



Identification and comparative analysis of differential proteins expression in rice under biotic stress by protein sequencing

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Abstract

Rice (*Oryza sativa* L.) plant is vulnerable to a number of pest and diseases. Among them sheath blight disease caused by *Rhizoctonia solani*, insect pest and brown plant hopper (BPH) (*Nilaparvata lugens*) are the most devastating agents and major challenge to rice cultivation. Plant growth-promoting rhizobacteria (PGPR) are associated with plant roots which augment plant productivity and immunity. Protein analysis was carried out to study the molecular mechanisms underlying PGPR mediated pest and disease resistance and growth promotion. Plants were treated with and without *Pseudomonas fluorescens* strain *Pfl* and challenged with pest and pathogen at 0, 6, 24, 48, 72, and 96 h after inoculation. The comparative analysis of relative abundances of protein bands between inoculated and non-inoculated samples was carried out. Five proteins were upregulated and 15 were differentially regulated in PGPR-primed plants challenged with BPH. In PGPR-primed plants challenged with pathogen, 27 proteins were upregulated. The differential protein bands were sequenced by Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS). Protein sequencing results showed high-percent homology with chloroplastic aldolase, fructose-bisphosphate aldolase, peroxidase, 2-cys peroxiredoxin bas1, chloroplastic-like, small subunit of ribulose-1,5-bisphosphate carboxylase, Os12g0291400 and hypothetical protein OsI_38046. Western blotting detected the presence of PR protein chitinase. The analysis confirmed the presence of chitinase of molecular weight 17, 20, and 35 kDa in PGPR primed plant challenged with *R. solani*. These results showed that the differentially expressed proteins possibly play role in biotic stress defense in plants challenged with biotic stress. Expression proteins remarkably influenced by *Pfl* colonization, which might be a key element for induced systemic tolerance by PGPR.

Keywords *Pseudomonas fluorescens* · *R. solani* · Brown plant hopper · Chitinase and MALDI-TOF/MS

Introduction

Rice (*Oryza sativa* L.) is one of the major food crops for about 65% of the world's population and is the staple food for an expansive part of the world, particularly in Asia (Chatterjee et al. 2021). Biotic stress including pest and diseases is the major concern that pulls down the productivity of rice. Among the pest, brown plant hopper (*Nilaparvata lugens*) is one of the most destructive pests of rice throughout Asia. In addition to reducing yields through the direct sucking of plant sap, it acts as a vector for viral diseases like rice grassy stunt and ragged stunt. Brown planthopper (BPH) is re-emerging as a key pest threatening rice production in tropical Asia (Otuka 2013). Furthermore, the soilborne Basidiomycete fungus *Rhizoctonia solani* (teleomorph: *Thanatephorus cucumeris*) is a large species complex containing related but genetically distinct groups of pathogens affecting many plants species worldwide. It causes sheath

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blight in rice, which is considered to be one of the most devastating diseases on a global scale (Li et al. 2021). A predisposing infection with a pathogen causing a hypersensitive reaction is the classic way to induce induced resistance. This elevated resistance response is known as systemic acquired resistance (SAR), which results from challenge inoculation of distant plant parts. SAR was first characterized in tobacco plants that expressed increased resistance systemically after infection by tobacco mosaic virus (Ross 1961). Pathogen-induced SAR is associated with an early increase in endogenously synthesized salicylic acid (SA) (Tripathi et al. 2019). Accumulation of SA is critical in the signaling pathway that controls SAR, since plants that do not accumulate SA are incapable of expressing induced resistance (Gruner et al. 2018). Furthermore, SAR is characterized by the activation of so-called SAR genes (Delaney et al. 1994), including genes that encode pathogenesis-related (PR) proteins (Ali et al. 2018), which are often used as markers for the state of induced resistance. Both PR genes and induced resistance are expressed in plants treated with SA (Molinari and Leonetti 2019). Plant growth-promoting rhizobacteria (PGPR) suppress a variety of root and vascular diseases caused by soilborne pathogens (Bhattacharyya and Jha 2012). PGPR suppress disease by antagonizing pathogens via different mechanisms such as antibiosis, competition for iron or carbon, and production of lytic enzymes. PGPR also activate plant defense resulting in systemic protection against different fungal, bacterial, and viral pathogens (Pieterse et al. 1996), a phenomenon termed induced systemic resistance (ISR). An alternative approach to inducing systemic resistance was reported by Alstrom (1991), Peer et al. (1991), and Wei et al. (1991). These authors independently demonstrated that selected strains of nonpathogenic plant growth-promoting rhizobacteria colonize the rhizosphere of the plant and are able to elevate plant resistance. Moreover, these bacteria, mainly fluorescent *Pseudomonas* spp., had been studied for their ability to control soilborne pathogens through competition for nutrients, siderophore-mediated competition for iron, or antibiosis (Ran et al. 2005). It appeared that these bacteria could also provide protection against disease by elevating systemic resistance within the plant. Induced systemic resistance (ISR) mediated by nonpathogenic rhizobacteria has been demonstrated in several plant species (Choudhary et al. 2007) and shown to be effective against bacterial, viral, and fungal diseases. Maurhofer et al. (1994) showed that ISR induced by *Pseudomonas fluorescens* strain CHA0 in tobacco is associated with PR protein accumulation, suggesting that nonpathogen-induced ISR and pathogen-induced SAR share similar mechanisms. Recent development of high-throughput techniques, omics data are providing opportunities for research into the molecular mechanisms of biological phenotypes (Kumar et al. 2015; Stephen et al. 2022). The present study was undertaken to analyze proteome with

special reference to biotic stress tolerance in a popular high-yielding rice variety Jyothi through application of *P. fluorescens*. Protein analysis was carried out to study the molecular mechanisms underlying the PGPR mediated pest and disease resistance and growth promotion.

