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Research article

Proteomic and transcriptomic approaches to identify resistance and susceptibility related proteins in contrasting rice genotypes infected with fungal pathogen *Rhizoctonia solani*

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ABSTRACT

The devastating sheath blight disease caused by *Rhizoctonia solani* Kuhn (teleomorph: *Thanatephorus cucumeris*) causes major yield loss in most rice growing regions of the world. In this study, two moderately tolerant and four susceptible genotypes of rice were selected for *R. solani* induced proteome analysis using two-dimensional polyacrylamide gel electrophoresis. Forty five differentially expressed proteins (DEPs) were identified and analyzed by Mass Spectrometry. Based on their functions, these proteins were classified into different groups, viz., photosynthesis, resistance and pathogenesis, stress, cell wall metabolism and cytoskeleton development associated proteins, and hypothetical or uncharacterized proteins. Expression of 14 genes encoding DEPs was analyzed by quantitative PCR which showed consistency in transcripts and genes expression pattern. Furthermore, the expression of 16 other genes involved in diverse biological functions was analyzed. Up-regulation of these genes in the tolerant genotype Pankaj during sheath blight disease suggested efficient genetic regulation of this cultivar under stress. Also, expression analysis of conserved microRNAs (miRNAs) and their target genes revealed important role of miRNAs in post-transcriptional gene regulation during development of rice sheath blight disease. Genome-wide discovery of miRNAs and further characterization of DEPs and genes will help in better understanding of the molecular events during sheath blight disease development in rice.

1. Introduction

Rice (*Oryza sativa L.*) is one of the primary staple foods and major calorie provider in the world. It is also considered as model cereal crop plant having immense socio-economic impact. Food security in India and other Asian countries largely depends on adequate rice production. With the reduction of farm resources, specifically land, labor and water, enhancing rice productivity is a major challenge. Biotic stresses are key constraints in achieving higher productivity of rice. Among the fungal diseases of rice, sheath blight (ShB) caused by *Rhizoctonia solani* Kuhn [Teleomorph: *Thanatephorus cucumeris* (Frank) Donk], causes significant loss in yield and quality (Lee and Rush, 1983; Singh et al., 2016). The severity of the disease depends upon the age of the plant at the time of infection, season, variety, and the quantity of applied nitrogenous fertilizer. The extent of yield loss due to this disease has been reported to vary from 5.9 to 69% (Tan et al., 2007; Venkatrao et al., 1990; Yellareddygari et al., 2014).

Despite extensive efforts, varieties having sufficient level of resistance to ShB could not be developed due to lack of complete resistance in the primary gene pool, and complex and polygenic nature of resistance. Most of the previous efforts have focussed on identification of QTLs (Quantitative Trait Loci) for disease tolerance (Channamallikarjuna et al., 2010; Silva et al., 2012) or developing transgenic rice lines using the host or pathogen genes conferring tolerance (Datta et al., 1999; Richa et al., 2016; Sridevi et al., 2008; Sripriya et al., 2008; Tiwari et al., 2017). However, present state of understanding of the molecular basis of *R. solani*-rice pathosystem and the major factors (genes/proteins) responsible for establishing the infection by *R. solani* in rice, are scanty. This information is required not only to unravel the susceptibility and resistance mechanisms in the host but also to open up new avenues for modifications of the identified genes either through transgenic or genome editing approaches.

Driven by plethora of innovations in mass spectrometry (MS)-based technologies and rapid development of quantitative methods,

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proteomics has emerged as a complementary technique to other approaches such as transcriptomics and metabolomics in the post-genomic era (Wienkoop et al., 2010). In recent years, this approach has become more sensitive and powerful due to improvements in two-dimensional polyacrylamide gel electrophoresis (2DE), protein detection, quantification, finger printing and partial sequencing of proteins by MS MALDI-TOF (Mass Spectrometry Matrix Assisted Laser Desorption Ionization-Time of Flight), and bioinformatics tools. Proteomic analysis can ideally provide direct functional information by exploring the global expression patterns of proteins in various states (Ghatak et al., 2017; Rakwal and Agrawal, 2003). Rice proteomics research has made considerable progress in providing functional information of proteins expressed in the various developmental stages, tissues, cells, and abiotic and biotic stress environments (Agrawal et al., 2006; Ghatak et al., 2017; Komatsu and Yano, 2006).

In this study, six rice genotypes (Pankaj, Tetep, TN1, BPT5204, N22, and Vandana) were chosen for analysis of the DEPs during *R. solani* infection. Among these genotypes, Pankaj and Tetep are moderately tolerant (Channamallikarjuna et al., 2010; Singh et al., 2016), while TN1, BPT5204, N22, and Vandana are highly susceptible to ShB disease (Dey et al., 2016; Yadav et al., 2015). Further, expression of some of the identified proteins was validated at the transcript level. This comprehensive study of *R. solani* infection in susceptible and tolerant rice genotypes at proteome and transcriptome level would decode wider avenues towards genomics assisted breeding and/or gene editing to combat this devastating disease.

2. Materials and methods

2.1. Plant materials and disease inoculation

Six rice genotypes were grown in pots with 3 replications (5 plants/replication). Homogenous conditions were maintained for all the genotypes and replications throughout the growth period. A virulent strain of *R. solani* (WGL-12-1) of AG-1 IA group was used for inoculation of rice plants (Yugander et al., 2015). The fungus was grown in potato dextrose agar (PDA) medium at room temperature (25 °C) for 72–96 h. Forty five days old rice tillers were inoculated with *R. solani* by placing a young sclerotium of the fungus beneath the rice leaf sheath. The inoculated plants were maintained in a glass house with high humidity (RH > 90%) and warm temperature (30 °C) to facilitate rapid pathogen infection. Plant tissue samples from the control (un-inoculated) and infected (inoculated) plants (5 days post inoculation) were collected and used for the proteomic and transcriptomic studies. Simultaneously, to study the reaction of these rice genotypes to the pathogen, healthy leaves were detached from plants of each genotype and cut into pieces (2 inches in length). These leaf bits were then put in 40 ppm benzimidazole solution in a sterile Petri plate to maintain the leaf pieces in green and healthy state. Fungal sclerotial bodies were placed on centre of the leaf pieces in the Petri plate for infection. Observations on ShB symptoms on these leaf bits were made after 3 days of incubation (Fig. 1).

