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ORIGINAL ARTICLE

Enhanced Resistance of PsbS-deficient Rice (*Oryza sativa* L.) to Fungal and Bacterial Pathogens

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Abstract The 22-kDa PsbS protein of Photosystem II is involved in nonphotochemical quenching (NPQ) of chlorophyll fluorescence. Genome-wide analysis of the expression pattern in *PsbS* knockout (KO) rice plants showed that a lack of this protein led to changes in the transcript levels of 406 genes, presumably a result of superoxide produced in the chloroplasts. The top Gene Ontology categories, in which expression was the most differential, included 'Immune response', 'Response to jasmonic acid', and 'MAPK cascade'. From those genes, we randomly selected nine that were up-regulated. Our microarray results were confirmed by quantitative RT-PCR analysis. The KO and *PsbS* RNAi (knockdown) plants were more resistant to pathogens *Magnaporthe oryzae* PO6-6 and *Xanthomonas oryzae* pv. *oryzae* than either the wild-type plants or *PsbS*-overexpressing transgenic line. These findings suggest that superoxide production might be the reason that these plants have greater pathogen resistance to fungal and bacterial pathogens in the absence of energy-dependent NPQ. For example, a high level of cell wall lignification in the KO mutants was possibly due to enhanced superoxide production. Our data indicate that certain abiotic stress-induced reactive oxygen species can promote specific signaling pathways, which then activate a defense mechanism against biotic stress in *PsbS*-KO rice plants.

Keywords: Biotic stress tolerance, Microarray, Nonphotochemical quenching, PsbS, Rice, Superoxide

Introduction

Although light energy can be converted to useful chemical energy during photosynthesis, excess light is harmful to plants. Most of the excess energy, when not required for photosynthetic CO_2 assimilation, is dissipated as heat via nonphotochemical quenching (NPQ) of chlorophyll fluorescence (Horton et al. 1996; Niyogi 2000; Zulfugarov et al. 2010; Zulfugarov et al. 2014a), through the provision of alternative electron acceptors (such as molecular oxygen) in the water–water cycle (Asada 1999; Asada 2006), or by photorespiration (Ort and Baker 2002; Müller-Moulé et al. 2003).

Energy-dependent quenching, qE, the major NPQ component, depends on i) a trans-thylakoid proton gradient (ΔpH) (Müller et al. 2001), ii) pigments involved in the xanthophyll cycle (Demmig-Adams et al. 1989), and iii) a 22-kDa PSII protein called PsbS (Li et al. 2000). These parameters control qE in an integrated way; the qE signal generally disappears when one of these parameters does not exist. The role of PsbS protein in qE was first established in the *npq4-1* mutants of *Arabidopsis thaliana* where lacking *PsbS* (Li et

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al. 2000). Although PsbS protein is evidently necessary for qE, Arabidopsis mutants completely lacking that protein show normal photochemistry without any visible phenotype (Li et al. 2000). The same situation is true for rice mutants that lack PsbS (Zulfugarov et al.2007; Murchie and Niyogi 2011; Zulfugarov et al. 2014a). When NPQ is blocked, more reactive oxygen species (ROS) are produced in the chloroplasts. One powerful ROS is the highly reactive singlet oxygen (Kearns 1971). One-electron reduction of molecular oxygen, in its triplet state, produces the superoxide anion radical (thereafter called superoxide), which is then rapidly dismutated by the superoxide dismutase enzyme. This two-electron reduction of molecular oxygen results in the production of hydrogen peroxide (H_2O_2), which has a longer lifetime than the superoxide (Fridovich 1997). Under many biotic- and abiotic-stress conditions, an imbalance occurs between ROS generation and scavenging, and the accumulated ROS damages neighboring cells (Hideg et al. 2002). Although ROS are scavenged by diverse antioxidative defense substances, e.g., antioxidant enzymes and non-enzymatic small molecules such as ascorbate, tocopherol, and glutathione (Foyer and Noctor 1999; Mittler 2002), the level of ROS in plants may rise rapidly in response to environmental changes (Blokhina et al. 2003). Because of the chemical nature of ROS, they react with a large array of molecules in biological organisms, possibly causing often fatal damage to those molecules (Jabs 1999). Although ROS are cytotoxic, plants also use them as signaling molecules to control processes such as programmed cell death, abiotic-stress responses, pathogen defenses, and systemic signaling (Mittler 2002). The development of rapid and localized cell death involves a ROS burst in the chloroplasts (Zurbriggen et al. 2009) and inhibits the spread of the pathogen (Camejo et al. 2016). In addition, ROS accumulations lead to signal transduction pathways that induce defense systems against the pathogen. Important intermediates within those signal transduction pathways include ethylene, salicylic acid (SA), and jasmonic acid (JA) (Neill et al. 2002; Sasaki-Sekimoto et al. 2005; Song et al. 2014).

We have previously shown that a *PsbS*-knockout (KO) rice mutant produces more superoxide and H_2O_2 when compared with the untransformed wild-type (WT) control (Zulfugarov et al. 2014a). Therefore, to understand the signaling role of ROS produced in the mutant, we have investigated the effects of ROS production on gene expression. In this study, we found that the KO rice plants had increased expression of genes involved in JA signaling and the defense mechanism, thereby suggesting that changes occur in the biotic-stress response by such mutants. We further demonstrated here that the mutants were more resistant to both bacterial and fungal pathogens than either the WT plants or *PsbS*-overexpressing (OX) lines. This indicated a plausible link among tolerance to biotic stresses, superoxide production in the chloroplasts, and changes in the mRNA levels of stress-responsive genes and those related to the defense mechanism.