Materials and methods

Study materials

The high-yielding rice variety of Kerala, Jyothi (PTB-39), susceptible to blight and BPH, was used in this study. A commercial *P. fluorescens* strain, *Pf1*, developed by Kerala Agricultural University (Thrissur, India) and found to exhibit plant growth promotional activity in rice under both laboratory and field conditions, was used.

Biopriming with *Pseudomonas fluorescens* Pf1 strain

Thirty-five-day-old rice plants were treated with *Pf1*, and untreated plants served as the control. The experiment was conducted in a completely randomized block design with five pots per treatment and three plants per pot. Plants were treated with *Pf1* by two different modes, first as seed treatment and second as foliar spray at 35 days after sowing. For the seed treatment, rice seeds were soaked in the sterile distilled water containing talc-based formulation (10 g/kg of seed). After 24 h, the suspension was drained off, and the seeds were dried under shade for 30 min and sown. For the foliar spray, the talc-based formulation was dissolved in water (20 g/L; 2%) and sprayed.

Isolation and culturing of *Rhizoctonia solani*

Rice sheath blight pathogen *Rhizoctonia solani* was isolated from infected rice plants collected from the fields of Agriculture Research Station, Mannuthy (Kerala, Thrissur). The portion of the leaf sheath showing typical lesions was cut into small pieces, surface sterilized with 0.1% sodium hypochlorite for 1 min, and washed thrice before placing on Potato Dextrose Agar (PDA) medium. The axenic culture of the pathogen was obtained by single hyphal tip technique and maintained in PDA slant at 30°C under laboratory conditions for further use.

Challenge inoculation with *Rhizoctonia solani*

Forty-five-day-old plants were inoculated with immature white sclerotia obtained from the 4-day-old *R. solani* culture. The immature sclerotia were placed between the leaf sheath and stem without any injury and wrapped with moist cotton; the inoculated plants were covered with polythene

to maintain humidity. Both Pf-treated and non-treated plants were inoculated with *R. solani* culture. The experiment was conducted in a completely randomized block design with five pots per treatment and three plants per pot. Sheath blight intensity 7 days after inoculation was calculated based on a 0–9 grading scale of Standard Evaluation System for rice, IRRI (1980). The percent disease incidence was calculated using the following formula Wheeler (1969).

$$\text{PDI} = \frac{\text{Sum of all numerical rating}}{(\text{Total number of tillers observed} \times \text{Maximum disease grade})} \times 100$$

Mass rearing of brown plant hopper

Mass rearing of BPH was done following the method described. Rice seedlings were raised in earthen pots, and the pots were kept partially immersed in a plastic tray containing water. These trays were kept in insect rearing cages (3 m × 2.5 m × 2.75 m) with netting. BPH adults were collected from the rice fields of the Regional Agricultural Research Station (Pattambi, Kerala) and released onto individual plants kept inside the rearing cages for oviposition; plants damaged by BPH were periodically replaced with fresh plants. The plants were always maintained free of spiders, mired bugs, and ants for effective BPH rearing. Nymphs emerged a week after oviposition.

Challenge inoculation with BPH

The efficiency of *Pfl* treatment on rice plants in protecting against BPH was assessed using five pots per control and *Pf* treatment. The fourth and fifth instar nymphs of BPH were carefully collected from the cage using an aspirator, starved for 5 h, and released (10 nymphs/pot) on to sheath of *Pseudomonas* treated rice plants covered with Mylar film and allowed to feed for 7 days. Percent mortality and damage caused by BPH was scored from undamaged (level 0) to complete hopper burn (level 9) based on Inger scale (Inger et al. 1996). Damage rating was done as per Standard Evaluation System for rice (IRRI 2002). The shoot length, root length, number of tillers, fresh weight, and dry weight of the control and treated plants were also measured 30 days after sowing. Statistical analysis was carried out using *t*-test.

Challenge inoculation with insect and pathogen

Forty-five-day-old rice plants were inoculated with the pathogen *R. solani* and the insect BPH. After 24 h of inoculation, pest/disease development was assessed based on symptoms. Leaf sheaths from different treatments, including PGPR treatment and challenged with *R. solani* (T1), PGPR treatment and challenged with BPH (T2), inoculation with *R. solani* alone (T3), inoculation with BPH alone (T4), PGPR

alone (T5), and absolute control (T6), were collected in liquid nitrogen.

Assay of defense-related enzymes

Samples collected from rice plants under different treatments were used to study the induction of defense enzymes in response to pest and pathogen attack. Leaf and sheath tissues from each treatment were collected at 0, 6, 24, 48, 72, and 96 h intervals and immediately homogenized with liquid nitrogen. One gram of the powdered sample was extracted with 2 mL of 0.1 M sodium citrate buffer (pH 5.0) at 4 °C, and the homogenate was centrifuged for 20 min at 10,000 rpm to collect the supernatant. Protein content in the supernatant was determined by the Bradford method using bovine serum albumin (Thermo Scientific) as the standard.