2.2. Proteome analysis

2.2.1. Protein extraction

Protein extraction from rice tissue was done as described in earlier reports (Damerval et al., 1986; Kamo et al., 1995) with minor modifications. One gram of rice tissue (mixture of leaf and sheath) from control and infected plants was ground into fine powder using liquid nitrogen and sterilized mortar and pestle. The infected tissue included section infected by fungus along with regions surrounding the infected area. This fine powder was transferred to an Oakridge tube containing 10% TCA extraction buffer (pre-cooled at −20 °C). These tubes were then incubated for 1 h at −20 °C. It was then centrifuged at 12,000 rpm for 15 min at 4 °C. Supernatant was discarded and 10 ml wash buffer (10 ml acetone, 0.07% DTT) was added and kept for 1 h incubation at −20 °C. Tubes were again centrifuged at 12,000 rpm for 15 min at 4 °C. Supernatant was discarded and washing was repeated two times. The pellet was lyophilized and stored in −80 °C.

2.2.2. Protein solubilisation

Fifteen microgram of lyophilized protein was suspended in 250 µl lysis buffer (9M Urea, CHAPS 4%, DTT 1%, IPG buffer 1%, 35 mM Tris) and incubated for 1 h at 37 °C with intermittent vortexing. It was then centrifuged at 12,000 rpm for 15 min at room temperature. The clear supernatant was transferred to Eppendorf tubes and stored in −80 °C. Quantification of protein was done with 2-D Quant Kit (GE Healthcare). All protein samples were normalized to 125 µg/125 µl. For IPG strips (pH 4–7), 125 µg of protein was loaded.

2.2.3. IPG strip rehydration

The solubilised protein was mixed with appropriate amount of 2D streak rehydration buffer (GE Healthcare) to make up concentration to 125 µg/125 µl. An aliquot of 125 µl was added in the rehydration tray. IPG strips of pH 4–7 were placed gel side down in the rehydration tray and mineral oil was added on the IPG strips to avoid dehydration.

2.2.4. Isoelectric focussing

The IPG strips were placed on focussing tray (GE Healthcare Ettan™ IPGphor™ 3) in gel side up configuration. Paper wicks were dipped in distilled water and placed on both ends of the channels covering the wire electrodes. The tray was placed in the PROTEAN IEF cell; mineral oil was added over the strips, and first dimension run was carried out as recommended (200Vhr, 300Vhr, 4000Vhr and 1250Vhr respectively). A three step protocol was programmed in the PROTEAN IEF cell. Default temperature as 20 °C and maximum current as 70 µA/strip was set.

2.2.5. SDS-PAGE and Coomassie Brilliant blue (CBB) staining

Equilibration buffer I (6M Urea, 30%w/v Glycerol, 2%w/v SDS, 1% w/v DTT and 1.5 mM Tris HCl pH 8.8) was added to the channels of fresh rehydration tray and IPG strips were placed on these channels. Later equilibration buffer II (6M Urea, 30%w/v Glycerol, 2%w/v SDS, 2%w/v iodoacetamide and 1.5 mM Tris HCl pH 8.8) was added to each of the strips. Meanwhile, 12% SDS-PAGE gel was prepared. The strips were removed from the tray and dipped in 1X TGS (tris-glycine-SDS) buffer. The strips were then inserted into plates containing the solidified SDS-PAGE gel. Over the strip, melted agarose mixed with bromophenol blue dye was poured. Electrophoresis was carried out in SE 600 Vertical Unit (GE Healthcare) at 20 mA for 6 h until the dye front reached 1 mm from the bottom of the gel. All gels were stained with colloidal Coomassie Brilliant blue G-250. The gel was transferred to staining solution and kept overnight with uniform shaking. The next day, gel was placed into destaining solution with uniform shaking for 90 min and the process was repeated at least twice until the background of the gel became colourless. Later the protein spots were viewed and documented in GE image scanner. All the steps of protein extraction and 2DE were performed in three replications to identify the DEPs.

2.2.6. Peptide mass fingerprinting and in-silico analysis

The control sample was compared with infected sample of respective genotype to identify the DEPs. In total, 45 differentially expressed protein spots were selected from comparison of control and infected sample of six genotypes. The DEPs showing either exclusive expression or up/down regulation (with fold-change cut-off of 2.0 for up-regulation and 1.5 for down-regulation) were selected and sent for peptide mass fingerprinting using commercial facility of Sandor Proteomics, Hyderabad. The peptides were identified by Matrix Assisted Lazer Desorption/Ionization – Time of Flight Mass Spectrometry (MALDI-ToF/MS). The peaks were analyzed by the online bioinformatics tool, peptide search engine MASCOT (Matrix Science) for the identification of proteins.

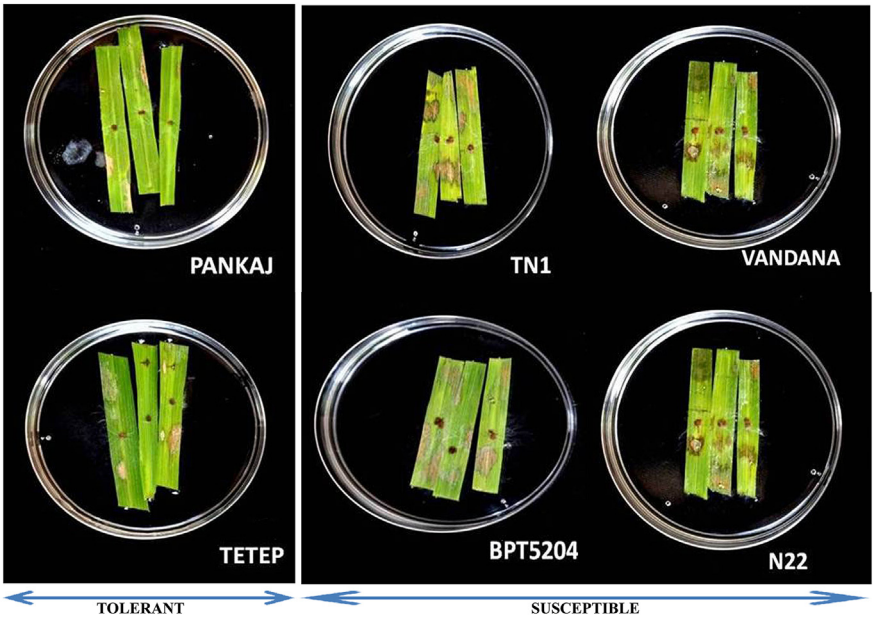

Fig. 1. Reaction of six rice genotypes against sheath blight disease pathogen, *R. solani*. The tolerant genotypes Pankaj and Tetep showed disease score of 3 (20–30%) and 5 (31–45%), respectively. Susceptible genotypes (TN1, BPT5204, N22 and Vandana) showed disease score of 9 (> 65%). Disease scoring was done based on the scale suggested in Standard Evaluation System for Rice given by IRRI (2014).