Results

Expression of Genes Involved in Jasmonate Signaling and the Defense Response is Enhanced in *PsbS*-KO Plant Leaves

To investigate the transcriptional changes in PsbS-deficient rice, we compared mRNA levels from *PsbS*-KO and WT plants, using a 60,727-oligonucleotide DNA microarray. Total RNAs were prepared from leaves sampled over 3 d from one-month-old greenhouse-grown seedlings cultured under natural sunlight (16-h photoperiod; 200 μmol photons m^{-2} s^{-1}). Differentially expressed genes (DEGs) were identified by applying B-statistics (Sjödin et al. 2006). A B-statistics value of zero corresponded to a 50:50 probability of the gene being differentially expressed. Although this was not a stringent criterion for differential expression, it was useful here for ranking and initial selection of genes of interest. We identified 406 genes that were differentially expressed (B>0) in *PsbS*-KO vs. WT plants (Table 1; see Table S1).

Of those 406 DEGs (Table S1), 315 (Table S2) had Arabidopsis homologues. Although many of the most significantly expressed differentially had no informative annotation (Table 1), we found that, among the top 30 genes (as ranked by B-values), two (LOC_Os12g14440 and LOC_Os12g09720) encode JA-induced proteins (Zhou et al. 2010; Gan et al. 2011) and another two encode proteins with annotations that indicate their roles in pathogen resistance – disease resistance protein RPS2 (LOC_Os09g14010) and SCP-like extracellular protein (LOC_Os01g28500; expressed). We also identified two transcription factors – heat shock protein DnaJ (LOC_Os06g44160; putative, expressed) and WRKY95 (LOC_Os12g02440; expressed) – plus two genes that target chloroplasts – polyphenol oxidase (LOC_Os01g58100; putative, expressed) and inosine-uridine preferring nucleoside hydrolase family protein (LOC_Os05g33630; putative, expressed). Several genes coding for kinases or annotated as retrotransposons were also up-regulated. One of them, LOC_Os08g01390, encodes a phosphatidylinositol-4-phosphate 5-kinase family protein that consumes ATP while LOC_Os08g10300 putatively encodes SHR5-receptor-like kinase (RLK). Genes that encode RLKs comprise a major class of up-regulated genes, including one that is expressed during a *Pi33/ACE1* interaction in rice, which is triggered by resistance gene *Pi33* in response to infection by ACE1, a virulent blast fungus (Vergne et al. 2007).

To verify our microarray data, we randomly selected eight genes that appeared to be up-regulated in *PsbS*-KO rice plants. Leaf RNA was used for quantitative RT-PCR (qRT-

Table 1. The 30 most significantly differentially expressed genes in *PsbS*-KO rice plants versus the WT under a moderate light intensity. Genes with positive M-values (log2 ratio) were up-regulated whereas the gene with a negative M-value was down-regulated in KO plants. The composite intensity, A, was calculated as log2((Channel 1 intensity)*(channel 2 intensity))$^{1/2}$; high/low values indicate a high/low level of expression for the respective gene

B-value	Oligo ID	TIGR Locus ID	TIGR putative function	M (log2 ratio)	A (mean intensity)
Up-regulated					
11.96	Os012955_01	Os12g14440	Jacalin-like lectin domain-containing protein, putative, expressed	2.66	14.27
11.27	Os023437_01	Os11g32820	expressed protein	3.16	13.27
11.02	Os033331_01	Os07g44220	hypothetical protein	1.96	11.35
10.97	Os049680_01	Os03g33480	retrotransposon protein, putative, Ty3-gypsy subclass	2.70	12.23
9.51	Os002086_01	Os06g44160	heat shock protein DnaJ, putative, expressed	2.74	10.31
9.29	Os008445_01	Os09g14010	disease resistance protein RPS2, putative	1.74	13.11
8.93	Os050925_01	Os01g61840	hypothetical protein	2.02	12.14
8.41	Os013495_01	Os08g01390	phosphatidylinositol-4-phosphate 5-Kinase, putative, expressed	1.36	11.43
8.40	Os012123_01	Os08g10300	SHR5-receptor-like kinase, putative, expressed	1.97	11.65
8.27	Os044745_01	Os05g39270	retrotransposon protein, putative, Ty3-gypsy subclass	1.39	12.60
8.03	Os045285_01	Os12g09720	dirigent, putative, expressed	4.30	11.90
8.01	Os048875_01	Os12g22470	retrotransposon protein, putative, Ty3-gypsy subclass, expressed	1.75	10.51
7.86	Os000537_01	Os12g23150	4-nitrophenylphosphatase-like, putative, expressed	2.58	10.38
7.70	Os028413_01	Os09g10990	transposon protein, putative, CACTA, En/Spm sub-class	1.83	12.31
7.51	Os014992_01	Os05g33630	inosine-uridine preferring nucleoside hydrolase family protein, putative, expressed	1.48	12.91
7.36	Os013962_01	Os01g74530	protein phosphatase 2C, putative, expressed	2.06	11.41
7.19	Os027111_01	Os01g28500	SCP-like extracellular protein, expressed	1.52	12.36
7.18	Os020172_01	Os12g02440	WRKY 95, expressed	1.33	12.66
6.86	Os056521_01	Os01g58100	polyphenol oxidase, putative, expressed	1.83	11.80
6.83	Os034831_01	Os06g15430	expressed protein	3.25	11.38
6.77	Os052914_01	Os11g45130	pollen-signalling protein with adenylyl cyclase activity, putative, expressed	2.12	9.10
6.67	Os015968_01	Os07g03110	OsFBX213 - F-box domain-containing protein, expressed	1.45	10.93
6.66	Os025840_01	Os02g42540	expressed protein	1.43	12.58
6.61	Os009835_01	Os12g36830	pathogenesis-related Bet v I family protein, putative, expressed	1.37	13.92
6.57	Os018245_01	Os12g36850	pathogenesis-related Bet v I family protein, putative, expressed	1.85	13.03
6.50	Os029243_01	Os05g17870	retrotransposon protein, putative, unclassified	1.24	11.58
6.31	Os016288_01	Os08g07430	expressed protein	1.51	11.71
6.30	Os042473_01	Os05g39920	retrotransposon protein, putative, unclassified	1.14	11.51
6.24	Os022932_01	Os04g44150	gibberellin 2-beta-dioxygenase 7, putative, expressed	1.96	12.17
Down-regulated					
8.50	Os011831_01	Os11g33394	plant protein of unknown function domain-containing protein, expressed	-2.40	12.78

