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ORIGINAL PAPER

A comparative proteomic approach to identify defence-related proteins between resistant and susceptible rice cultivars challenged with the fungal pathogen *Rhizoctonia solani*

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Abstract

Rice sheath blight, caused by *Rhizoctonia solani*, is a major worldwide rice disease for which little is known about the molecular mechanisms of host immunity to infection. In the present study, a comparative proteomic analysis of two rice cultivars, Teqing (resistant) and Lemont (susceptible), inoculated with *R. solani* was conducted using an eight-plex iTRAQ (isobaric tags for relative and absolute quantitation) technique, resulting in the identification and quantification of 6560 proteins. A total of 755 proteins showed significant changes in abundance between plants infected with *R. solani* and control plants, based on an error factor < 2 and a more than 1.5-fold or less than 0.67-fold quantitative difference. The differentially abundant proteins were mainly involved in glyoxylate and dicarboxylate metabolism; glycine, serine and threonine metabolism; unsaturated fatty acid biosynthesis; and glycolysis/gluconeogenesis regulation pathways ($p < 0.01$). In addition, the expression levels of the genes encoding selected proteins were tested by qRT-PCR, and their functions were tested in *Nicotiana benthamiana* via agroinfiltration. Based on these proteomic and experimental data, a putative model of the regulation of rice immunity under *R. solani* infection is proposed. The proteins identified in the present study provide a basis for elucidating the molecular mechanisms underlying rice immunity to infection by *R. solani*.

Keywords Rice · *Rhizoctonia solani* · Protein network · Immunity · Proteomics · iTRAQ

Abbreviations

2-DE	Two-dimensional electrophoresis
DEPs	Differentially expressed proteins
EF	Error factor
EV	Empty vector
FDR	False discovery Rate
GC–MS	Gas chromatography–mass spectrometry
GO	Gene ontology
iTRAQ	Isobaric tags for relative and absolute quantification
KEGG	Kyoto encyclopedia of genes and genomes
LC–MS	Liquid chromatography–tandem mass spectrometry
MDH	Malate dehydrogenase
TFA	Trifluoroacetic acid
PDA	Potato dextrose agar
PR	Pathogenesis-related
qRT-PCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
SA	Salicylic acid
TCA	Tricarboxylic acid cycle

Hongyu Ma and Cong Sheng have contributed equally to this work.

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Introduction

Rice sheath blight is caused by the soil-borne necrotrophic fungus *Rhizoctonia solani*, which causes disease in a broad spectrum of plants such as rice, wheat, potato, bean, cotton, and sugar beet (Anderson 1982). The fungus invades

the roots, stems and other aerial parts of the plant, causing disease symptoms such as root rot, sheath blight, banded leaf, aerial blight and brown patch (Ogoshi 1987; Wrather et al. 1997). *R. solani* is divided into 14 anastomosis groups (AG1 to AG13 and AGBI) (Ogoshi 1987), in which AG1 IA is one of the most important plant pathogens causing the most serious economic agricultural losses.

Rice sheath blight is considered one of the major rice diseases that leads to significant yield and quality losses (Lee and Rush 1983). The general symptoms of sheath blight include necrotic, dark, reddish-brown, elliptical or oval lesions on the leaf sheath, leaf blade and culm (Kumar et al. 2009; Ghosh et al. 2014). Sheath blight is difficult to manage because of the wide host range, rapid variability, and long survival time in the soil (Taheri 2007). In addition, attempts to control sheath blight by traditional breeding processes have not succeeded due to the lack of strongly resistant cultivars (Bonman et al. 1992). Hence, investigating and utilizing innate rice immune components is an important step toward resistant variety development or breeding.

