Two Homologous Putative Protein Tyrosine Phosphatases, OsPFA-DSP2 and AtPFA-DSP4, Negatively Regulate the Pathogen Response in Transgenic Plants

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Abstract

Protein phosphatases, together with protein kinases, regulate protein phosphorylation and dephosphorylation, and play critical roles in plant growth and biotic stress responses. However, little is known about the biological functions of plant protein tyrosine dual-specificity phosphatase (PFA-DSP) in biotic stresses. Here, we found that OsPFA-DSP2 was mainly expressed in calli, seedlings, roots, and young panicles, and localized in cytoplasm and nucleus. Ectopic overexpression of OsPFA-DSP2 in rice increased sensitivity to Magnaporthe grisea (M. grisea Z1 strain), inhibited the accumulation of hydrogen peroxide (H$_2$O$_2$) and suppressed the expression of pathogenesis-related (PR) genes after fungal infection. Interestingly, transgenic Arabidopsis plants overexpressing AtPFA-DSP4, which is homologous to OsPFA-DSP2, also exhibited sensitivity to Pseudomonas syringae pv. tomato DC3000 (Pst DC3000), reduced accumulation of H$_2$O$_2$ and decreased photosynthetic capacity after infection compared with Col-0. These results indicate that OsPFA-DSP2 and AtPFA-DSP4 act as negative regulators of the pathogen response in transgenic plants.

Introduction

Plants face a variety of biotic stresses in nature, including bacteria and fungi, which can strongly affect growth and production [1]. In response to these biotic stresses, plants have evolved many defense mechanisms [2]. In one such defense mechanism, reactive oxygen species (ROS) (H$_2$O$_2$, especially) play a central role in plants by controlling many biological processes, including gene expression, activation of transcription factors, redox balance, programmed cell death (PCD) and regulation of the mitogen-activated protein kinase (MAPK) pathway [3,4,5,6]. Several studies have reported that high levels of ROS lead to the oxidative destruction of cells, but moderate levels can act as signaling molecules to regulate plant growth and the biotic stress response [7]. H$_2$O$_2$ is an important component of ROS; endogenous and exogenous H$_2$O$_2$ directly or indirectly killed pathogen cells or inhibited their growth, penetration and proliferation at the infection site by inducing PCD [3,8,9].

Reversible protein phosphorylation, mediated through the MAPK signalling cascade, plays a key role in determining the response to many external stimuli in plants, including biotic stresses [10,11]. The process of reversible phosphorylation is controlled by a balance between the activities of protein kinases and protein phosphatases in vivo [12]. Plant MAPKs are phosphorylated (and activated) by a series of substrate-specific kinases (e.g., MAPKK, MAPK), MAPks are dephosphorylated, and hence deactivated, by dual-specificity MAP kinase phosphatases (DSPs) [12,13]. The study by Asai et al. [14] reported the identification of the components in a MAPK signalling pathway in Arabidopsis. These authors demonstrated that in response to the flagellin-derived peptide flg22, AtMEKK1 (a MAPKKKs) activates AtMKK4 and AtMKK5 (two MAPKks), which in turn activate the functionally redundant MAPks AtMPK3 and AtMPK6. This cascade results in transcription of defense-related genes, and was shown to play an important role in resistance to both bacterial and fungal pathogen [14]. More recent studies have shown that a transcription factor in Nicotiana benthamiana, WRKY8, is phosphorylated by several MAPks (SIPK, NT4 and WIPK), which in turn results in transcription of defense-related genes, and also that phosphorylation of pathogen-inducible WRKY33 by PP2C phosphatases as well as protein kinases play critical roles in plant biotic stress. For example, the rice protein phosphatase XB15 (belonging to the PP2C subfamily), phosphorylated and inactivated protein kinase XA21, negatively regulating XA21-mediated innate immunity [17]. AtMPK2, a MAPK phosphatase, interacts differentially with AtMPK3 and AtMPK6 in the negative regulation of specific

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defense responses [18,19]. Similarly, knockout mutants in AtMPK1 displayed elevated resistance to *Pst* DC3000 by regulating AtMPK6 activity, again suggesting that phosphatases play key roles in the biotic stress responses [20].