PCR) analyses of those genes (Fig. 1). All tested genes, except for one, showed higher expression in the *PsbS*-KO plants. In addition, the level of *psbS* expression was examined as a control for the KO plants and none of *psbS* expression was found in *PsbS*-KO plants by RT-PCR (Fig. S1). Thus, our gene expression data confirmed that the microarray results were reliable.

Gene Ontology Analysis

Having demonstrated that the microarray data were generally reliable, we calculated Gene Ontology (GO) term enrichments with GoMiner (Ashburner et al. 2000; Zeeberg et al. 2003; http://www.geneontology.org/, http://discover.nci.nih.gov/gominer/) to obtain information about the classes or groups of genes that were possibly regulated differentially. Among the 60,727 oligomers on the microarray, 44,704 could be matched to rice pseudomolecules v7.0 (http://rice.plantbiology.msu.edu/) with BLASTx scores of at least 100. We also found that 19,216 rice genes with scores ≥100 had Arabidopsis homologues. Those were used a complete gene set for GoMiner analysis. For that examination, GoMiner first categorizes each gene according to its GO terms and mode of expression, i.e., down- or up-regulated, as denoted by 'Under', 'Over', and 'Change'. The program then calculates *P*-values based on a one-sided Fisher exact test for the total number of categorized

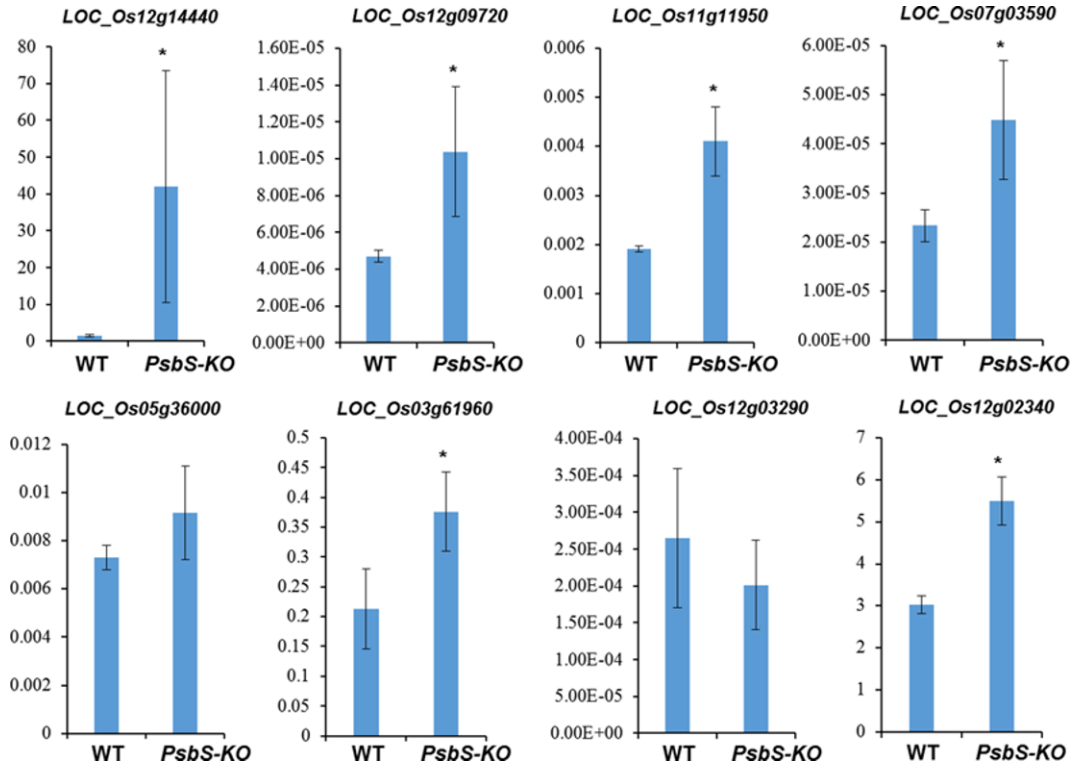

Fig. 1. Relative expression of stress- and defense-related genes in WT (cv. Hwayoung) and *PsbS*-KO rice plants. Quantitative RT-PCR was performed with cDNA derived from WT and KO leaf samples. LOC_Os12g14440, Jacalin-like lectin domain-containing protein, putative, expressed; LOC_Os12g09720, Dirigent, putative, expressed; LOC_Os11g11950, disease resistance protein RPM1, putative, expressed; LOC_Os07g03590, SCP-like extracellular protein, expressed; LOC_Os05g36000, FabA-like domain-containing protein, expressed; LOC_Os03g61960, 2Fe-2S iron-sulfur cluster binding domain-containing protein, expressed; LOC_Os12g03290, AP2 domain-containing protein, expressed; LOC_Os12g02340, LTPL14 - protease inhibitor/seed storage/LTP family protein precursor, expressed. Rice Ubiquitin5 (*OsUBQ5*; LOC_Os01g22490) was used as internal control. Each data point represents mean ±SD from at least 3 experiments. Statistical significance of correlation coefficients is indicated by * ($P < 0.01$).