Assay of peroxidase enzyme

Leaf- sheath sample (1 g) was homogenized in 2 mL of 0.1 M phosphate buffer (pH 7.0) at 4 °C. The homogenate was centrifuged at 16,000 g at 4 °C for 15 min, and the supernatant was used as the enzyme source. The reaction mixture consisting of 1.5 mL of 0.05 M pyrogallol, 0.5 mL of enzyme extract, and 0.5 mL of 1% H₂O₂ was incubated at room temperature (28 ± 2 °C). The change in absorbance at 420 nm was recorded every 30 s for 3 min. The enzyme activity was expressed as the change in the absorbance min⁻¹ mg⁻¹protein.

Protein profiling by SDS-PAGE

Fresh leaf tissue ground using pestle and mortar in liquid nitrogen reconstituted with 0.2 M phosphate buffer (pH 7.3) and extracted total proteins from both treated and untreated plants. The protein extracted from different treatment samples was normalized to 1000 µg/mL, and the proteome in these samples was analyzed using SDS-PAGE. The protein profile was visualized using a white light transilluminator (Biorad) and analyzed using Quantity 1 software.

Development of polyclonal antibody for chitinase enzyme

The polyclonal antibodies was developed in 1-year-old New Zealand rabbits using crude protein extracted from *Trichoderma harzianum* following the method described by Acharya (2016). The pre-immune and post-immune serum collected from the animal was purified and used to detect the antibody titer by Outcherlony double immunodiffusion assay. The antibody was further used for Western blot analysis.

Western blot analysis

Approximately 15 µg of total protein from each sample was run on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electro blotted onto nitrocellulose membrane (Millipore, India) in western transfer buffer (25 mM Tris, 150 mM glycine, 20% methanol and 0.04% SDS) for 3 h at a constant voltage of 50 V. A commercially available NAP (non-animal protein) blocker (G-Biosciences) was used to accomplish the blocking. The membrane was hybridized with a 1:3000 dilution of specific primary antibody and then with 1:6000 dilutions of horseradish peroxidase-conjugated secondary antibody. The membrane was washed four times (10 min each time) with 1× PBST after blocking with the secondary antibody. After incubation with secondary antibody, the substrate TMB (3,3',5,5'-Tetramethylbenzidine) was added and incubated in dark for about 30 min to form blue colored bands.

Characterization of differentially expressed proteins

The upregulated and the differentially expressed protein bands were sequence analyzed by MALDI-TOF/MS at the Sandor Proteomics Pvt. Ltd. (Hyderabad, India). The obtained peaks were analyzed with the bioinformatics tool, MASCOT/MS peptide search engine ([https://www.matrix](https://www.matrixscience.com/search_form_select.html)

[science.com/search_form_select.html](https://www.matrixscience.com/search_form_select.html)) was used to characterize proteins.

Results

Pathogenicity test for *R. solani*

Under favorable conditions, *R. solani* inoculation in rice resulted in typical sheath blight symptoms within 7 days. Initially, minute specks and small lesions were found, which gradually enlarged to form lesions with a grayish white center with brown margin. As the disease progressed, several lesions coalesced to form blights. Infection started near the water line and slowly spread to the upper leaf sheath and leaf blades. Severely affected plants dried up completely after 96 h (Fig. 1). A comparison of the control and *Pfl*-primed plants showed that the PDI of the control plants (47.33%) was remarkably higher than the plants treated with *Pfl* (14.19%; Tables 1 and 2). The significantly low PDI of the *Pfl*-primed plants indicated that *Pfl* induces resistance to *R. solani* in rice.

Percent mortality and damage due to BPH

The application of *Pfl* highly influenced the BPH feeding preference and extent of damage on rice plants. The damage assessment 7 days after pest release revealed 28% BPH

Fig. 1 Symptoms appearance on *Pfl* treated and control plants after different intervals of inoculation with *R. solani* A: 24 h after inoculation, B: 48 h after inoculation, C: 72 h after inoculation D: 96 h after inoculation

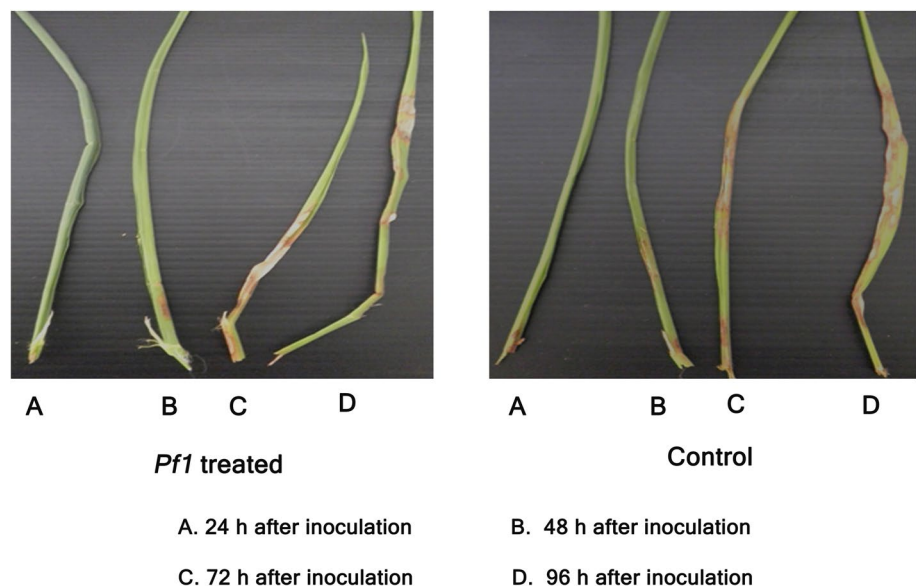


Table 1 Percent mortality of BPH in *Pfl* treated and control plants

Treatments	R1 (%)	R2 (%)	R3 (%)	R4 (%)	R5 (%)	Mean (%)
<i>Pfl</i> +BPH	30	20	30	40	20	28
Control+BPH	0	0	0	0	0	0