Based on substrate specificity, protein tyrosine phosphatases (PTPs) can be divided into many groups, such as those that utilize phosphoproteins, lipids, deoxyribonucleic acids and carbohydrates [21]. In land plants, the lipid phosphatases are classified into three groups: tumor suppressor phosphatase and tension homologue (PTPs) can be divided into many groups, such as those that utilize phosphatidylinositol as substrate [22]. We previously demonstrated that OsPFA-DSP1 is an active tyrosine-specific phosphatase, and that it acts as a negative regulator in the abiotic stress response [23]. In addition, the AtPFA-DSP1 protein from *Arabidopsis thaliana* has been shown to possess phosphatase activity [24]. However, the biological function of PFA-DSPs in the biotic stress response is unknown. Here, we used overexpression and RNA-interference knockdown of OsPFA-DSP2 and AtPFA-DSP4 in transgenic rice and Arabidopsis plants to analyze their biological function during biotic stress. We also assayed the expression patterns and subcellular localization of OsPFA-DSP2, and used genetic techniques to study its biological functions. Our results indicate that OsPFA-DSP1 is an active transgenic rice plant, which is resistant to the fungal pathogen *Magnaporthe grisea*.

**Materials and Methods**

**Generation of transgenic plants**

The overexpression construct for *OsPFA-DSP2* (LOC_Os02g53160) from *Oryza sativa* ssp. *japonica* cv. Nipponbare was created by inserting a complete cDNA sequence into the vector pCXUN-flag, which contained a maize (*Zea mays*) ubiquitin gene promoter [25]. *OsPFA-DSP2* primer sequences are as follows: (sense primer) 5'-ATGCAAGCTGAGAATTTCG-3' and (antisense primer) 5'-TTAACACTGTGAGGCCGTC-3'. The *OsPFA-DSP2* interference construct was created by inserting a genomic sequence fragment (containing 125 bp of the 3'UTR and 125 bp from the translation stop codon) into the binary vector pCAMBIA1301, which carries the cauliflower mosaic virus (CaMV) 35S promoter. The recombinant plasmids were introduced into *Agrobacterium tumefaciens* (strain C58) using a binary vector pCAMBIA1301, which carries the cauliflower mosaic virus (CaMV) 35S promoter. The recombinant plasmids were introduced into *Arabidopsis thaliana* and *Oryza sativa* by electroporation. Transformation mediated by *A. tumefaciens* was performed as described [29]. The transgenic plants were selected by spraying 1-week-old seedlings with 0.01% Glufosinate (Basta).

**Examination of transgenic plants and knockout mutants**

Transgenic rice plants generated with the overexpression and interference recombinant vectors were examined by RT-PCR using two *OsPFA-DSP2* gene-specific primers, 5'-ATGCAAGCTGAGAATTTCG-3' and 5'-TTAACACTGTGAGGCCGTC-3'. The expression level of the *OsPFA-DSP2* gene was assayed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) using the *OsPFA-DSP2* sequence-specific primers, 5'-CCAGTTCGATTTGAGCCGTC-3' and 5'-TTAAGCCGTTCCACATCTGTTTGGAC-3'. The expression level of the rice *β*-actin gene was assayed with *actin*-specific primers and used to standardize the RNA sample for each qRT-PCR. *β*-actin primers were 5'-GGTTATTGTTAAGCAACTGCTGAG-3' and 5'-GATGAAA-GAGGGCTGGAAGAAG-3', respectively. The *OsPFA-DSP2* mutant was identified from a rice mutant collection (International Rice Functional Genomic Consortia; http://irfg.iris.org) harboring a *Tos17* insertion into the 2nd intron of *Os02g53160* (accession number NG8341) by PCR with the gene-specific primers, 5'-ATCGGATGTTGAGAATTTCG-3' and 5'-CCAGTTCGATTTGAGCCGTC-3'. The overexpression in transgenic Arabidopsis was quantified using RT-PCR with the primer pair 5'-ATCGGATGTTGAGAATTTCG-3' and 5'-CCAGTTCGATTTGAGCCGTC-3'. The expression of the *OsPFA-DSP2* gene was assayed by qRT-PCR using the primers: 5'-ATGCAAGCTGAGAATTTCG-3' and 5'-TTAACACTGTGAGGCCGTC-3'. The *OsPFA-DSP2* gene was assayed with *actin*-specific primers and used to standardize the RNA sample for each qRT-PCR.