Table 2. Assignment of differentially expressed genes into Gene Ontology classes. Values within parentheses represent the total number of genes in the rice genome annotated to that class. Frequency denotes the number of differentially expressed genes in *PsbS*-KO versus WT plants grown under a moderate light intensity that were assigned to a particular class

GO class	Frequency
Response to acid chemical (1514)	43
Immune response (909)	34
Response to temperature stimulus (950)	26
Response to jasmonic acid (449)	22
Cell death (627)	22
Protein targeting to membrane (409)	17
Terpenoid metabolic process (315)	13
MAPK cascade (278)	12
Isopentenyldiphosphate biosynthetic process (228)	10
Coenzyme biosynthetic process (192)	9
Pinocytosis (120)	7
Lipid transfer (188)	7
Porphyrin-containing compound biosynthetic process (158)	7
Glycolipid metabolic process (149)	6
Lipoate metabolic process (44)	4
Other classes (frequency < 10)	*95*

GO terms. Any term that has an adjusted *P*-value <0.05 is chosen for further analysis. Here, all genes were classified according to their functions.

In all, 108 biological processes (Table S3) were enriched according to the GoMiner analysis. The top 15 DEGs were then assigned to GO classes (Table 2). The most common categories (i.e., showing greatest differential expression) were 'Immune response', 'Response to jasmonic acid', and 'MAPK cascade'. They included JA-signaling genes (Table 1). Because our analyses of both single genes and GO categories indicated that genes involved in the response to biotic interaction were over-represented among upregulated genes, we decided to investigate possible changes in the biotic-stress responses by *PsbS*-KO plants.

PsbS-KO Plant Leaves Show Increased Pathogen Resistance

To examine the role of PsbS in plant defenses, we evaluated the level of resistance that the KO mutant, RNAi knockdown, and overexpression plants had to the virulent *Magnaporthe oryzae* race PO6-6. Using the spot-inoculation method, we consistently found that the null mutant and RNAi line

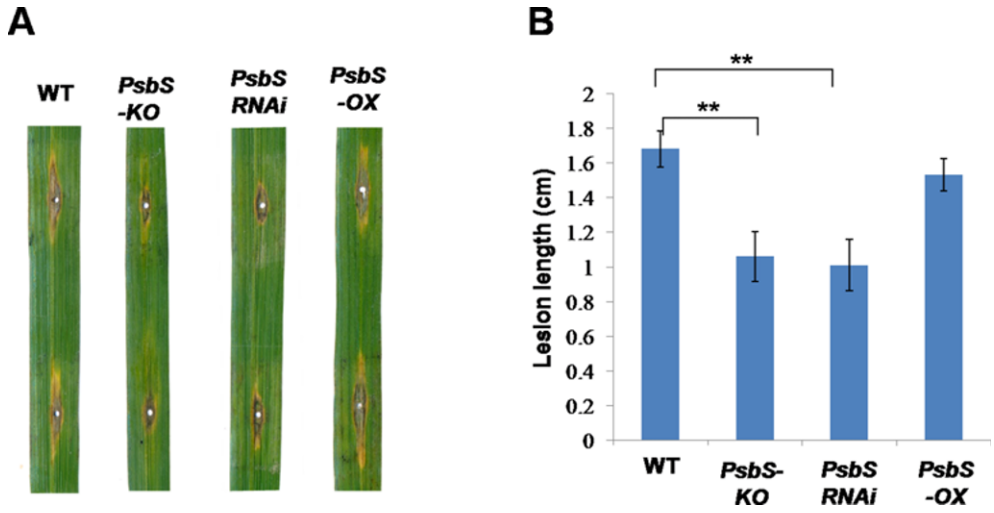

Fig. 2. Phenotypes of *PsbS*-KO, *PsbS* RNAi, and *PsbS*-OX transgenic plants exposed to *Magnaporthe oryzae* PO6-6. (A) Disease response 8 d after punch-inoculation of WT 'Hwayoung', KO, knockdown, and OX leaf samples. (B) Quantitative lesion lengths measured at 8 DAI. Each data point represents average and standard deviation of at least 20 samples. Statistical significance of correlation coefficients is indicated by ** (P <0.01).