Table 2 Damage scale rated by SES on control and *Pf1* treated plant by BPH

Treatments	R1	R2	R3	R4	R5	Mean
control + BPH	3	3.7	4.7	3.6	4.1	3.82
<i>Pf</i> +BPH	0	0	0	0	0	0

Table 3 Measurement of biometric parameters

Characters	<i>Pf1</i> Treated	Control	T value
Shoot length(cm)	33.26	31.98	6.74**
Root length(cm)	39.41	28.37	3.54**
Number of tillers	3.50	3.04	3.73**
Fresh weight(kg)	36.19	30.82	7.7**
Dry weight(kg)	27.64	22.68	10.47**

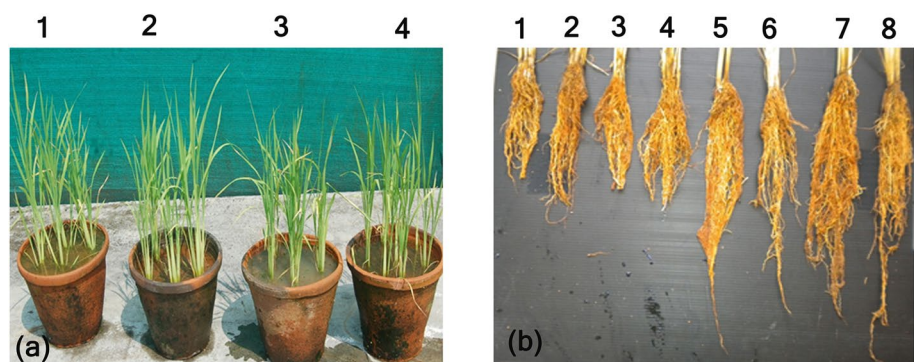
**Significant at 1% level

mortality in *Pf1*-treated plants, whereas no BPH mortality was noticed in the control plants (Tables 2 and 3). BPH actively fed the control plants, resulting in damage, whereas the pest caused no noticeable damage to plants treated with *Pf1*. The *Pf1*-treated plants exhibited a convincingly high level of resistance to BPH attack, unlike the control plants, which were moderately susceptible.

Measurement of biometric parameters

The growth parameters such as shoot length, root length, number of tillers, fresh weight, and dry weight of treated and control rice plants were measured 30 days after sowing. The observed average shoot length, root length, fresh weight, and dry weight were significantly higher in *Pf1*-treated plants than the control plants; 4.08, 28.01, 13.14, 14.83, and 7.06% increase in shoot length, root length, number of tillers, and dry weight of *Pf1*-primed plants was observed, while the control plants failed to attain any gain in these parameters. Thus, the indisputable role of PGPR in plant growth promotion was upheld in rice plants (Table 3 and Fig. 2).

Fig. 2 **a** Plants 30 Days after sowing. 1 and 2 indicates *Pf1* treated plants 3 and 4 are control plants; **b** enhanced root growth in rice seedlings treated with *Pf1*. 1–4 indicate the roots of control plants, 5–8 are the roots of *Pf1* treated plants



Analysis of *Pf1*-induced protein expression profiles

The protein bands of rice plants treated with *Pseudomonas* bacteria strain *Pf1* were analyzed after challenge inoculation with BPH. The banding pattern of *Pf1*-treated plants subsequently challenged with BPH was more predominant than the uninoculated control plants. Analysis of the differences in proteome profile between the *Pf1* treated and non-treated rice leaves challenged with BPH revealed that 100 proteins were upregulated while 5 were down regulated in BPH challenged plants, and 15 differential proteins were noticed in PGPR-primed plants challenged with BPH compared to unprimed plants challenged with BPH (Figs. 3 and 4).

Expression analysis of *Pf1*-treated rice plants challenged with *R. solani*

The protein extracted from the rice plants treated with *Pf1* and inoculated with *R. solani* was analyzed. The protein banding pattern was more prevalent in *Pseudomonas*-treated plants challenged with *R. solani* than the uninoculated control plants. Detailed analysis revealed that 27 protein bands were upregulated in *R. solani* challenged samples, whereas the banding pattern remained the same in both *Pf1*-primed and nonprimed plants. However, no difference was observed in *R. solani*-treated *Pf1* plants compared with BPH challenged + *Pf1*-treated plants (Figs. 3 and 4). In BPH challenged plants, 100 proteins were upregulated while 5 were downregulated. Fifteen differential proteins were noticed in PGPR-primed plants challenged with BPH. Two proteins bands of 19 and 30 kDa, differentially expressed at 24 and 48 h, in *Pf1*-primed plants were selected for sequence analysis out of the 15 differentially expressed bands. In *R. solani* treated plants, 27 proteins were upregulated, out of

Fig. 3 Differential protein expression analysis from BPH and *Pf* treated plants using SDS-PAGE