2.3. RT-PCR analysis

2.3.1. Quantitative real time PCR analysis (qRT-PCR) of genes

Some of the identified proteins with annotated functions were randomly chosen for expression analysis at transcription level. The corresponding gene sequences were retrieved from NCBI (http://www.ncbi.nlm.nih.gov) and RGAP (http://rice.plantbiology.msu.edu/). The primers were designed using Primer 3 software (Table 1). In addition to 14 genes encoding DEPs, we analyzed the expression of 16 other genes involved in important biological functions (Table 2). Same plant samples (earlier used for protein extraction) were used for RNA isolation by RNeasy Plant Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized by using Improm-II Reverse Transcription System (Promega). cDNA was normalized and used for qRT-PCR using SYBR Premix Ex-Taq (Takara). RNA extraction and qRT-PCR were performed in three biological replicates. The actin gene was used as internal control and all the reactions were kept in triplicates in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, USA). The qRT-PCR profile was 50 °C at pre holding stage for 10 min, 95 °C at holding stage for 10 min, denaturing at 95 °C for 15s and annealing/extension at 60 °C for 30s for 40 cycles, followed by a dissociation stage (melting curve analysis).

2.3.2. Quantitative real time PCR analysis of microRNAs and their target genes

RNA and small RNA were isolated from control and infected leaf and sheath tissues of 45 days old plants collected at 5 days post inoculation. RNA and small RNA were isolated from same sample using the mirVana miRNA isolation kit (Ambion) according to the manufacturer's instructions. cDNA synthesis of small RNAs was performed using the miScript Reverse Transcription Kit (Qiagen). cDNA of target genes was synthesized from 2 µg of RNA using ImProm-II™ Reverse Transcription System (Promega) and oligo dT primers. We followed the methods of expression analysis of miRNAs and their target genes as reported earlier (Mangrauthia et al., 2017; Sailaja et al., 2014).

2.3.3. Analysis of real time PCR data

Relative transcript levels were obtained using the comparative Ct method. The mean threshold cycle (Ct) value of a gene or miRNA was normalized to the Ct value of internal control gene or small RNA to obtain ΔCT value. Further, ΔΔCT value was calculated using the formulae ΔΔCT = ΔCT of infected sample − ΔCT control sample, and then fold difference was calculated from $2^{-\Delta\Delta Ct}$. Similarly, ΔCT standard deviation was calculated as reported earlier (Mangrauthia et al., 2017; Sailaja et al., 2014).

Table 1
Primer sequences of genes corresponding to differentially expressed proteins identified through 2D gel electrophoresis.

S. No	Protein Name	Gene Accession No.	Forward primer	Reverse primer
1	CASP 2B1 like- protein (CASP2B1)	Os12g0514300 (Chr 12)	AAGCCAGACGAGGACCTTCTT	GAGGACGAGTGCCTTCATGTC
2	Putative beta transducin (Transducin)	BAD37438 (Chr 6)	GTAGCAATGAAAATGGTCCGTAG	CCCATATTCTAAGAGTCCCATCC
3	Disease resistance protein RPM1 isoform X2 (RPM1X2)	BioProject: PRJNA122 (Chr 9)	GGACACTAAGGTGACACATCTCC	TTACACTGAAGTGGCCTTGTTCT
4	Probable receptor-like protein kinase At5g56460 isoform X2 (Kinase2)	BioProject: PRJNA122 (Chr 3)	ATAGTTTCCAGGGGCATAGAGAG	CTCTGTGGTCATCTTCACAACAA
5	Serine/threonine protein kinase (OSK3)	OSK3	CCTTTGTGGTACTCTTCCATTTG	GAGCAGATAAATGACTTGGGAGA
6	NBS-LRR-like protein (NBS LRR)	Os01g0721200 (Chr 1)	CAGTCCATTAAAGCGGTTCTGTA	GCATGTCTGAAACTACGGTTTTC
7	SNF1-related protein kinase (SNF1)		TATCCTCAGGCCACTGATATGTT	GCAGATACTTCTCGTCTCTGCTC
8	Calcium-dependent protein kinase 21 (CDPK21)	Os08g0540400 (Chr 8)	CGAGATCAGGATGCTACTCGAAG	GTACTCGTCGTTGCTCATCTTCT
9	Thioredoxin O, mitochondrial (Thioredoxin)	Os06g0665900 (Chr 6)	GTGTTCTACTACACGGCGGTATG	CTTAGTTTGCTTCCAACACCATC
10	Endo-1,4-beta-xylanase A (Xylanase)	BioProject: PRJNA122 (Chr 1)	GTGAAGGTGATGGATCTCCAAAT	CACCTTGTCAGTCTTCTTCCTGA
11	Similar to Histone H1 (Histone)	Os04g0253000 (Chr 4)	CCTTATTTCGAGATGATCAAGGAG	GGAGAGCATCTTCTTGTAGTTGG
12	Glyceraldehyde-3-phosphate dehydrogenase B (G3PD)	LOC4331495 (Chr 3)	GGTGTCAACGAGGGAGACTACT	TAGGAGTGAGTTGTGGTCATGGT
13	Similar to ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RUBISCO)	Os12g0207600 (Chr 12)	GTTTACTTCCATTGTGGGTAACG	CCTTTCAACTTGGATACCATGAG
14	Peroxisomal (S)-2-hydroxy-acid oxidase GLO1 (GLO1)	OsI_13800	GTGTACAAGGACAGGAATGTGGT	AACCTGTTCTTGATGTCAGCTTC

Table 2
Details of genes and primers used for real time PCR expression analysis in rice genotypes under control and disease infected samples.