**Pathogen culture and plant infection**

*Magnaporthe oryzae* strain Z1 was cultured on PDA medium for 5 days, then transferred to spore production medium and cultured for 24 h at 24°C with 16 h light/8 h dark. Conidia were suspended in sterilized distilled-water at a concentration of ~3×10^5 conidial/mL. Seedlings at the three-to five-leaf stage were inoculated with *M. grisea* by the spraying and immersed infection method [17,29]. Transgenic rice plants after inoculation were initially cultured for 24 h in the dark at 25°C with humidity >95%, followed by the same conditions for 6 days with 16 h light/8 h dark, after which they were observed for disease reaction phenotype. Disease was scored by measuring the lesion area at 7 days after infection. For all the disease evaluations, the mock-inoculated control was treated under identical conditions.

*Arabidopsis* plants were grown in a chamber with a 12 h light/12 h dark photoperiod at 22°C for 24 days before bacterial inoculation. Bacterial infections and growth assays with *Pst* DC3000 were described previously [30]. *Pst* DC3000 was cultured overnight at 28°C in King's B medium supplemented with 50 μg rifampicin/mL. The cells were pelleted, washed, resuspended, and diluted in H_2O to a concentration of 10^5 cfu/mL. Arabidopsis leaves were syringe-inoculated. Plant leaves were harvested at the indicated time for ROS detection, F_0/F_m measure and bacterial...
counting. Bacterial growth was assessed by plating a dilution series of leaf discs ground in H$_2$O on King’s B plates containing 50 µg rifampicin/mL. The colony-forming units (cfu) were counted after incubation at 28°C for 2 days. Each data point is shown as triplicates.

**Gene expression analysis**

To examined the influence of rice blast infection on resistance gene (OsPR1a and OsPR5) expression, RNA was extracted from leaves with TRIzol reagent (Invitrogen, USA), and cDNA was synthesized by reverse transcription using Prime Script RTase (Takara, Japan). For qRT-PCR assays, SYBR Green I (Takara, Japan) was added to the reaction system and run on a LC480 real-time PCR detection system according the manufacturer’s instruction (Roche, Switzerland). Data were analyzed using optical monitor software (Roche). The expression level of the rice β-actin gene was used to standardize the RNA (20 ng) sample for each qRT-PCR. The assays were repeated at least three times, with each repetition having three replications; similar results were obtained in repeated experiments. The SD was calculated for each data point. The sequence-specific primers were as follows: OsPR1a, 5'-GGGTCGCTGCTGCACTAC-3' and 5'-CATGCTAAACACGTAGCATA-3'; OsPR5, 5'-GGGTCTGCTGCCGACGCTTT-3' and 5'-ACGACTTGGTAGTTGTT-3'; β-Actin, 5'-GGTATTTGTTAGCAACTG-3' and 5'-GATGAAAGAGGCGTGGAA-3'.

**Expression pattern analysis**

Total RNA was extracted from different tissues of wild-type rice using TRIzol reagent (Invitrogen, USA). The cDNA was synthesized by reverse transcription using Prime Script RTase (Takara, Japan) according to the manufacturer’s protocol, and the expression of OsPFA-DSP2 was detected using SYBR Premix Ex Taq (Takara, Japan). For qRT-PCR assays, SYBR Green I (Takara, Japan) was added to the reaction system and run on a LC480 real-time PCR detection system according the manufacturer’s instruction (Roche, Switzerland). Data were analyzed using optical monitor software (Roche). The expression level of the rice β-actin gene was used to standardize the RNA (20 ng) sample for each qRT-PCR. The assays were repeated at least three times, with each repetition having three replications; similar results were obtained in repeated experiments. The SD was calculated for each data point. The sequence-specific primers were as follows: OsPR1a, 5'-GGGTCGCTGCTGCACTAC-3' and 5'-CATGCTAAACACGTAGCATA-3'; OsPR5, 5'-GGGTCTGCTGCCGACGCTTT-3' and 5'-ACGACTTGGTAGTTGTT-3'; β-Actin, 5'-GGTATTTGTTAGCAACTG-3' and 5'-GATGAAAGAGGCGTGGAA-3'.