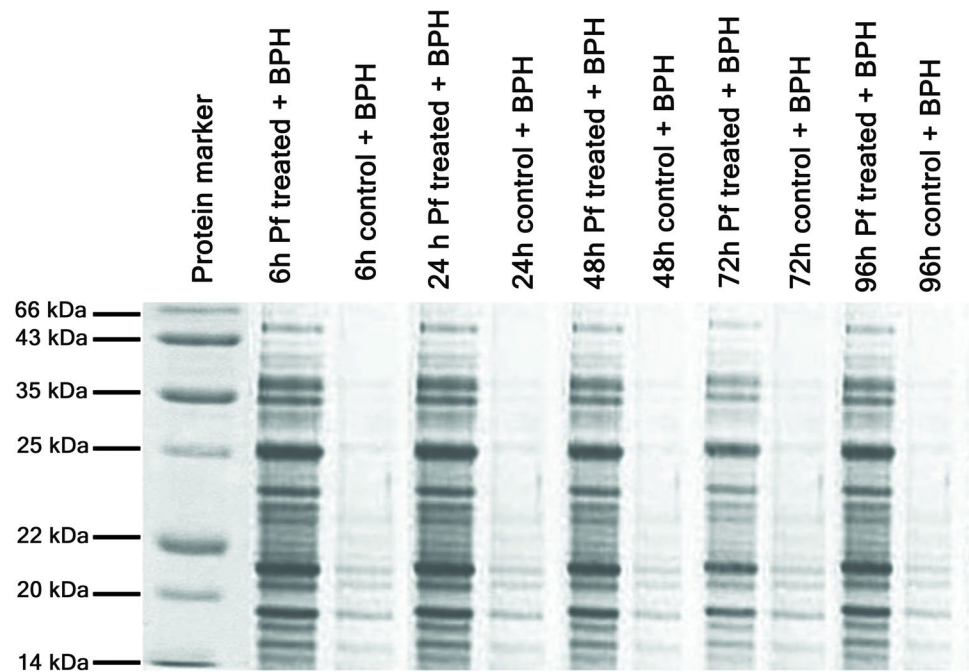
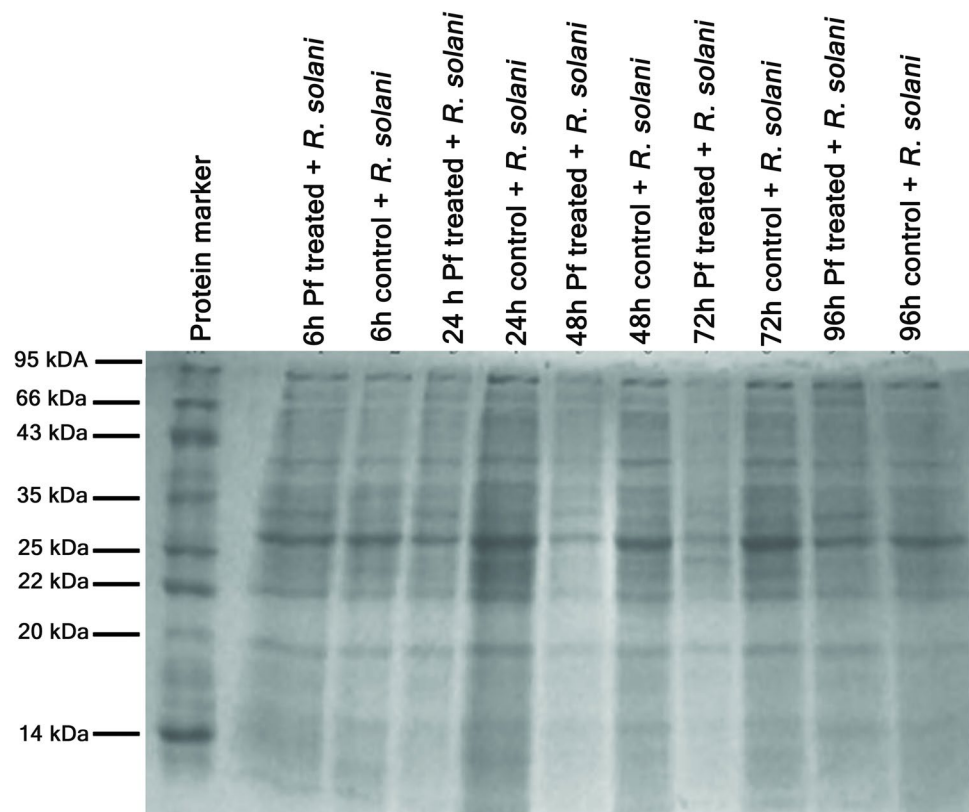


Fig. 4 Differential protein expression analysis from *R. solani* and *Pf* treated plants using SDS-PAGE



which one protein band of 29 kDa upregulated after 4 h in *Pf*-primed plant was selected. The sequences of the three selected proteins were annotated to protein databases by NCBI BLASTp. One among the identified proteins showed 81% similarity with peroxidase of *Oryza sativa Indica*

group. Remaining two protein sequences showed homology with the rice protein of japonica cultivars. The identified proteins were fructose-bisphosphate aldolase, Os02g0537700-*Oryza sativa (Japonica Cultivar-Group)*, and Rubisco complexed with 2-carboxyarabinitol-1,5- bisphosphate. Protein

band of 19 kDa (denominated 1) was selected from the *Pfl*-primed plant challenged with BPH. The sequence alignment revealed 99, 94, and 81% homology with chloroplastic aldolase, fructose-bisphosphate aldolase, and peroxidase, respectively. Protein band 2, of 29 kDa obtained from the *Pfl*-treated plants challenged with the pathogen, showed 84 and 86% homology with 2-cys peroxiredoxin bas and 2-cys peroxiredoxin basI, chloroplastic-like, respectively. The protein band 3 selected from the *Pfl*-primed plant infected with BPH was 30 kDa and exhibited 99% to the small subunit of ribulose-1,5-bisphosphate carboxylase and 100% similarity to Os12g0291400 and a hypothetical protein OsI_38046. Details of MALDI-TOF search alignment are given in Table 4 and the MALDI-TOF search alignment of protein bands are provided in supplementary file 1.