Gene Accession No.	Description	Forward primer	Reverse Primer
LOC_Os11g48000.1	ZOS11-11 - C2H2 zinc finger protein	CACCACCTCCACCGATAATAC	CTCGTCCTGATCTGCAACTAC
LOC_Os12g02330.2	LTPL13 -Protease inhibitor/seed storage/LTP family protein precursor	CACCATCAAGTCTCTCAACCTC	GGATTAATCGATCTAGCTGACCTG
LOC_Os09g29480.2	2-aminoethanethiol dioxygenase	CACGAGTGCGACGATTTCT	TGCTCAGCACCACCATTT
LOC_Os03g53690.1	oxidoreductase, short chain dehydrogenase/reductase family domain containing protein	GTGGAGGTGATGACCAAGAT	GTCTTGCCGGTGTACATCAT
LOC_Os06g45890.1	MYB family transcription factor	AGGCTCTAGCAACAGCAATC	GTAAATGCAGCGATCTTCACTATTC
LOC_Os02g02424.1	ZOS2-02 - C2H2 zinc finger protein	GACTGCCCTTTCAGCTACATTA	CTTCCTACCACAACCATCCATAG
LOC_Os12g44010.1	purple acid phosphatase precursor	ACGGCACCAAGTACCACTA	CGATGAGCCCGAACTTGTA
LOC_Os04g43290.3	ARPC2B	GCTGCAAGTGAGGAAGAGAA	CCGCTTGATCTTGTCGATGA
LOC_Os02g53970.1	OsSub24 - Putative Subtilisin homologue	CTCGGAAATGGCGAAACTTATG	CTTCCCAGGTTCACACAATCTA
LOC_Os04g46980.1	cis-zeatin O-glucosyltransferase	GCCTGAGAAAGGGATGACAATA	TAGCCAACGAAGTCATCCAAG
LOC_Os10g33370.1	3-ketoacyl-CoA synthase precursor	CAAGTTCCTCCTCAAGGTCATC	TCTCGTCCATCTCCTCCATC
LOC_Os06g01200.1	zinc finger, C3HC4 type domain containing protein	GGCTGAGAGAAGGTTCAGGATA	GCATCAGTAGAACCACCAACAG
LOC_Os01g67980.1	cysteine proteinase EP-B 1 precursor	GACCAGTCCTTCCAGTTCTAC	CACGAGTTCTTGACGATCCA
LOC_Os09g12790.1	potassium channel protein	CGCGAAGCTCTTCTCAATCT	CTCCTGAAGGTCAAGCACATAG
LOC_Os03g05420.2	MT-A70 domain containing protein	CTGGAACGGTGAGGAGATTATG	TGTTCAAGACCTACACCATCAC
LOC_Os10g13960.1	retrotransposon protein	TCCTTCTTGACCAACGCTTATC	GTTCTGTTTGCCTGGCTTATATTC

3. Results

To understand the changes in rice proteome after *R. solani* infection, six genotypes were used in this study. The phenotypic assay suggested Pankaj as the most tolerant followed by Tetep (Fig. 1). Remaining four genotypes - TN1, BPT5204, N22, and Vandana showed susceptible reaction.

3.1. Differentially expressed proteins during R. solani infection

To decipher the differential regulation of proteins during sheath blight disease in rice, 2DE was performed (Fig. 2). Among hundreds of protein spots appeared in each sample, few showed differential regulation in response to fungal infection. Protein spots constantly showing differential expression in three biological replicates were picked for Mass Spectrometry analysis. Functional annotation of the DEPs suggested these proteins as kinases, transposon proteins, disease resistance proteins, histone proteins, proteins associated with photosynthesis and metabolism, and hypothetical or uncharacterized proteins. Interestingly, 10 DEPs were encoded from rice chromosomes 11 and 12 (Table 3).

Based on their function, 45 DEPs were classified into different groups: Group 1 consists of photosynthesis related proteins like glyceraldehyde-3-phosphate dehydrogenase B chloroplastic (XP_015630808.1); glyceraldehyde-3-phosphate dehydrogenase cytosolic (G3PC_SINAL), soluble starch synthase 1 (SSY1_ORYSI), Accumulation And Replication Of Chloroplasts 5/ARC5 (BAT16130.1), ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (SBO07520.1 and BAT16303.1), and peroxisomal (S)-2-hydroxy-acid oxidase GLO1 (GLO1_ORYSI). Group 2 consists of proteins related to resistance and pathogenesis such as disease resistance protein RPM1 isoform X2 (XP_015612237.1), NBS-LRR-like protein (BAB84426.1), beta transducin (BAD37438.1), spermidine hydroxycinnamoyl transferase 1 (SHT1_ORYSJ), and cysteine protease XCP2 (XCP2_ARATH). Group 3 includes stress related proteins which are also known for signal transduction, e.g. SNF1-related protein kinase (AAB05457.1), serine/threonine protein kinase OSK3 (OSK3_ORYSI), calcium-dependent protein kinase 21 (CDPKL_ORYSJ), probable receptor-like protein kinase At5g56460 isoform X2 (XP_015629879.1) and phosphatidylinositol 4-kinase beta 1 (P4KB1_ARATH). Group 4 consists of proteins related to cell wall metabolism and cytoskeleton development such as endo-1,4-beta-xylanase A (XP_015626728.1), CASP-like protein 2B1(CSPLE_ORYSJ), and P4KB1_ARATH. Group 5 included 15 proteins which are either hypothetical or uncharacterized. This forms the largest group.

Among the 13 DEPs identified in the tolerant genotype Pankaj, ARC5, serine/threonine protein kinase, retrotransposon protein and five hypothetical or uncharacterized proteins showed exclusive expression in infected tissue. Similarly, soluble starch synthase and SBO07520.1 showed expression in control tissue only. While, putative transposase, putative beta transducin, and CASP like protein 2B1 were down-regulated in infected tissue as compared to control. In Tetep, two proteins similar to histone H1, thioredoxin 1 (TRX-1), uncharacterized protein LOC4346319, and a hypothetical protein OsI_09415 showed exclusive expression in infected tissue.

Six DEPs were identified in the susceptible genotype Vandana, among which two hypothetical proteins and histone H1 showed

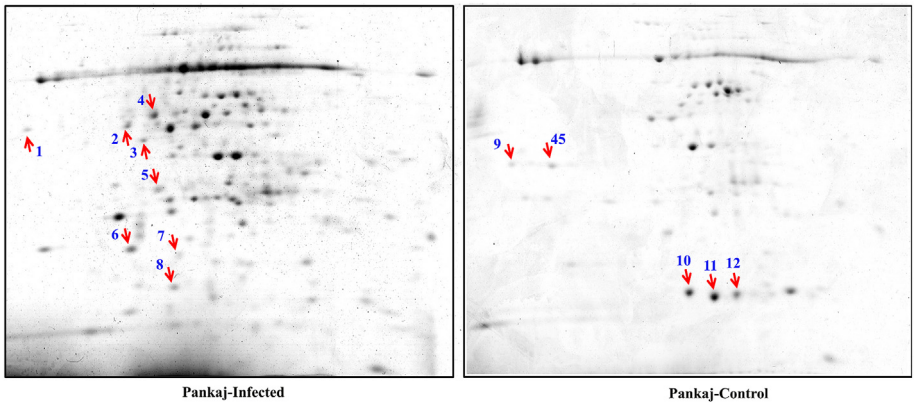

Fig. 2. A representative gel picture showing 2D gel electrophoresis of proteins isolated from control and *R. solani* infected Pankaj cultivar. Selected spots have been marked with arrow and spot number on the gel image.

Table 3
Differentially expressed proteins identified through 2D gel electrophoresis in six rice genotypes under control and *R. solani* infected conditions. In column Expression levels: + indicates up-regulation, - indicates down-regulation, no symbol indicates exclusive expression.