**Results**

Expression pattern and subcellular localization of OsPFA-DSP2

OsPFA-DSP2 is a 205 amino acid protein that has been annotated as a putative dual-specificity phosphatase (DSPs) belonging to the PFA-DSP subfamily; it contains four conserved motifs characteristic of members of the PFA-DSP subfamily [23]. To determine the expression pattern of OsPFA-DSP2, qRT-PCR and GENEVESTIGATOR analysis were carried out, and transgenic plants expressing the GUS gene under control of the OsPFA-DAP2 promoter were generated. Expression of OsPFA-DSP2 was highest in young panicles, followed by roots, calli, old leaves, stem and leaves (10-days-old) as determined by qRT-PCR (Figure 1A). The probe Os.Affx24843.1.S1_at from OsPFA-DSP2 was used to analyze an O. sativa microarray database (OS_51K: Rice Genome 51K array) in GENEVESTIGATOR V3 [34]. The results of this analysis indicated that OsPFA-DSP2 was mainly expressed in seedlings, at the tillering stage and milk stage, and was similar to results of the qRT-PCR assay (Figure 1B).

In order to further demonstrate the expression pattern of OsPFA-DSP2, the activity of the Escherichia coli β-glucuronidase (GUS) gene under control of the OsPFA-DSP2 promoter region (1750 bp) was examined in transgenic rice plants. A total of 15 independent transgenic lines were analyzed, and all exhibited the same pattern of GUS staining. GUS activity was strongest in calli, young roots and seedling, with very little expression detected in old roots, stems and old leaves (Figure 1C–H). Based on these results, we concluded that OsPFA-DSP2 is mainly expressed in young tissues.

To investigate the subcellular localization of the OsPFA-DSP2 protein in rice protoplasts, we created constructs for expression of OsPFA-DSP2 fused to the green fluorescence protein (GFP) under the control of the 35S-CaMV promoter. The fluorescence emission of GFP was monitored in rice protoplasts using an Olympus fluorescence microscope. Nuclei were counter-stained with DAPI dye. The GFP fluorescence was distributed in the cytoplasm and nucleus (Figure 1I). OsPFA-DSP2-GFP fusion protein fluorescence was mainly localized to the cytoplasm and nucleus, which could be seen by blue DAPI staining. This result is in agreement with several previous studies that showed protein dual-specificity phosphatases such as AtIBR5, AtMPK2, AtPP2C5, OsIBR3 and OsPFA-DSP1, also localized to the cytoplasm and nucleus when analyzed as protein fusions with fluorescent protein tags [18,23,35,36,37]. These data indicate that OsPFA-DSP2 may target to both the cytoplasm and nucleus.

OsPFA-DSP2 is involved in the host response to M. grisea infection

To demonstrate the function of the OsPFA-DSP2 gene in rice, the OsPFA-DSP2 deficiency mutant (seed stock number: NG8142, named as ospfa-dsp2) was chosen from the Tos17 rice database [38]. In this mutant, a retrotransposon (Tos17) has inserted into the second intron. Transcription of OsPFA-DSP2 in the mutant was not detected by semi-reverse transcription polymerase chain reaction (RT-PCR) (Figure 2A). We also generated RNAi transgenic plants to specifically silence OsPFA-DSP2, expression of...
OsPFA-DSP2 in the mutant and silenced plant (named RNAi2) was suppressed approximately 95% and 89% compared with that in wild type (WT) rice as detected by qRT-PCR (Figure 2B). The expression level of OsPFA-DSP2 in overexpression lines (named OX6, OX13 and OX21) was increased about 102.5-fold, 83-fold and 112.7-fold, respectively, as compared to WT (Figure 2C). To observe the phenotypes, the F2 generation of transgenic rice plants was treated with an M. grisea conidial suspension, containing 0.01% Tween 20, by two different methods. Compared with CK, which was transformed with the empty vector (pCXUN-flag vector), the overexpression lines OX13 and OX21 were sensitive to M. grisea at 7 days post inoculation (dpi); the lesion area was approximately 4.5-fold and 6-fold larger than that observed on CK ($p < 0.01$) (Figure 2D and E). However, the symptoms of the ospfa-dsp2 mutant and RNAi2 plant did not differ appreciably from those observed on CK at 7 dpi, with the lesion area being similar (Figure 2D and E). Many more hypersensitive response (HR) spots were observed on the surface of OX6 and OX21 leaves than on CK, ospfa-dsp2 mutant and RNAi2 rice leaves at 7 days after spraying with the M. grisea conidial suspension; the lesion area of OX6 and OX21 leaves were ~5.7-fold and 6.2-fold ($p < 0.01$) larger than the area on CK (Figure 2F and G). The number of HR spots on the ospfa-dsp2 mutant was less than that of CK, and the area of the lesions was reduced by about half ($p < 0.05$) (Figure 2F and G). However, the HR spots and lesion area observed on the RNAi2 line was not obviously different from CK (Figure 2F and G). These results indicated that OsPFA-DSP2 could be considered as a negative regulator involved in the plant response to M. grisea.