Analysis of peroxidase activity

The enhanced activities of defense-related enzyme peroxidase (PO) in the *Pfl*-treated rice plants indicate the development of induced systemic resistance against sheath blight disease and BPH insect in rice. An increase in PO activity was detected between 6 to 96 h after challenge inoculation in *Pfl*-primed plants. Exactly 43.62% boost in PO activity was observed in pathogen-challenged plants over the control and 21.9% in BPH challenged *Pfl*-primed plants.

Western Blot

Western blot analysis confirmed that the 17, 20 and 35 kDa proteins were expressed in *Pfl*-treated plants challenged with

R. solani inoculation after 96 h, and there was no detection in control plant protein sample (Fig. 5). These banding patterns indicate the presence of chitinase in PGPR-primed plants challenged with *R. solani* and the expressed chitinase possibly provides resistance against the pathogen.

Discussion

Broad conglomeration of PGPR frequently colonizes the rhizosphere of plant species and provides beneficial effects, such as enhanced plant growth and reduced disease susceptibility. In addition, plants respond to PGPR by enduring physiological or biochemical changes, commonly referred to as ISR. This PGPR-induced ISR has suppressed various plant diseases under both greenhouse and field conditions (Yi et al. 2013). In the present study, rice plants that had been treated with *Pfl* were resistance to sheath blight than controls. Significantly lower PDI (14.19) was observed in *Pfl*-treated plants than control plants (47.33), indicating that *Pfl* induced resistance to *R. solani* infection in rice through ISR. These observations are reliable with the earlier reports. Insect or pathogen attack triggers an endogenous defense mechanism in all plants. Various defense genes are induced or activated in response to the stimuli or signals. Researchers have proven that the self-defense mechanisms in plants can be instigated by applying a biological inducer, which is used as a novel and efficient plant protection strategy. The fluorescent *Pseudomonas* that has been efficiently controlled various soilborne pathogens. *Pseudomonas fluorescens* successfully demonstrated to control the damping-off of cucumber (Rezzonico et al. 2005), red rot of sugarcane

Table 4 Proteins identified by sequence analysis in response to PGPR priming and biotic stress in rice by MS-MALDI-TOF

Spot no	Name of protein	Identity	Organism	Nature of gene regulation	Accession
1	Fructose-bisphosphate aldolase	100	<i>Oryza sativa Indica Group</i>	DE	ABA91631.2
1	chloroplastic aldolase	98	<i>Oryza sativa Japonica Group</i>	DE	NP 001065862.1
1	fructose-bisphosphate aldolase	94	<i>Zea mays</i>	DE	ACG36798.1
1	peroxidase	81	<i>Oryza sativa Indica Group</i>	DE	ACM17593.1
2	Hypothetical protein OsI_07554	88	<i>Oryza sativa Indica Group</i>	DE	EEC73346.1
2	2-cys peroxiredoxin basI	84	<i>Zea mays</i>	DE	NP 001137046.1
2	2-Cys peroxiredoxin BAS1, chloroplastic-like	86	<i>Brachypodium distachyon</i>	DE	NP 001148975.1
2	2-Cys peroxiredoxin BAS1, chloroplastic-like	86	<i>Zea mays</i>	DE	ACG 35092.1
3	Rubisco Complexed With 2-Carboxyarabinitol-1,5-Bisphosphate	100	<i>Oryza sativa Japonica Group</i>	UP	NP 001066606.1
3	Os12g0291400	100	<i>Oryza sativa Japonica Group</i>	UP	NP 001066607.1
3	hypothetical protein OsI_38046	100	<i>Oryza sativa Indica Group</i>	UP	EAY 82834.1
3	Os12g0291100 [100	<i>Oryza sativa Japonica Group</i>	UP	NP001066604.1
3	small subunit of ribulose-1,5-bisphosphate carboxylase	99	<i>Oryza sativa Japonica Group</i>	UP	BAA00539.1

DE Differentially expressed; UP upregulated

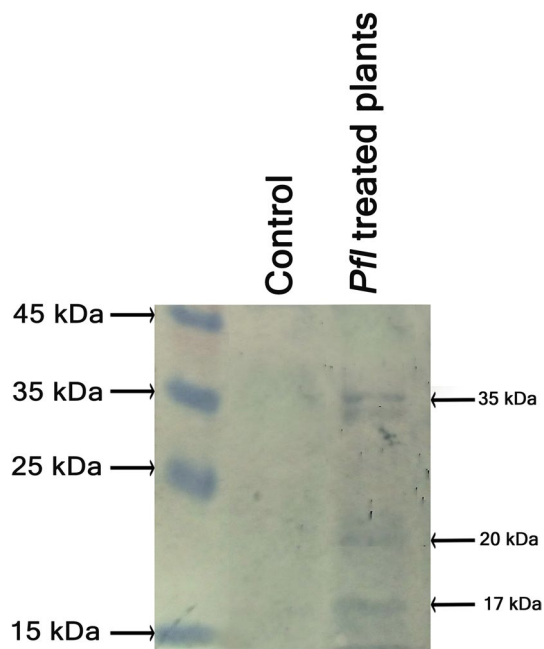


Fig. 5 Western blot analysis showing detection of R proteins at 17, 20 and 35 kDa