Proteomic analysis is a valuable tool in dissecting plant-pathogen interactions due to the direct involvement of proteins in molecular processes and biological functions (Kim et al. 2004; Neilson et al. 2011a, b). Recently, proteomic research has made considerable progress in providing functional information regarding abiotic and biotic stress responses (Komatsu and Tanaka 2005). For example, proteomic responses to salinity, drought and cold have been studied in various plants including rice (Neilson et al. 2011a, b; Sarhadi et al. 2012; Wu et al. 2016), canola (Bandehagh et al. 2011), soybean (Ma et al. 2012; Tian et al. 2015), wheat (Guo et al. 2012; Faghani et al. 2015), grape (Delaunois et al. 2013), and barley (Fatehi et al. 2012; Chmielewska et al. 2016). Biotic stresses caused by living organisms (Delaunois et al. 2014), such as fungi (Li et al. 2012; Zhao et al. 2014), bacteria (Gonzalez et al. 2012), viruses (Chen et al. 2014; Berard et al. 2015), and insects (Du et al. 2015) have also been investigated. Zhao et al. reported a total of 27 differentially abundant proteins in response to *Pst* (*Pseudomonas syringae* pv. *Tomato*) inoculation using the two-dimensional electrophoresis (2-DE) technique. Most of these proteins fall into the category "response to stimulus" and are involved in basic resistance processes, such as glycerol-3-phosphate and hydrogen peroxide signaling (Zhao et al. 2014). Li et al. (2012) observed that salicylic acid (SA)-treated rice leaves contained 36 differentially abundant proteins implicated in various functions, including defense, antioxidative enzymes, and signal transduction.

Proteins responsive to *R. solani* in sheath tissue were investigated in two rice cultivars, Labelle (resistant) and LSBR-5 (susceptible) (Lee et al. 2006). Moreover, an array of antifungal proteins, including chitinases (Datta et al. 2000; Sridevi et al. 2003; Sripriya et al. 2008), thaumatin-like proteins (Datta et al. 1999), nonspecific lipid transfer proteins (Patkar 2006) and plant defense proteins (Jha and Chattoo 2010), have been biotechnologically exploited to generate transgenic plants with resistance to sheath blight. Constitutive overexpression of the *OsWRKY4* gene increases resistance to *R. solani* concomitant with elevated expression of jasmonate (JA)- and ethylene (ET)-responsive pathogenesis-related (PR) genes, implying that the defensive response to rice sheath blight occurs through the JA/ET-dependent signal pathway (Wang et al. 2015). More recently, gas chromatography–mass spectrometry (GC–MS) and RNA sequencing analyses showed that modulating host photosynthesis, respiration, phytohormone signaling, and secondary metabolism is crucial in rice during *R. solani* infection (Ghosh et al. 2017).

Most rice varieties grown around the world are susceptible to *R. solani*, although moderate to high levels of resistant have been reported (Pan et al. 1999). Lemont is highly susceptible to sheath blight disease, while Teqing is moderately resistant (Bollich et al. 1985; Tabien et al. 2000; Zheng et al. 2011; Pinson et al. 2012). Although sheath blight disease is one of the most destructive diseases worldwide, limited information is available regarding the mechanisms of rice response to infection. Understanding the genetic mechanisms of plant disease resistance against this pathogen will benefit the development of improved varieties with *R. solani* resistance enormously. To investigate the rice—*R. solani* interaction, we conducted a proteomic analysis in resistant and susceptible rice cultivars after *R. solani* infection The primary objectives of this research were to: (1) identify proteins significantly expressed in response to *R. solani* infection in rice; (2) analyze the proteins that were differentially expressed between resistant and susceptible rice cultivars; (3) reveal the interaction network and defense mechanism between rice and *R. solani.* The knowledge gained from this study will be instrumental in understanding the molecular mechanisms involved in the response to *R. solani* infection in rice.

Materials and methods

Plant materials and inocolalion with *R. solani*

Rice cultivars Teqing (resistant) and Lemont (susceptible) were provided by National Mid-term Genebank for Rice of China National Rice Research (Tabien et al. 2000). After soaking and germination of rice seeds with water, they were planted in soil and placed in the growth room. After then, rice seedlings were transferred into 500 mL pots containing a vermiculite-potting soil mixture. Plants were cultivated in a growth room maintained at 26 °C and 70% relative humidity with a 12/12 h day (200 μE m^{-2} s^{-1})/night. *R. solani*

AG1 IA was used in this study. Inoculation was performed using a mycelial plug (5 mm in diameter) containing the actively growing mycelium of *R. solani* from a culture that had almost covered the surface of the potato dextrose agar (PDA) media in a 90 mm plate. Plugs were inoculated onto the stems of rice seedlings at the four-leaf stage at 20 mm above the surface of the culture liquid. At the same time, the control samples were inoculated with PDA. The samples of each experimental groups were collected 24 h post inoculation (hpi) for proteomic analysis. The stems of 60 plants were collected as one independent biological replicate and two biological replicates were produced in our work, which resulted in eight samples. The eight samples of two biological repeats were analyzed using 8-plex iTRAQ experiment and by running the LC–MS/MS analysis twice. LC–MS/MS analysis for twice resulted in two technical replicates in this study. All samples were immediately frozen in liquid nitrogen and stored at − 80 °C until used. The pictures of phenotype in two rice cultivars infected with *R. solani* were taken at 24 and 192 hpi.