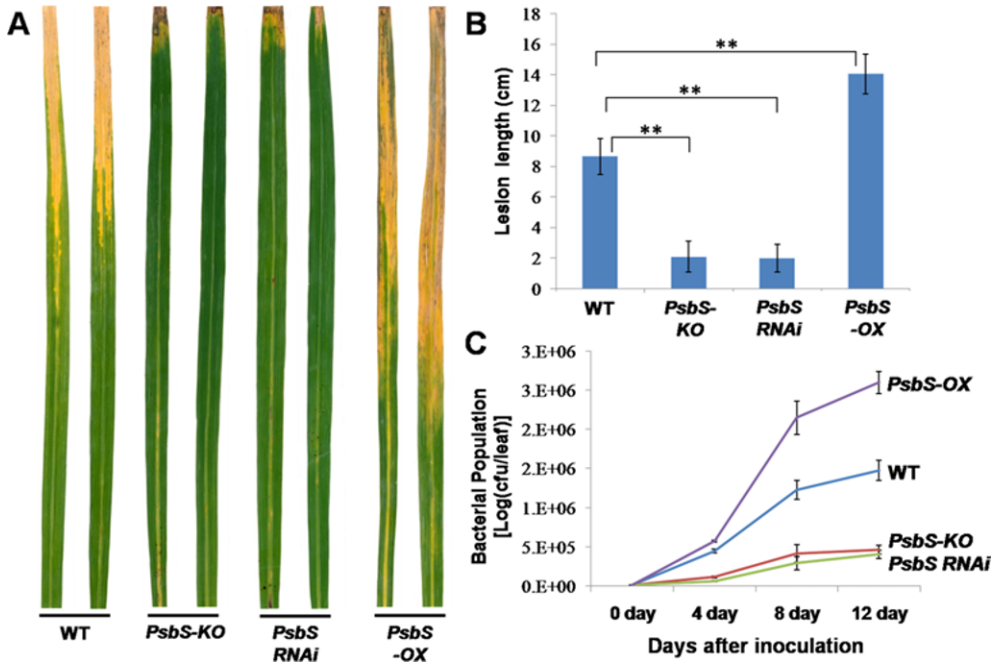

Fig. 3. Phenotypes of *PsbS*-KO, *PsbS* RNAi, and *PsbS*-OX transgenic plants exposed to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strain K3a. (A) Representative water-soaked lesions of KO, knockdown, OX, and WT 'Hwayoung' control plants. Pictures were taken at 12 DAI. (B) Leaf lesion lengths were measured at 12 DAI, using 3 leaves sampled separately at a similar growth stage from 3 different plants. Each bar represents average and standard deviation of 3 leaves. (C) *Xoo* growth curve analysis of KO, knockdown, OX, and WT 'Hwayoung' control plants, conducted at 0, 4, 8, and 12 DAI. For each time point, bacterial population size was determined separately for 3 leaves at a similar growth stage from 3 different plants. Each bar represents standard deviation of 3 leaves. Statistical significance of correlation coefficients is indicated by ** (P < 0.01).

displayed enhanced resistance (Fig. 2A), with the average lesion on leaves of those plants being much shorter than lesions on the WT leaves at 8 d after inoculation (DAI) (Fig. 2B). We also analyzed the responses of transgenic overexpression plants to *M. oryzae*. Based on symptom development (Fig. 2A) and lesion lengths (Fig. 2B), constitutive expression of *PsbS* had no significant effect on the plant response to this pathogen. These results, therefore, suggested that knocking out *PsbS* increases resistance to this rice blast fungus.

To elucidate how resistance might also apply to other types of rice pathogens, we challenged 10-week-old plants from each line with the bacterial pathogen *Xoo*, strain K3a

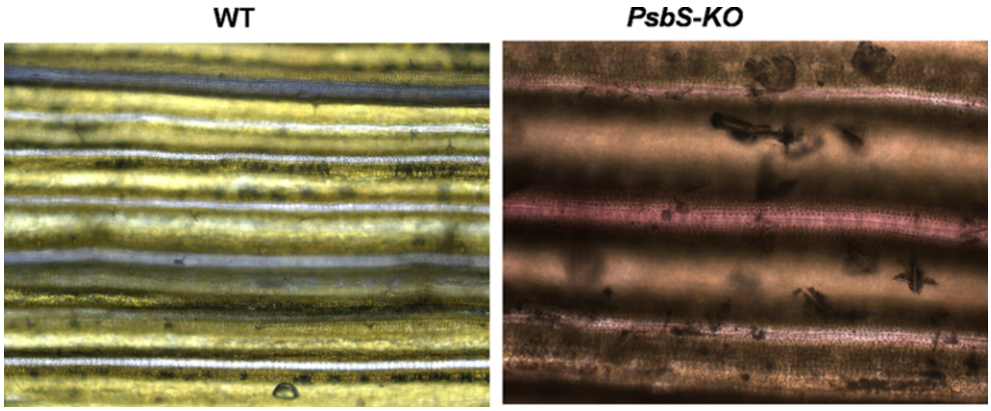

Fig. 4. Histochemical analysis of rice genotypes. Leaf sections from WT 'Hwayoung' and *PsbS*-KO plants were stained with phloroglucinol–HCl. Photographs were taken under microscope.

(Fig. 3A). At 12 DAI, leaves from *PsbS*-KO and *PsbS* RNAi plants had shorter water-soaked lesion areas when compared with the WT control 'Hwayoung' samples. However, those symptoms were drastically increased in the OX line, which was much more susceptible than the WT (Fig. 3B). The numbers of *Xoo* cells growing on the leaves of both mutant and control plants were studied with a bacterial growth curve analysis, which revealed a clear positive correlation between lesion lengths and *Xoo* population sizes in the WT, KO, RNAi, and OX plants (Fig. 3C). This suggested that the lack of PsbS also increased resistance against bacterial blight disease. Taken together, our findings indicated that the loss of function of *PsbS* and, subsequently, the reduced qE portion of NPQ were responsible for the improved resistance of rice to fungal blast and bacterial blight.

