2* were significantly higher in *RAVL1* OX and *EIL1* Ri5/*RAVL1* OX (F3) than in WT and *EIL1* Ri5 (Fig. 7B). Further, *BRI1* mutation enhanced resistance to *R. solani* AG-1 in a *RAVL1* OX background, with *d61-1/RAVL1* OX showing a phenotype similar to that of *d61-1* (Fig. 7C, D, and E), indicating that the susceptibility of *RAVL1* OX to *R. solani* AG-1 depends on BR signaling.

DISCUSSION

Sheath blight disease caused by *R. solani* is a major rice disease, which severely reduces grain yield; however, the host resistance mechanisms remain unknown. Previous studies have shown that BR signaling is closely associated with host–pathogen interaction (Ali et al. 2013; Goddard et al. 2014). The present study showed that the *BRI1* mutant *d61-1* and the key BR biosynthesis gene mutant *d2* are highly resistant to *R. solani* AG-1, whereas the *BRI1* overexpression line *bri1-D* is more susceptible than WT plants, indicating that BR signaling supports rice susceptibility against sheath blight disease. Furthermore, analyses of the key BR signaling transcription factor RAVL1 in response to sheath blight disease showed that *ravl1* mutants exhibited similar symptoms to WT plants, whereas *RAVL1* OX plants showed enhanced susceptibility, suggesting that *ravl1* mutants might regulate other genes besides BR signaling genes, or that a protein functionally redundant to RAVL1 might modulate the development of sheath blight disease together with RAVL1.

Fig. 8. Brassinosteroid and ethylene signaling negatively regulate rice response to sheath blight disease. RAVL1 regulation of brassinosteroid and ethylene signaling in response to sheath blight disease. RAVL1 directly activates brassinosteroid- (*BRI1* and *D2*) and ethylene- (*EIL1*) related genes.

In the present study, transcriptome analysis of WT and *rav1l* plants revealed that the expression of few ethylene-related genes, including *EIL1* and *ACO2*, was altered in *rav1l*. However, no BR-related gene showed more than twofold changes in expression in *rav1l*. Further verification via qRT-PCR demonstrated that *EIL1* was positively regulated by RAVL1. The promoter sequence analysis identified two E-box elements located within 1.5 kb of the *EIL1* promoter. Additionally, the ChIP assay suggested that RAVL1 can bind to P2 elements, which are located 688-bp downstream of the start codon, but not to the P1 region. The in vitro EMSA performed to verify if RAVL1 directly bound to P2 indicated that the RAVL1 recombinant protein bound to P2, but not to E-box element sequences mutated to TTTT. Further competition EMSA experiments suggested that RAVL1 specifically bound the P2 region. The transient promoter transactivation assay supported that RAVL1 can directly activate expression of *EIL1* via promoter binding. The levels of *EIL1*, *Os07g06190*, and *Os09g20350* were lower in *rav1l* mutants and higher in RAVL1 OX than in WT, while the levels of *ACO2* and *Os07g06300* were higher in *rav1l* than in WT and RAVL1 OX. Although *EIL1* is known as a positive regulator, the role of *Os07g06190*, *Os09g20350*, and *Os07g06300* in ethylene signaling is unclear. RAVL1 activates *EIL1*, and *rav1l* mutants are less sensitive while RAVL1 OX is more sensitive to ACC than WT, suggesting that RAVL1 might positively regulate ethylene signaling.

Previous studies revealed the interaction between BR and ethylene signaling (Gendron et al. 2008), and showed that BR treatment elevates ethylene production by increasing the stability of acetyl-coenzyme A synthetase (ACS), suggesting a potential role for BRs in the regulation of ethylene biogenesis (Hansen et al. 2009). However, direct associations between BR and ethylene signaling have not been identified. Our data suggest that BR and ethylene signaling are linked by RAVL1. A previous study reported that the overexpression of *OsACS2*, an ethylene biosynthetic gene, enhances rice resistance to rice blast and sheath blight disease (Helliwell et al. 2013). Because *EIL1* is a major regulator of ethylene signaling, the response of *EIL1* RNAi lines against *R. solani* AG-1 was tested. The expression levels of *EIL1* in RNAi plants were significantly suppressed, and thus, the plants were more susceptible to *R. solani* AG-1 than WT. Additionally, the ethylene-hypersensitive *ers1* mutant was examined, and data showed that it was more resistant than WT. Therefore, ethylene signaling positively regulates rice resistance to sheath blight disease.

We found that RAVL1 transcriptionally regulates both BR and ethylene signaling genes and that BR and ethylene signaling oppositely regulated plant resistance to sheath blight disease. Whereas *rav1l* exhibited infection symptoms similar to those of WT, *d61-1* was resistant to *R. solani* AG-1. Thus, a potential redundant protein of RAVL1 might exist or other RAVL1 downstream gene functionally opposite to *BR11* might respond to *R. solani* AG-1. Therefore, we examined whether these effects were caused by downregulation of both *BR11* and *EIL1*. To this end, we genetically combined *BR11* and *EIL1* by crossing *d61-1* and *EIL1* RNAi 5 (*EIL1* Ri5) mutants and found that *d61-1/EIL1* Ri5 had a similar phenotype to that of WT. Thus, combined BR and ethylene signaling modulate rice defense against sheath blight disease. To examine if the RAVL1 activation of *EIL1* depends on BR signaling, the expression levels of *EIL1* were examined in RAVL1 OX and *d61-1/RAVL1* OX. Results showed that *EIL1* expression was significantly higher in both RAVL1 OX and *d61-1/RAVL1* OX than in WT, indicating that RAVL1 transcriptionally activates *EIL1* independently of BR signaling. Similarly, the expression levels of *BR11* and *D2* examined in RAVL1 OX and *EIL1* Ri5/RAVL1 OX indicated that *BR11* and *D2* were highly expressed in RAVL1 OX and *EIL1* Ri5/RAVL1 OX. Thus, RAVL1 in BR and ethylene signaling are involved rice defense against sheath blight disease (Fig. 8).

Both *rav1l* mutants were WT-like and RAVL1 OX was more susceptible than WT to *R. solani* AG-1, suggesting that overexpression of RAVL1 might transcriptionally activate genes that do

not change their action in *rav1l*. Further, the RAVL1 OX phenotype disappeared in *d61-1/RAVL1* OX, suggesting that *BR11* is epistatic to RAVL1 OX in response to *R. solani* AG-1. Furthermore, *BR11* and *D2* expression was not affected upon *R. solani* infection, suggesting that RAVL1 might contribute to BR and ET signaling pathway differentially under different conditions, such as plant development and resistance. Overall, the present study provides information on the role of BR and ethylene signaling in response to sheath blight disease, and identifies RAVL1 as the linkage between BR and ethylene signaling in rice.

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