Spot No	Mascot Score	Protein Description	Gene Accession No	Fold change expression	Protein Accession No.	Sample[a]
1	101	Hypothetical protein	Os04g0429850 (Chr 4)		BAS89247.1	Pankaj Trt
2	94	Uncharacterized protein	LOC4346319 (Chr 8)		XP_015649750.1	Pankaj Trt
3	131	Similar to ARC5 (ACCUMULATION AND REPLICATION OF CHLOROPLAST 5); GTP binding/GTPase	Os12g0178700 (Chr 12)		BAT16130.1	Pankaj Trt
4	102	Hypothetical protein	OsJ_35649 (Chr 12)		EEE52981.1	Pankaj Trt
5	135	Serine/threonine protein kinase	OSK3		OSK3_ORYSI	Pankaj Trt
6	138	Uncharacterized protein	LOC107276951 (Chr 11)		XP_015616377.1	Pankaj Trt
7	191	Retrotransposon protein, putative, Ty1-copia subclass	BioProject: PRJNA16949 (Chr 12)		ABA97637.1	Pankaj Trt
8	145	Hypothetical protein	OsI_35764 BioProject: PRJNA361 (Chr 11)		EEC67990.1	Pankaj Trt
9	182	Soluble starch synthase 1	SSY1_ORYSI Chloroplast		SSY1_ORYSI	Pankaj Control
10	151	Putative transposase	AC025098_4 (Chr 10)	−2.86801	AAM08737.1	Pankaj Trt
11	193	CASP-like protein 2B1	Os12g0514300 (Chr 12)	−1.95462	CSPLE_ORYSJ	Pankaj Trt
12	169	Putative beta transducin	BAD37438 (Chr 6)	−3.25783	BAD37438.1	Pankaj Trt
13	95	Hypothetical protein OsI_09427	EEC74234 (Chr 2)		EEC74234.1	Vandana Trt
14	194	Disease resistance protein RPM1 isoform X2	BioProject: PRJNA122 (Chr 9)	+5.45871	XP_015612237.1	Vandana Trt
15	178	Probable receptor-like protein kinase At5g56460 isoform X2	BioProject: PRJNA122 (Chr 3)	+2.16487	XP_015629879.1	Vandana Trt
16,43	147	Histone H1	BioProject: PRJNA122 (Chr 4)		XP_015637216.1	Vandana Trt, Tetep Trt
17	103	Hypothetical protein	LOC_Os10g16580 (Chr 10)		ABB47098.1	Vandana Trt
18	207	Glyceraldehyde-3-phosphate dehydrogenase B	LOC4331495 (Chr 3)		XP_015630808.1	Vandana Control
19	161	Spermidine hydroxycinnamoyltransferase 1	LOC_Os12g27220 (Chr 12)		SHT1_ORYSJ	BPT5204 Trt
20	89	Cysteine protease XCP2	At1g20850 (Arabidopsis)	+2.25460	XCP2_ARATH	BPT5204 Trt
21	75	CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,3-sialyltransferase 2	Brassica napus	−1.53248	XP_013669182.1	BPT5204 Trt
22	183	Hypothetical protein	BioProject: PRJNA361 (Chr 11)		EEC68528.1	BPT5204 Trt
23	109	Hypothetical protein	BioProject: PRJNA361 (Chr 6)	+2.63237	EEC80646.1	BPT5204 Trt
24	89	BnaA10g06850D (Cytochrome b5-like Heme/Steroid binding domain)	*B.napus*		CDY14370.1	BPT5204 Trt
25	165	Conserved hypothetical protein	Os02g0833250 (Chr 2)		BAH91951.1	N22 Trt
26	169	DNA polymerase subunit Cdc27 domain containing protein	Os01g0204000 (Chr 1)	+2.45872	BAS70931.1	N22 Trt
27	94	Phosphatidylinositol 4-kinase beta 1	At5g64070 (Arabidopsis)	+2.14346	P4KB1_ARATH	N22 Trt
28	86	Uncharacterized protein isoform X1	*B. rapa* BioProject: PRJNA249065	+2.23694	XP_009134338.1	N22 Trt
29	161	Glyceraldehyde-3-phosphate dehydrogenase	G3PC_SINAL Sinapis alba	+2.58632	G3PC_SINAL	N22 Trt
30	168	NBS-LRR-like protein	Chr 1	+2.84725	BAB84426.1	N22 Trt
31	103	Similar to ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Os12g0207600 Chr 12	−3.52761	BAT16303.1	TN1 Trt
32	167	H0725E11.3 (Transposon protein)	Chr 4		CAH66172.1	TN1 Trt
33	168	Uncharacterized protein	LOC4346319, Chr 8		XP_015649750.1	TN1 Trt
34	168	SNF1-related protein kinase		−2.05719	AAB05457.1	TN1 Trt
35	165	Calcium-dependent protein kinase 21	Os08g0540400 (Chr 8)	+2.66906	CDPKL_ORYSJ	TN1 Trt
36	162	Thioredoxin O, mitochondrial	Os06g0665900 (Chr 6)	+2.42672	TRXO_ORYSJ	TN1 Trt
37	170	Endo-1,4-beta-xylanase A	BioProject: PRJNA122 Chr 1	+2.75331	XP_015626728.1	TN1 Trt
38	163	Peroxisomal (S)-2-hydroxy-acid oxidase GLO1	OsI_13800	+2.15433	GLO1_ORYSI	TN1 Trt
39	169	Hypothetical protein OsI_36817	BioProject: PRJNA361 Chr 11		EEC68528.1	TN1 Control
40	113	Similar to Histone H1	Os04g0253000 Chr 4		BAF14234.1	Tetep Trt
41	150	Similar to Thioredoxin 1 (TRX-1)	Os04g0530600 Chr 4		TRXM2_ORYSJ	Tetep Trt
42	173	Uncharacterized protein LOC4346319	BioProject: PRJNA122 Chr 8		XP_015649750.1	Tetep Trt
44	172	Hypothetical protein OsI_09415	BioProject: PRJNA361 Chr 2		EEC74228.1	Tetep Trt
45	174	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial	chloroplast Oryza glaberrima		SBO07520.1	Pankaj Control

[a] Trt-samples infected with *R. Solani*.

exclusive expression, while, RPM1 isoform X2 and receptor protein kinase isoform X2 showed increased expression in infected tissue. Glycereldehyde-3-phosphate dehydrogenase B showed expression only in control tissue of Vandana. In another susceptible genotype BPT5204, spermidine hydroxycinnamoyltransferase 1, cytochrome b5-like heme/steroid binding domain and a hypothetical protein showed exclusive expression, while cysteine protease XCP2 and hypothetical protein showed increased expression in infected tissue. Infected tissue of N22 showed exclusive expression of a hypothetical protein and increased expression of DNA polymerase subunit Cdc27 domain containing protein, phosphatidylinositol 4-kinase beta 1, uncharacterized protein isoform X1, glyceraldehyde-3-phosphate dehydrogenase, and NBS-LRR-like protein. In fungal infected TN1, H0725E11.3 (transposon protein) showed exclusive expression while peroxisomal (S)-2-hydroxy-acid oxidase GLO1, endo-1,4-beta-xylanase A, thioredoxin O, and calcium-dependent protein kinase 21 showed increased expression. Expression of SNF1-related protein kinase and ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit was decreased in infected tissue of TN1.