(Anita and Samiyappan 2012), and sheath blight in rice (Vidhyasekaran and Muthamilan 1999). The studies also reported that the type III protein secretion system present in *P. fluorescens* effectively counter the effectors in pathogens. In the present study, the *P. fluorescens* isolate *Pfl* reduced the intensity of sheath blight in rice. Jaleel et al. (2007) had indicated that *P. fluorescens* serves as a strong elicitor of plant defense reactions. Studies have suggested that the pre-application of fluorescent *Pseudomonas* toughens host cell wall structure, thereby restricting pathogen invasion into plant tissues (Preston 2004). For instance, T3SS gene *hrcV* a type III protein secretion system present in *P. fluorescens* reduced polygalacturonase activity in *Pythium ultimum* thus provided tolerance to cucumber from pathogen mediated damping-off (Couillerot et al. 2009). Our previous transcriptome studies demonstrated the effect of *Pfl* in enhancing abiotic stress tolerance (Saakre et al. 2017). In contrast, the present study was performed to validate the effect of *Pfl* against biotic stress at the protein level. The study clearly depicts that *Pfl* induces the activity of enzymes associated with phenylpropanoid metabolism and PR protein accumulation in rice in response to *R. solani* challenge inoculation. Seed treatment with PGPRs has triggered the plant to synthesize small amounts of defense-related compounds, which occurred as an immune response to pathogen inoculation. The production of defense-related proteins in *Pfl*-primed plants before challenge inoculation and greater accumulation of defense compounds in *Pfl*-treated plants after pathogen challenge indicates that biopriming can trigger ISR at time

of infection. Thus, the present study proposes the application of fluorescent *Pfl* in rice as a promising approach to induce resistance against sheath blight.

The application of *Pfl* highly influenced the feeding preference and damage caused by BPH in the rice plants. The highest mortality of BPH occurred on plants treated with *Pfl*, whereas the BPH released on control plants remained alive. Consequently, the damage index was also significantly higher on control plants than the plants treated with *Pfl*. These observations suggest that the application of *P. fluorescens* bioformulation significantly reduced the pest incidence in rice. The ISR or SAR mechanisms or a combination of both may be involved in pest resistance of rice, consistent with numerous earlier reports. Management of insect pests by various *Pseudomonas* strains either as a bacterial suspension or other formulations have been reported (Senthilraja et al. 2013). It is evident that feeding pattern of various insect pests has altered due to the application of *Pseudomonas* strains on plants. Karthiba et al. (2010) reported 20% weevil mortality in chestnuts sprayed with *P. fluorescens* suspension. Previous investigations have also proven that *Pseudomonas*-treated rice leaves altered the feeding behavior of leaf folder, reduced larval and pupal weight, and increased the larval mortality and incidence of malformed adults under in vitro conditions (Radjacommare et al. 2002). In addition, the PGPR possess the ability to produce plant growth hormones like auxins, cytokinins, gibberellins, and ethylene (Cassán et al. 2014; Nysanth et al. 2019). Rhizobacteria produced auxins promote plant growth. Ahirwar et al. (2015) reported an increase in root and shoot fresh weight of tomato, cucumber, lettuce, and potato after bacterization with *Pseudomonas* strains. Furthermore, direct growth stimulation occurred as a result of ACC deaminase enzyme secreted by PGPR that has the ability to lower plant ethylene level during biotic and abiotic stress (Glick 2014). In the present study, the plants treated with *Pfl* showed an increase in all growth parameters, including plant height, active tillers, root length, wet weight, and dry weight, which confirms that the application of fluorescent *Pseudomonas* in rice promotes growth.

Further sequence analysis of the differentially expressed protein band 1 revealed significant homology with chloroplastic aldolase, fructose-bisphosphate aldolase, and peroxidase. The chloroplastic aldolase and fructose-bisphosphate aldolase belonging to class I adolases possess a key lysine residue in the catalytic site required for the formation of a covalent intermediate in the form of a Schiff base with its substrate prior to the aldol cleavage (LowKam et al. 2010). They play vital roles in photosynthesis, especially in the Calvin cycle. In the present study, the high expression of the protein band 1 in the PGPR-treated rice leaf sheath tissues compared to control indicates that the photosynthetic

activity might have facilitated attain greater growth and better defense.