Levels of Cell Wall Lignin are Increased in *PsbS*-KO Plant Leaves

Genes related to terpenoid metabolic processes were up-regulated in our modified plants (Table 2). Lignification of the cell wall can help plants become more resistant to pathogens (Hijwegen 1963; Barros-Rios et al. 2015). Moreover, enhancing superoxide production can induce lignification (Ogawa et al. 1996). That process can also be regulated by interactions between ROS and JA (Denness et al. 2011). Our investigation of lignification included phloroglucinol-staining of leaves from WT and *PsbS*-KO rice plants, which demonstrated that KO tissues had more cell wall lignin than did the WT.

Discussion

Our study results provide evidence that rice mutants, lacking an important defense mechanism against abiotic stress, can become more tolerant to two rice-specific pathogens. We previously reported that such mutants generate more ROS, specifically superoxide, than the WT under abiotic-stress conditions (Zulfugarov et al. 2014a). Therefore, we speculated that superoxide may act as a direct or indirect signal that triggers the expression of genes related to biotic-stress resistance.

After plants are attacked by a pathogen, ROS signaling is widely regarded as central to the induction of disease resistance (Mehdy 1994; Bolwell and Wojtaszek 1997; Wojtaszek 1997; Fobert and Després 2005; Torres et al. 2006). For example, ROS can elicit systemic acquired resistance through the activity of SA (Kachroo and Robin 2013; Wrzaczek et al. 2013). However, the signaling network components that lead to pathogen resistance are not very well understood and little detailed information is available for how disease resistance develops in response to abiotic stress-induced ROS production in plants.

If ROS act as a signal that stimulates various stress responses, their biological activities should exhibit a high degree of specificity (Laloi et al. 2004). However, the results of only a few case studies have suggested that a given ROS can have a selective signaling effect (Apel and Hirt 2004; Mittler et al. 2004). It is also difficult to determine the signaling pathways mediated by individual ROS because they are generated concurrently in plants under environmental stresses. That is, ROS-mediated pathways appear to be controlled by the localized and temporal generation of specific ROS as well as by changes in the steady-state level of each ROS that is affected by scavenging systems (Mittler et al. 2004).

The type, dose, and duration of ROS action and the site of ROS production are critical for signaling (op den Camp et al. 2003; Apel and Hirt 2004; Mittler et al. 2004; Gechev and Hille 2005; Gechev et al. 2006; D'Autréaux and Toledano 2007; Zulfugarov et al. 2011; Poudyal et al. 2016). The most frequently discussed ROS involved in pathogen resistance is

H_2O_2 (Neill et al. 2002). However, superoxide can also participate, either directly or indirectly via H_2O_2, in determining the degree of resistance. Our microarray data were generated using plants adapted to a moderate light intensity. Under such conditions, superoxide was produced most rapidly in the *PsbS*-KO rice mutant, but H_2O_2 production did not differ significantly between this mutant and the WT (Zulfugarov et al. 2014a). Singlet oxygen also can act as a signaling molecule (Apel and Hirt 2004). In PsbS-deficient rice, however, the ROS primarily produced is superoxide (at the site of PSII), rather than singlet oxygen (Zulfugarov et al. 2014a). Therefore, we might assume that superoxide production at the PSII site could trigger specific changes in the levels of gene expression. However, the elucidation of superoxide-dependent expression by specific genes in plants requires further study.

Photosynthesis also play important roles in the plant response to pathogen attack because photosynthetic assimilates serve as a carbon source in the synthesis of defense compounds (Hammerschmidt 1999). Additionally, during the process of infection, pathogens withdraw sugars, leading to the consumption of carbon compounds. Therefore, strong cross-talk exists between abiotic- and biotic-stress responses. Although NPQ plays a role in photoprotection and probably also affects the pathogen response, it has not been well characterized in the plant immune response to bacteria, fungi, and viruses, as well as to insects. A few researchers have demonstrated a correlation between NPQ fluctuations and resistance to pathogen attack (Berger et al. 2004; Bonfig et al. 2006; Frenkel at al. 2009; Göhre et al. 2012; Jänkänpää et al. 2013). For example, the Arabidopsis *PsbS*-KO mutant *npq4-1* is less attractive for herbivores (Jänkänpää et al. 2013) because it has altered levels of metabolites, particularly those for JA metabolism (Frenkel at al. 2009). Furthermore, NPQ generation is negatively correlated with ROS production under excess light (Horton et al. 1996; Rodríguez-Moreno et al. 2008; Demmig-Adams et al. 2014).