3.2. Expression analysis of genes encoding differentially expressed proteins

Genes encoding 14 DEPs were selected for analysis of their expression pattern through qRT-PCR. Expression of genes was compared in control and infected tissue of source genotypes or samples (Table 3) where DEPs were identified. Notably, 11 of the 14 genes showed expression pattern similar to the proteins identified through 2DE. Three proteins- OSK3, G3PD, and Histone showed exclusive expression in 2DE while qRT-PCR analysis showed up-regulation of the corresponding genes (Fig. 3). The maximum up-regulation of 12.12 fold was detected in case of the gene encoding RPM1 isoform X2 in infected tissue of Vandana. The maximum down-regulation of 18.12 and 17.14 fold was recorded for genes encoding putative beta transducin and ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, respectively, in infected tissue of Pankaj and TN1, respectively (Supplementary Table 1).

3.3. Expression analysis of genes associated with plant growth and metabolism

We analyzed the expression of 16 other genes including transcription factors (zinc finger proteins and MYB family transcription factor), protease inhibitor, cysteine proteinase, retrotransposon protein, potassium channel protein, cis-zeatin O-glucosyltransferase, and putative subtilisin etc. These genes were selected based on their diverse roles in plant growth and metabolism. Also, these genes represented 8 different chromosomes of rice (Table 2). The expression analysis was restricted to 2 susceptible (TN1 and BPT5204) and 2 tolerant (Pankaj and Tetep) rice genotypes. Fold change expression of these genes in infected samples (as compared to respective control) at 5 days after inoculation is shown as heat map (Fig. 4). Here, the level of up-regulation ranged from 1.09 fold (LOC_Os03g05420.2 in BPT5204) to 139.10 fold (LOC_Os04g46980.1 in Pankaj) while down-regulation ranged from 1.02 fold (LOC_Os01g67980.1 in Pankaj) to 212.30 fold (LOC_Os09g29480.2 in Tetep). Most of the genes showed up-regulation in Pankaj and down-regulation in BPT52014, TN1 and Tetep during sheath blight disease (Supplementary Table 1).

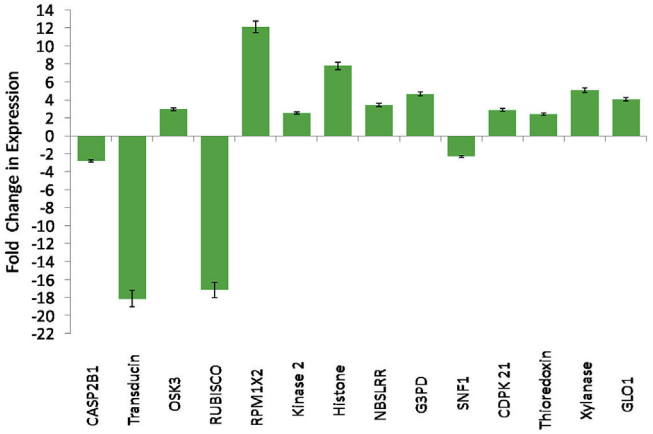

Fig. 3. Gene expression analysis of 14 genes corresponding to differentially expressed proteins in rice after inoculation with *R. solani*. Expression of genes was performed in control and infected tissue of source genotypes which showed differential expression of that particular protein. The y-axis shows the fold change expression of genes in infected tissue. Bars represent the mean ± SE of three biological replicates.

3.4. Expression analysis of conserved miRNAs and their targets genes

In order to understand the miRNA mediated post-transcriptional gene regulation in rice during *R. solani* infection, expression of four most conserved miRNAs and their target genes was analyzed at 5 days after inoculation (Fig. 5). miR162 and miR168 are involved in biogenesis of miRNAs while miR171 and miR166 are involved in development and stress response. Negative expression correlation was observed in all the four rice miRNAs and their target genes during *R. solani* infection. Pankaj showed up-regulation while BPT5204 showed maximum degree of down-regulation of target genes of miRs162, 171, and 396.

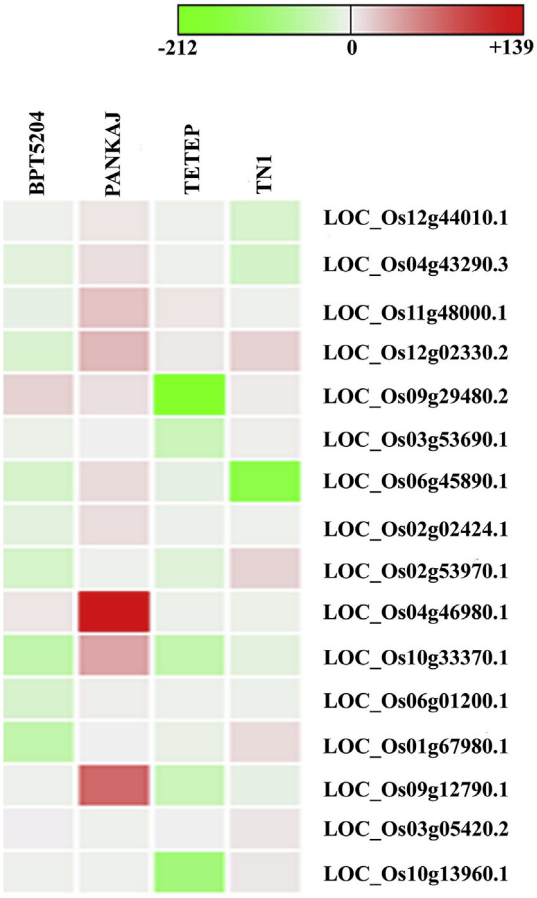

Fig. 4. A heat map representation of fold change genes expression in rice cultivars infected with *R. solani*. The expression of genes was compared with control sample of respective genotype. The values shown in bars at the top indicate fold change regulation in sheath blight disease infected samples in comparison with control.

4. Discussion

A combinatorial approach of proteome and transcriptome was followed to decipher the defence responses in tolerant and susceptible genotypes of *O. sativa* against *R. solani*. Such approaches are essential to identify the crucial players of the underlying molecular mechanism of plant response against various stresses. It provides correlation between transcript expression with the protein profile that can indicate possible gene regulation mechanism. These studies are particularly important for *R. solani,* a complex pathogen having a wide host range with more than 100 plant species (Ogoshi, 1987; Zheng et al., 2013). We identified the DEPs during *R. solani* infection in two tolerant and four susceptible rice genotypes. These DEPs were associated with major plant functions such as photosynthesis, stress response, pathogenesis, resistance and other metabolic pathways.