OsPFA-DSP2 inhibits accumulation of H$_2$O$_2$ and expression of PR genes in response to M. grisea infection

In order to further study the biological function of phosphatase OsPFA-DSP2, DAB staining was used to detect H$_2$O$_2$ accumulation in the leaves after infection. Leaves were stained for H$_2$O$_2$ at 2 hours post inoculation (hpi) with M. grisea ($3 - 5 \times 10^5$ conidia/mL containing 0.01% Tween 20) and distilled-water containing 0.01% Tween 20 (control treatment). We observed that the production and accumulation of H$_2$O$_2$ in rice leaves was not detected by DAB staining in the control, a low level of H$_2$O$_2$ accumulated in the leaves of CK, the ospfa-dsp2 mutant and RNAi2 plants at 2 hpi, and H$_2$O$_2$ accumulation in OX13 and OX21 was minimal (Figure 3A). Also, when compared with CK, the expression level of OsPR1a in the OX13 and OX21 plants was reduced by ~88.2% and 93% at 6 hpi ($p < 0.01$), but the expression level of OsPR1a in...
the *ospfa-dsp2* mutant and RNAi2 plant was increased by approximately 1.5-fold at 6 hpi (Figure 3B). The expression profile of *OsPR5* in the *ospfa-dsp2* mutant and RNAi2 plants was also elevated, approximately 1.2-fold compared with CK at 12 hpi, but its expression in OX13 and OX21 was reduced by 68% and 88% compared with CK (*p*, 0.01) (Figure 3B). These results indicated that OsPFA-DSP2 inhibited the production and accumulation of H₂O₂ and suppressed the transcription of PR genes, further demonstrating that OsPFA-DSP2 plays a key role in the host response to *M. grisea* infection.

**AtPFA-DSP4** is involved in the host response to *Pst* DC3000 infection

The homolog of *OsPFA-DSP2* in Arabidopsis is *AtPFA-DSP4*; their protein sequences share a high degree of similarity (identities = 67%). Because OsPFA-DSP2 is involved in the pathogen response, we were interested to know whether AtPFA-DSP4 has similar function. An AtPFA-DSP4 deficiency mutant (stock name: salk_016876, named as *atfpa-dsp4* mutant) was obtained from the Arabidopsis Biological Resource Center (ABRC) at The Ohio State University. The T-DNA was determined to be inserted in the fifth exon by analyzing the information from database and the gene sequence; transcription of *AtPFA-DSP4* in the mutant was not detected by RT-PCR. We also generated *AtPFA-DSP4*-overexpressing transgenic plants; two independent overexpression lines, OX8 and OX11, were used in our experiments. The expression levels of *AtPFA-DSP4* in OX8 and OX11 were increased, 55.5-fold and 74.5-fold compared with Col-0, respectively (Figure 4B). The Arabidopsis plants were treated with a *Pst* DC3000 suspension in distilled water. Compared with Col-0, OX8 and OX11 lines were sensitive to *Pst* DC3000 at 3 dpi; based on color, the lesions on OX8 and OX11 leaves which were injected with the *Pst* DC3000 suspension were more extensive than those observed on Col-0 (Figure 4C). However, the lesion area on the *atfpa-dsp4* mutant was not appreciably different than Col-0 (Figure 4C). We also calculated the amount of *Pst* DC3000 per unit area in leaves infected with *Pst* DC3000. The bacterial cell counts in OX8 and OX11 plants were 1.14-fold and 1.13-fold higher, respectively, than in Col-0 at 3 dpi, but the bacterial cell count in the mutant was not distinct from Col-0 at 3 dpi (Figure 4D). These results...
indicated that AtPFA-DSP4 is involved in Pst DC3000 response and negatively regulated the process.