The two putative JA-induced protein genes induced in *PsbS*-KO (LOC_Os12g14440 – jacalin-like lectin domain containing protein and LOC_Os12g09720 – dirigent) (Table 1) encode JA-induced proteins (Zhou et al. 2010; Gan et al. 2011). Both show very high sequence similarities with TA-JA1, a JA-regulated disease response protein in *Triticum aestivum* (Wang and Ma 2005). Other known JA-regulated proteins with diverse physiological functions include a proteinase-inhibitor, pathogenesis-related proteins, phytoalexin-synthesizing enzymes, cell wall proteins, osmotin, and lipoxygenase (Wasternack and Parthier 1997). Therefore, JA may play a role in stress adaptations by rice plants lacking PsbS. The overlap between the response to a lack of qE and treatment with JA has been investigated in greater detail in Arabidopsis, where the absence of PsbS leads to a metabolic shift -- probably away from growth and toward defense – that even seems to affect herbivore preferences (Frenkel et al. 2009, Frenkel et al. unpublished). However, the transcriptional response is not identical to the one that follows from JA treatment, i.e., plants appear to be primed to evoke stronger JA responses upon herbivore attack (Frenkel et al. 2009). Our present data suggest that this priming is the consequence of signaling from superoxide production at PSII.

Other genes of interest that were up-regulated in our KO mutant were those involved in defenses (e.g., disease resistance protein RPS2 and pathogenesis-related Bet v I family proteins) or the kinase cascade, including SHR5-RLK and WRKY 95 (Table 1). We were intrigued to learn that the genes induced in PsbS-deficient rice plants seemed to have a degree of functional overlap with those induced in corresponding field-grown Arabidopsis mutant plants (Frenkel et al. 2009). Thus, both species appear to have an over-representation of genes involved in those defense and JA responses.

Upregulation of genes related to processes of terpenoid metabolism (Table 2) suggest that cell wall lignification occurs in *PsbS*-KO mutant plants. Evidence also exists that dynamic interactions between JA and ROS are involved in cell expansion; i.e., loosening the wall allows cells to expand and lignify (Gapper and Dolan 2006; Denness et al. 2011). Hydrogen peroxide can serve as a substrate in the peroxidative cross-linking reactions of lignin precursors and induce cross-linking of cell wall proteins (Brisson et al. 1994; van Breusegem et al. 2001; Ros-Barceló et al. 2002; Ros-Barceló 2005). However, Ogawa et al. (1996) have shown that increased superoxide levels may also promote cell wall lignification. Although we cannot say definitely which type of ROS participates in determining pathogen resistance in our KO plants, we can assume, based on our current research, that superoxide is the first substance involved in that process.

Taken together, we propose that superoxide, either directly or indirectly through H_2O_2, causes changes in the expression of genes related to the pathogen resistance. It can also affect JA metabolism and cell wall lignification in *PsbS*-KO mutant plants, ultimately making those plants resistant to pathogen attack. When superoxide is released from PSII under conditions of excess excitation energy in those KO plants, another abiotic-stress protection system (cyclic electron flow) is accelerated to compensate for a qE deficiency (Zulfugarov et al. 2014b). Our current study provides some novel insight into the relationship among qE, cyclic electron flow, and the ROS signaling network which – in Arabidopsis, rice, and *Populus* (Külheim et al. unpublished) - seems to activate defense response pathways. Whether this influences the susceptibility of PsbS-lacking rice mutants to other stresses, as is the case with Arabidopsis, remains to be established. Nevertheless, given the similarities of their qE machinery and the consequences of qE losses between species, it is

possible that this will turn out to be true.

Materials and Methods

Plants and Growth Conditions

Rice (*Oryza sativa* L.) plants of various genotypes were grown either in soil in a greenhouse (natural sunlight) or in a culture room (200 μmol photons m^{-2} s^{-1} white light; 16-h photoperiod), at a temperature of 28±2ºC (28ºC/22ºC temperature cycle). The WT rice cultivar Hwayoung served as our control, and *PsbS*-KO and *OsPsbS*-RNAi lines (generated based on 'Hwayoung') (Zulfugarov et al. 2014a) were used as the PsbS knockout and knockdown genotypes, respectively.

In addition, a *PsbS* overexpression line (*PsbS*-OX) was generated based on 'Hwayoung' rice. Briefly, a *PsbS* overexpression construct was obtained via PCR by amplifying the entire ORF of *PsbS* (Clone No. AK058284; Rice Genomics Resource Center, Japan) and cloning into the *Hin*dIII-*Kpn*I sites of pGA1611 (Lee et al. 1999). This construct was then introduced into *Agrobacterium tumefaciens* strain PC2760 by the freeze-thaw method (Höfgen and Willmitzer 1988). Calli derived from mature seeds of 'Hwayoung' rice were transformed as previously described (Lee et al. 2009a). Sequences of primers used for cloning were 5'-AGAAAGCTTAGCAGCTAATCGCGCACGTAC-3' and 5'-GAAGGTACCATTCTACTCTTCGTCGTCGTC-3'. Primers used for semi-quantitative RT-PCR were *Actin* forward, 5'-TCA-TGAAGATCCTGACGGAG-3'; *Actin* reverse, 5'-ACTCAGCCT-TGGCAATCCAC-3'; *PsbS* forward, 5'-CTGTTCGGCAGGTCC-AAGAC-3'; and *PsbS* reverse, 5'-GTTCAGCTGCGCCAGGATTC-3'. Figure S2 presents the vector construction map and level of *psbs* expression, as well as the protein level of PsbS determined by commercially available antibody against PsbS protein (Agrisera), and the NPQ generation of *PsbS*-OX lines.