Significant number of DEPs emanating from chromosome 11 and 12 of rice were identified (Table 3). Earlier study by Rice Chromosomes 11 and 12 Sequencing Consortia (2005) reported that Chr 11 and 12 are rich in disease resistance genes. The abundance of resistance associated and defense response genes in these chromosomes indicate that they may be potential targets for breeding of durable disease resistance in rice. The tolerant genotype Pankaj showed down-regulation of putative beta transducin and CASP like protein 2B1 after fungal infection. Transducin beta-like gene FTL1 is essential for pathogenesis in *Fusarium graminearum*. FTL1 appears to be a component of well-conserved protein complex that plays a critical role in the penetration and colonization of wheat tissues (Ding et al., 2009). It would be interesting to decipher the role of plant encoded putative beta transducin protein in pathogenesis of *R. solani*. CASP-like protein 2B1 (iron-sulfur cluster binding) belongs to the Casparian Strip Membrane Proteins (CASP) family. Besides making a plasma membrane diffusion barrier, CASPs have an important role in directing the modification of the cell wall juxtaposing their membrane domain. By interacting with secreted peroxidases, they mediate the deposition of lignin and the building up of the Casparian strips (Lee et al., 2013; Roppolo et al., 2011, 2014). The down-regulation of CASP protein could be due to degradation of plant cell wall by saprophytic *R. solani* fungus or it may be a strategy of plant to develop hypersensitive response.

The susceptible genotype Vandana showed increased expression of RPM1 isoform X2 after *R. solani* infection. RPM1, a peripheral membrane R-protein of Arabidopsis confers resistance to *Pseudomonas syringae* expressing either avrRpm1 or avrB. RPM1 triggers a robust hypersensitive resistance response upon recognizing the *P. syringae* effector AvrRpm1. Degradation of RPM1 occurs with the onset of the hypersensitive response, which suggests that this protein may be involved in the negative feedback loop controlling the extent of cell death and overall resistance response (Boyes et al., 1998; Kim et al., 2009; Russell et al., 2015). In another susceptible genotype BPT5204, cysteine protease XCP2 showed increased expression during sheath blight disease. XCP2 is stabilized by PRN2 (a member of the functionally diverse cupin protein superfamily) through inhibition of its autolysis. Prn2 and Xcp2 mutants displayed decreased susceptibility to *R. solanacearum*. Therefore, stabilization of XCP2 by PRN2 may be required for full susceptibility to *R. solanacearum* in Arabidopsis (Zhang et al., 2014). It would be important to functionally characterize the XCP2 in rice for its possible role in susceptibility response to *R. solani*. In the absence of resistance genes for sheath blight disease, resistance development by mutating the susceptibility genes through genome editing tools seems to be a viable strategy. Spermidine hydroxycinnamoyl transferase1 expressed only in infected tissue of BPT5204. Spermidine hydroxycinnamoyl transferases are responsible for biosynthesis or modification of alkaloids, terpenoids and phenolics. Stress-induced phenylamides have antimicrobial activities against rice bacterial and fungal pathogens (Cho and Lee, 2015).

Phosphatidylinositol 4-kinase beta 1 (PI4Kbeta1) and NBS-LRR-like protein showed increased expression in infected tissue of the susceptible genotype N22. Davis et al. (2007) showed that phosphatidylinositol phosphate kinase 1 binds F-actin and recruits PI4Kbeta1 to the actin cytoskeleton. In another study, Krinke et al. (2007) reported that phosphatidylinositol 4-Kinase activation is an early response to salicylic acid in Arabidopsis. Induction of PI4Kbeta1 after *R. solani* infection may be associated with regulation of cell morphogenesis and defence response during sheath blight disease. To date, the disease resistance (R) genes were characterized by NBS-LRR domains (Belkhadir et al., 2004; Jones and Jones, 1997) in plants. NBS-LRRs recognize Pathogen-Associated Molecular Patterns (PAMPs) responsible for virulence. Highly susceptible rice genotype TN1 showed increased expression of Endo-1,4-beta-xylanase A and Calcium-dependent protein kinase 21 in infected tissue. Endo-1,4-beta-xylanase A degrades the linear polysaccharide beta-1,4-xylan into xylose, thus breaks down hemicellulose, one of the major components of plant cell walls. This is the crucial enzyme produced by fungi to degrade the plant cell wall. Induction of this enzyme in TN1 is an interesting observation which indicates its possible role in susceptibility response to *R. solani*. Siah et al. (2010) showed correlation of endo-beta-1,4-xylanase activity with the necrotrophic phase of the hemibiotrophic fungus *Mycosphaerella graminicola.* Calcium-dependent protein kinases (CPKs) have essential role in plant defence response (Romeis et al., 2001). CPK21 functions in abiotic stress response in *A. thaliana* (Franz et al., 2011) and also confers salt tolerance in rice (Asano et al., 2011). Induction of CPK21 after *R. solani* suggests cross talk of molecular pathways of biotic and abiotic stresses. SNF1 (sucrose non-fermenting 1)-related protein kinase (SnRK) was down-regulated during fungal infection in TN1. SnRKs act within an intricate network that links metabolic and stress signalling in plants (Halford and Hey, 2009). Several studies reported SnRK1 as a key component in plant response to pathogens (Hulsmans et al., 2016; Ke et al., 2017).

Relative expression level of mRNAs of 14 selected DEPs was analyzed using qRT-PCR to compare the pattern of proteins expression with their corresponding genes expression. The expression pattern of majority of these genes at the transcript level showed consistency with the protein expression, confirming their differential expression during *R. solani* infection. Earlier study in rice showed consistency of genes expression with proteomic analysis (Yang et al., 2014). Few genes did not show the desired consistency with protein expression level which might be due to several regulatory processes of these genes such as mRNA stability, splicing, post-transcriptional gene regulation, translational regulation, and protein degradation, as reported earlier by Liao et al. (2013). Here, we demonstrated that post-transcriptional gene regulation is noticeable during Rice-*R. solani* interaction. Expression analysis of miRNAs and their target genes indicated that post-transcriptional processing of mRNAs may have significant impact on protein expression during sheath blight disease in rice. In addition to the 14 DEP genes, expression of 16 other genes involved in diverse biological processes was analyzed (Fig. 4). Majority of these genes showed up-regulation in tolerant genotype Pankaj, suggesting efficient gene regulation mechanism in this cultivar during *R. solani* infection. Up-regulation of target genes and down-regulation of the corresponding miRNAs was also observed in Pankaj which further confirms its robust genetic regulation. It should be noted that Pankaj showed least damage due to *R. solani* infection (Fig. 1) and it has been one of the parents of many rice varieties which were reported to be moderately resistant/tolerant to rice sheath blight disease in All India Coordinated Rice Improvement Project trials (Rani et al., 2008).