**AtPFA-DSP4 inhibited accumulation of H₂O₂ and photosynthesis in the Pst DC3000 response**

We examined the effects of AtPFA-DSP4 on the photosynthetic capability of Arabidopsis after Pst DC3000 infection. The results of this study showed that AtPFA-DSP4 inhibited photosynthesis in all plants at 3 dpi, but the inhibition observed in OX8 and OX11 plants was reduced by approximately 29% and 41% compared with Col-0 (Figure 5A). We also detected the generation and accumulation of H₂O₂ in the leaves at 2 hpi using DAB staining. The mock treatment (distilled water) did not have an obvious affect on the production and accumulation of H₂O₂ in leaves at 2 hpi. However, infection with Pst DC3000 resulted in a marked increase in the accumulation of H₂O₂ in leaves of Col-0 and the atfpa-dsp4 mutant, but not in leaves of the OX8 and OX11 plants (Figure 5B). Based on these results, we concluded that AtPFA-DSP4 inhibited photosynthesis and suppressed production and accumulation of H₂O₂ during the response to bacterial infection.

**Discussion**

Protein phosphatases dephosphorylate activated protein kinases, and play important roles in plant processes such as growth and the response to abiotic and biotic stresses [12,39,40,41]. The results presented here show that OsPFA-DSP2 negatively regulates the pathogen response in rice. In the atfpa-dsp2 mutant and RNAi2 plants, the expression profiles of OsPR1a and OsPR5 were enhanced in the early stages of M. grisea infection compared with CK, and the lesion area was similar to that of CK at 7 dpi (Figure 2D-G and 3B). The phenotype of the atfpa-dsp2 mutant and RNAi2 plants was not obviously different from the CK at 7 dpi, leading us to suggest that the homologous genes exist in rice. The expression of OsPR1a and OsPR5 in OX13 and OX21 plants was decreased compared with CK, and the lesion area was more extensive than in CK, because of overexpression of OsPFA-DSP2 in rice (Figure 2D-G and 3B). A previous study showed that a mutant deficient in AtMKP1 expression suppressed the proliferation of Pst DC3000 and elevated the resistance to Pst DC3000 through the SA-mediated signalling pathway [20]. The function of OsPFA-DSP2, which negatively regulates the response to M. grisea infection, was consistent with the function of AtMKP1 [20]. However, rice plants lacking the phosphatase XB15 (a member of the PP2C subfamily) were sensitive to M. grisea, and its function was contrary to that of phosphatase OsPFA-DSP2 [17].

We also demonstrated that AtPFA-DSP4 acts as a negative regulator in the host response to Pst DC3000 infection. The AtPFA-DSP4 mutant showed reduced bacterial proliferation and enhanced resistance to Pst DC3000, but OX8 and OX11 plants were susceptible to Pst DC3000 at 3 dpi (Figure 4C and D). Several studies have shown that AtMKP1 and AtPTP1 negatively regulate the AMPK3/6 signaling pathway, and act to inhibit biosynthesis of salicylic acid (SA). However, the AtMKP1 mutant displayed growth defects and enhanced resistance to pathogen Pst DC3000 [20,42]. The function of AtPFA-DSP4 was found to be similar to that of AtMKP1, and was contrary to the function of AtMKP2 [18,20].

Pathogen infection leads to the generation and accumulation of ROS in plants; H₂O₂ is an important component of ROS, and plays key roles in plants, such as regulating growth and development, signal transduction and oxidative damage.
Figure 4. Phenotypes of atpfa-dsp4 mutant, Col-0 and AtPFA-DSP4-overexpression plants following Pst DC3000 infection. (A) Genomic structure and transcription of AtPFA-DSP4 detected using RT-PCR. The T-DNA was inserted into the fifth exon. (B) Expression of AtPFA-DSP4 in Col-0 and overexpression plants was quantified using qRT-PCR. (C) Phenotype of Col-0, atpfa-dsp4 mutant, and AtPFA-DSP4-overexpression plants at 3 days after Pst DC3000 infection. (D) Bacterial counts in leaves of Col-0, atpfa-dsp4 mutant, and AtPFA-DSP4-overexpression plants at different days after inoculation with Pst DC3000. The experiment was repeated with three biological replicates *P<0.05.