60K Rice Whole-genome DNA Chip analysis

One-month-old WT and *PsbS*-KO rice plants grown in a greenhouse were first adapted for 3 d in a culture room (200 μmol photons m^{-2} s^{-1} light intensity). Total RNA (100 mg) was prepared from leaf tissues of those genotypes (5–10 plants each), as reported previously (Oh et al. 2005), and the mRNA was purified from total RNAs using a Qiagenoligotex column (Qiagen, Valencia, CA, USA). Fluorescence-labeled probes and microarray hybridizations were prepared with an expression detection Array50™ kit (v. 2, 3DNA Array 900, Genisphere, Hatfield, PA, USA). The microarray was scanned with Genepix 4000B (Axon Instruments, Union City, CA, USA) and the quality of the chip data was analyzed with statistical R language and the Limma package (Smyth et al. 2005) in the Bioconductor project (http://www.bioconductor.org/), implemented on the Linux platform. To assess the reproducibility of our microarray analysis, we repeated the experiment three times with independently prepared total RNA. The rice 60K Oligomeric DNA microarray, representing all of the organism's genes, was obtained by GreenGene Biotech (details at www.ggbio.com/rice60kchip.html). Experiments were conducted with a dye swap to adjust for biases due to intrinsic intensity differences between Cy3 and Cy5. All gene annotations came from the TIGR pseudomolecule V.6 database (Michigan State University, Lansing, MI, USA) (ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_6.0/all.dir/). Genes that showed statistically significant differential expression were determined using B-statistics in UPSC-BASE (Sjödin et al. 2006). Gene product names were confirmed via MSU Osa1 Release 7 Annotation at the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/). The set of Arabidopsis homologues comprised genes with BLASTx scores ≥100.

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted using RNAiso Plus according to the manufacturer's protocol (Takara Bio, Kyoto, Japan). Reverse-transcription was performed according to the manufacturer's protocol, using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Japan). Quantitative RT-PCR was conducted with a Qiagen 154 Rotor-Gene Q real-time PCR cycler, following a thermal cycling procedure of 95°C for 40 s, 57°C for 40 s, and 72°C for 40 s. Primers for selected genes related to the defense mechanism and stress responses (Table S4) were designed to detect expression. *Rice ubiquitin 5* (*OsUbi5*, *LOC_Os01g22490*) (Jain et al. 2006) served as an endogenous control to normalize any variances in the quality of RNA and the amount of cDNA.

Pathogen Inoculation and Disease Evaluation

Magnaporthe oryzae PO6-6, to which 'Hwayoung' is susceptible, is commonly used to evaluate fungal disease resistance. All inoculations and disease evaluations were conducted in the greenhouse facilities at Kyung Hee University using the following inoculation methods. This fungal strain was first grown on a medium (20 g L^{-1} rice flour powder, 10 g L^{-1} dextrose, and 12 g L^{-1} agar) in the dark for 2 weeks at 22ºC (Lee et al. 2009b). To quantify the resistance response to *M. oryzae* PO6-6, we placed spore-covered agar blocks on 2.0-mm-diameter leaf discs that had been damaged with a punch prior to inoculation (Takahashi et al. 1999). The second fully expanded leaves from the top of five-week-old plants were used. Inoculated plants were placed in sealed containers to maintain humidity and were exposed to darkness for 24 h at 24ºC. They were then incubated at the same relative humidity under a 14-h photoperiod. For disease evaluations, the lengths of the blast lesions (cm) were measured at 8 DAI.

For *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) inoculations, well-expanded leaves from five-week-old plants were infected with a suspension of the *Xoo* strain K3a, which is compatible with the rice background 'Hwayoung'. The *Xoo* cells had been grown at 28°C for 3 d on peptone sucrose agar plates (10 g L^{-1} peptone, 10 g L^{-1} sucrose, 16 g L^{-1} agar, and 1 g L^{-1} glutamate; pH 7.5) that also contained 15 mg L^{-1} cephalexin. Rice leaves were inoculated using the scissor clip method (Han et al. 2013), with cells suspended in distilled water at a density (OD_{600}) of 0.8. Reactions to the disease were scored at 12 DAI by measuring lesion lengths (cm). Growth of *Xoo in planta* was monitored on inoculated leaves at four time points (0, 4, 8, and 12 DAI) using a previously described method (Han et al. 2013).

Histochemical Phloroglucinol-staining of Lignin

For determining the degree of cell wall lignification, histochemical staining was performed accordingly to the method of Zhong et al. (1998). Briefly, leaf segments of all rice genotypes were immersed in a 1% (w/v) potassium permanganate solution for 5 min at room temperature and then washed twice with 3% hydrochloric acid until the color turned from black or dark brown to light brown. The phloroglucinol–HCl reagent was prepared by mixing two volumes of 2% (w/v) phloroglucinol in 95% ethanol with one volume of concentrated HCl. All photographs were taken within 30 min of staining.

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Author's Contributions

ISZ, AT, CYK, KTXV, SYK, and HYS conducted experiments; MH, YKK, and OS performed data mining and bioinformatics analyses; ISZ, YHM, SJ, JSJ, and CHL designed the experimental plan, and wrote and edited the manuscript. All authors agreed on the contents of this paper and posted no conflict of interest.

Supporting Information

Fig. S1. Expression analysis of the PsbS gene in *PsbS*-KO mutant. *OsUBQ5* is PCR control.
Fig. S2. Generation of *PsbS*-OX. (A) Schematic map of *PsbS-OX* binary vector.
Table S1. Results of microarray analysis revealing 406 differentially expressed genes.
Table S2. The 315 differentially expressed genes among 406 genes (Table S1) of interest that had Arabidopsis homologues.
Table S3. Results of GoMiner analysis showing enrichment of 108 biological processes.
Table S4. Stress- and defense mechanism-related genes and their primer sequences.

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