The biochemical and molecular analysis of biomolecules associated with systemic resistance induced via *Pfl* priming revealed an increase in the activity of the defense-related enzyme peroxidase (PO) against sheath blight disease and BPH insect in rice. Peroxidases are commonly associated with shielding plants from pathogens. They are responsible for the radical dehydrogenation of sinapyl alcohol and coniferyl alcohol during lignin synthesis. Peroxidase polymorphism could be used as a biochemical marker indicating different levels of field resistance (Lebeda et al. 2001). The enzyme participates in insolubilization of the extracellular matrix processes in the extracellular matrix (Singh et al. 2010). Their association with the cell wall has also been confirmed. Peroxidases remove the toxic hydrogen peroxide from tissues and participate in synthesis of phenolic compounds and in the building of intermolecular bonds during the organization of the cell wall at the sites of infection by pathogens (Kerchev et al. 2015). The peroxidase enzyme is also involved in the synthesis of ethylene, the concentration of which increases during pathogenesis (Prasannath 2017). The present study found increased peroxidase activity in *Pf*-treated plants challenged with the BPH. Anand et al. (2010) reported the higher PO activity in cucumber roots challenged with *P. corrugate* as compared with control plants. The present study also found that a PO was prominently expressed in *P. fluorescens* isolate *Pfl* treated tissues against *R. solani*. This unique PO induced by *Pfl* might have contributed to induced defense in rice sheath against BPH infection.

The sequence analysis of protein band 2, which was differentially expressed in *Pfl* treated plants challenged with *R. solani*, was 29 kDa and showed 84 and 86% homology to 2-cys peroxiredoxin bas and 2-cys peroxiredoxin bas1, chloroplastic-like, respectively. 2-Cys peroxiredoxins are a large family of peroxidases that reduce alkyl hydroperoxides and hydrogen peroxide. These enzymes were identified in plants as ubiquitously occurring group of enzymes (Tripathi et al. 2009). However, *Bas1* expression is restricted to green tissues of the shoot (Baler and Dietz 1997) and *bas1* gene expression is regulated by cellular redox state, which depends on the antioxidant function of the enzyme while oxidative stressors increase the expression only slightly. Researchers have correlated peroxiredoxins and signaling networks under various stress responses, including pathogen elicitors, insect feeding, wounding, high temperature, and ABA-associated stomatal closure (Larkindale and Knight 2002; Apel and Hirt 2004; Peng et al. 2004; Mateo et al. 2006; Lakshmi et al. 2023). Moreover, peroxiredoxins are generally expressed more under oxidative stress, which is one of the most rapid defence reactions to pathogen attack in plants (Apel and

Hirt 2004). Therefore, the differential expression of protein band 2 in the PGPR-treated rice leaf sheath tissues challenged with pathogen indicates that 2-Cys peroxiredoxins was a part of the defense mechanism during the pathogen attack. The analysis of protein band 3 differentially expressed in *Pfl*-treated plants challenged with BPH (30 kDa) revealed high level of homology with the small subunit of ribulose-1,5-bisphosphate carboxylase (99%) and 100% similarity was observed to both Os12g0291400 and hypothetical protein OsI_38046; however, their roles in defense mechanism are not known. The level of RuBisCO proteins, one of the important enzymes in photosynthesis, is known to be reduced in infected plant cells under pest attack (Bilgin et al. 2010). RuBisCO proteins were also reported to be high in rice leaf sheath under wounding stress (Lee et al. 2006). Differential expression of band 3 proteins in the PGPR-treated rice leaf sheath tissues indicates increased photosynthetic activity that helped to attain greater growth and possibly related to plant defense. The chitinase enzyme was isolated from *Trichoderma hazianum* and purified by salt separation and dialysis. The purified chitinase was injected to rabbit to develop polyclonal antibody for chitinase. The presence of an antibody for chitinase was detected post-immune serum collected from rabbit by Ouchterlony double diffusion. The antibody developed was used to detect target protein in Western blot analysis. Results of the Western blot analysis also revealed that about 17, 20 and 35 kDa protein were developed in *Pf*-primed plants challenged with *R. solani* after 96 h of inoculation. These observations confirmed the upregulation of chitinase in PGPR-primed plants challenged with *R. solani*, indicating the role of chitinase in developing resistance against pathogen. Most chitinases have a molecular mass ranging between 15 and 43 kDa. Chitinase has been isolated from chickpea (Zarei et al. 2011), cucumber, barley (Kirubakaran and Sakthivel 2006), tobacco (Pu et al. 1996), black turtle bean (Chu and Ng 2005), tomato (Wu and Bradford 2003), and grapes (Sluyter et al. 2005). Few studies also found expression of chitinase gene in response to cold stress (−2 to −5 °C). The chitinases also have demonstrated significant antifungal activities against plant pathogenic fungi like *Alternaria sp.* of rice, *Bipolaris oryzae* of rice, *Botrytis cinerea* of tobacco, *Curvularia lunata* of clover, *Fusarium oxysporum*, *F. udum*, *Mycosphaerella arachidicola*, *Pestalotia theae* of tea, and *R. solani* of rice (Chu and Ng 2005; Saikia et al. 2005; Kirubakaran and Sakthivel 2006). The mode of action of PR-3 proteins is relatively simple and characterized. Chitinases cleave the cell wall chitin polymers in vivo, making the fungal cells osmotically sensitive (Prasannath 2017).

Conclusion

The present study found that the colonization of *P. fluorescens* influenced the differential expression of various proteins in rice plants; the differentially expressed chitinase, RuBisCO, and 2-cys peroxiredoxins provide resistance against the fungal pathogen, and chloroplastic aldolase, fructose-bisphosphate aldolase, and peroxidase provide resistance against BPH. Thus, *P. fluorescens* might be a key element for induced systemic resistance. The application of *P. fluorescens* will facilitate the rice plants to tolerate biotic stress via the expression of defense-related genes.

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Declarations

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