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Figure 5. Photosynthetic inhibition and accumulation of H2O2. (A) The effects of AtPFA-DSP4 on inhibition of photosynthesis. The photosynthetic capacity of Col-0, atpfa-dsp4 mutant and AtPFA-DSP4-overexpression plants was detected using the IMAGE-PAM system at days zero, two and three following Pst DC3000 inoculation. The experiment was repeated with three replicates (plants: n = 10), and averaged, **P<0.01, compared with Col-0 under same treatment condition. (B) Detection of H2O2 in leaves at 2 hpi after mock (distilled water) treatment and Pst DC3000 inoculation. The experiment was repeated with three independent replicates (leaves: n = 4).

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The results of our study showed that OsPFA-DSP2 and AtPFA-DSP4 inhibited the production and accumulation of H$_2$O$_2$ in leaves of overexpression lines in the early stages of infection. Recent studies have reported that endogenous and exogenous H$_2$O$_2$ inhibited pathogen cell growth, penetration and proliferation at the infection site by inducing PCD [3,8,9]. H$_2$O$_2$ levels in transgenic plants overexpressing OsPFA-DSP2 or AtPFA-DSP4 did not accumulate to control levels, which lead to inactivation of downstream signaling molecules and reduced expression of defense-related genes, such as PR genes. Pst DC3000 grew and proliferated rapidly in the overexpression lines, which were more susceptible to pathogens. However, because the appearance of black spots on the leaf surface of OX13 and OX21 plants was observed at 7 dpi, we suggest that H$_2$O$_2$ in the overexpression lines is produced during the later stages of fungal infection, leading to the appearance of HR symptoms (Figure 2F).

The results presented here show that AtPFA-DSP4 and OsPFA-DSP2 (data not shown) inhibited the photosynthetic capacity during the later stages of infection. Pathogen inoculation may result in the production and accumulation of H$_2$O$_2$, but a certain percentage of H$_2$O$_2$ is derived from the chloroplast [4,5,7,45]. H$_2$O$_2$ as a signaling molecule can negatively regulate photosynthetic capacity [46]. Therefore, we presumed that the integrity of chloroplasts was impaired by intense H$_2$O$_2$ production during the later stages of infection, leading to the observed decrease in photosynthesis in the overexpression lines. However, the production and accumulation of H$_2$O$_2$ in overexpression plants was not synchronous with the inhibition of photosynthesis. On the one hand, as a first defense system, the burst of H$_2$O$_2$ is rapid; on the other hand, chloroplast act as a semi-independent organelle, have the ability of auto-repair, resulting in delaying the inhibition of photosynthetic capacity. H$_2$O$_2$ is an upstream signaling molecule, and is involved in regulating the MAPK cascade [20,42,47].

In conclusion, we have demonstrated that two novel homologous genes from rice and Arabidopsis, OsPFA-DSP2 and AtPFA-DSP4, function as negative regulators in the pathogen response through the H$_2$O$_2$-mediated pathway. However, H$_2$O$_2$ plays critical roles in different signaling cascades, and which H$_2$O$_2$-mediated pathway is modulated by OsPFA-DSP2 and AtPFA-DSP4 is not clear. To further understand the functions of OsPFA-DSP2 and AtPFA-DSP4 in response to biotic stress, it will be critical to identify the upstream and downstream effectors which function in the OsPFA-DSP2 and AtPFA-DSP4 signaling pathways, and to demonstrate whether OsPFA-DSP2 and AtPFA-DSP4 function in biotic response.

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Author Contributions

Conceived and designed the experiments: HH JS JW HW. Performed the experiments: HH SS. Analyzed the data: BL DF. Wrote the paper: HH SS HW. Transformed and obtained plants: HH JS YZ. Isolated rice protoplast and observed the fluorescence: YZ YA. Corrected the paper: HH SS YZ HW.

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