In an earlier study, efforts were made to identify defence related proteins in rice against *R. solani* (Lee et al., 2006) using japonica cultivars Labelle (susceptible) and its somaclone LSBR-5 (tolerant). In this study, we have attempted a comprehensive proteomic analysis using two tolerant and four susceptible genotypes which include indica and aus (N22) rice cultivars. Role of hypothetical proteins in the interaction between rice and *R. solani* needs to be deciphered as they constitute the largest class of DEPs identified in this study. This also signifies the

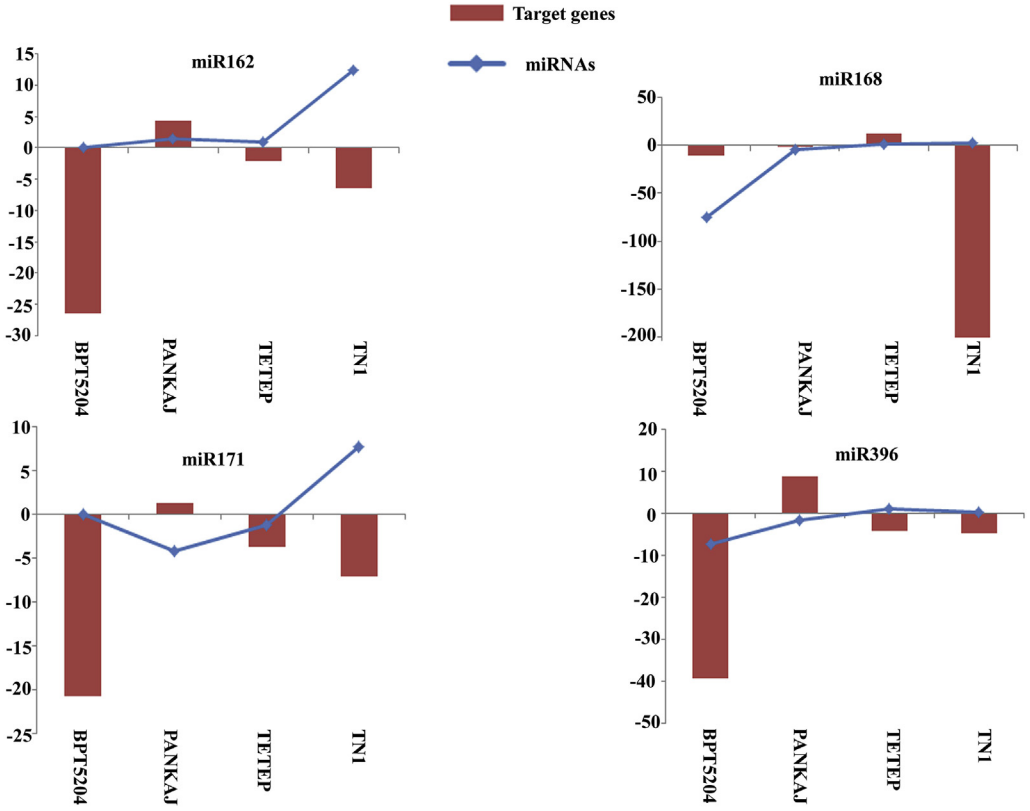

Fig. 5. Correlation of expression of four conserved miRNAs and their target genes. The y-axis shows the fold change expression of miRNAs and target genes in diseased samples of tolerant and susceptible rice genotypes in comparison with their respective control sample.

importance and necessity of functional genomics of such uncharacterized genes in rice. Functional characterization of the hypothetical DEPs would be more important as very less information is known about genes involved in disease development and resistance response against recalcitrant fungi *R. solani*. It would also be interesting to unravel the role of transposon and retrotransposon proteins during sheath blight disease development in rice. Importance of retrotransposons in re-functionalization of the rice blast disease resistance gene *Pit* has been demonstrated (Hayashi and Yoshida, 2009). A recent study suggests significant role of transposon-derived proteins in major gene regulation pathways (Duan et al., 2017). Initial study with four conserved miRNAs indicates major role of miRNA mediated gene regulation in rice-*R. solani* interaction which needs to be extrapolated at genome-wide scale in further studies.

Overall, this study with six rice genotypes including two tolerant and four susceptible cultivars revealed important role of DEPs in rice-*R. solani* interaction. Genes and miRNAs expression analysis helped in understanding the genetic regulation during sheath blight disease in rice. Based on their function, 45 identified DEPs were classified into five different groups, i.e. photosynthesis related proteins, proteins related to resistance and pathogenesis, stress related proteins, proteins related to cell wall metabolism and cytoskeleton development, and hypothetical or uncharacterized proteins. Interestingly, significant number of DEPs emerged from rice chromosomes 11 and 12. Most of the genes showed consistency in protein and transcript expression pattern identified through 2 DE and qRT-PCR, respectively. 2 DE analysis in six genotypes facilitated the identification of important DEPs such as CASP-like protein 2B1, Putative beta transducin, RPM1 isoform X2, cysteine protease XCP2, Spermidine hydroxycinnamoyl transferase1, Phosphatidylinositol 4-kinase beta 1, Endo-1,4-beta-xylanase A, and Calcium-dependent protein kinase 21, which may have critical role in susceptibility or resistance response of rice against *R. solani* pathogen. Gene expression analysis showed significant change in expression of LOC_Os12g44010.1, LOC_Os04g43290.3, LOC_Os11g48000.1, LOC_Os09g29480.2, LOC_Os06g45890.1, LOC_Os04g46980.1, and LOC_Os09g12790.1 genes after *R. solani* infection. These genes and DEPs are the key candidates for deciphering the molecular basis of sheath blight disease and developing resistance against *R. solani* in rice. 2DE and transcriptomic analysis suggested Pankaj as the most efficient genotype in terms of its genetic regulation during sheath blight disease, which was supported by its most tolerant phenotype through artificial inoculation by *R. solani*. More extensive efforts are required to identify the QTLs, genes, miRNAs and proteins at whole genome level which might be contributing in tolerance trait of Pankaj.

Author's contributions

SKM, PNB, SMB, GSL, MSM, RMS designed the experiment, PNB, PP, PM, RM,GSL, performed the experiment, SKM, PNB, PM, PP analyzed the data, SKM,PNB, PP, RMS, GSL wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.plaphy.2018.07.012.

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