Extraction of total proteins from the rice stem

The stems from three plants (approximately 0.5 g) were immersed in liquid nitrogen, ground to a fine powder, and then suspended in 10% w/v tricarboxylic acid/acetone containing 0.1% dithiothreitol (DTT) in 50 mL centrifuge tube at − 20 °C for 2 h. Thereafter, the tubes were centrifuged at 40,000×*g* for 20 min and the supernatants discarded. Finally, the protein pellets were washed twice with 30 mL of ice-cold acetone and lyophilized. The vacuum dried pellets were dissolved in 300 µl lysis solution containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 65 mM DTT and 1 mM phenylmethylsulfonyl fluoride. Insoluble materials were removed by centrifugation, and the protein concentration of the sample was quantified using the Bradford method with bovine serum albumin as standard protein.

Protein digestion, iTRAQ labeling and strong cation exchange

Proteins (100 µg of each sample) were first reduced, alkylated and then labeled using iTRAQ reagents as follows Proteins of each sample were first dissolved in 20 µL of dissolution buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS and 65 mM DTT), reduced with 120 µL of reducing reagent (8 M urea, 0.1 M Tris–HCl and 10 mM DTT), incubated at 36 °C for 1 h, alkylated with 120 µL of cysteine blocking reagent (8 M urea, 0.1 M Tris–HCl and 50 mM IAA), and incubated at room temperature for 10 min (Berger et al. 2007). After then, the samples were digested with trypsin at a 20:1 mass ratio at 37 °C for 14 h, then labeled using the iTRAQ Reagents 8-plex kit according to the manufacturer's instructions (AB Sciex Inc.). Eight samples of two biological repeats was labeled with iTRAQ tags, as follow: the untreated Teqing replicates were labeled with iTRAQ tags 113 and 114, and the *R. solani*-treated Teqing replicates were labeled with tags 115 and 116, while the untreated and *R. solani*-treated Lemont samples were labeled with 117/118 and 119/121, respectively. The labeled samples were then pooled and dried in an Eppendorf vacuum concentrator. Then, the samples were mixed and lyophilized before dissolving in 4 mL of strong cation exchange (SCX) buffer A (25 mM NaH_2PO_4 in 25% acetonitrile, pH 2.7). The peptides fractionated on Ultremex SCX column (4.6 × 250 mm) using an Agilent 1200 HPLC were fractionated into ten fractions. An Exigent Nano LC-Ultra 2D system (AB Sciex) was used for sample separation. At last, a Triple TOF 5600 mass spectrometer and a Nano Spray III Source (AB Sciex) were used to perform mass spectrometer data acquisition twice, which resulted in two technical replicates (Berger et al. 2007). In brief, peptides were eluted from the C18 column into the mass spectrometer using a linear gradient (5–80%) of acetonitrile (ACN) at a flow rate of 250 µL min^{-1} for 1 h. The buffers used to create the ACN gradient were: Buffer A [98% H_2O, 2% ACN, 0.1% formic acid, and 0.005% trifluoroacetic acid (TFA)] and Buffer B (100% ACN, 0.1% formic acid, and 0.005% TFA). MS/MS data were acquired in a data-dependent manner in which the MS1 data was acquired at a 250 ms at *m/z* of 400 to 2500 Da and the MS/MS data were acquired from *m/z* of 50 to 2000 Da. The independent data acquisition parameters were as follows: MS1-TOF acquisition time of 250 ms, followed by 50 MS2 events of 48 ms acquisition time for each event. The ion had the charge state + 2, + 3 and + 4. The ion exclusion time was set to 4 s. The collision energy was set to iTRAQ experiment setting. Finally, the collected data were analyzed using ProteinPilot™ 4.2 (ABSCIEX) for peptide identification.

Database search and iTRAQ quantification

ProteinPilot™ software (Version 4.2) was used for raw data processing against the database of *Oryza sativa* from UniProt (http://www.uniprot.org). The main database search parameters were as follows: the instrument was TripleTOF 5600, iTRAQ quantification, cysteine modified with iodoacetamide; and biological modifications were selected as ID and trypsin digestion. Peptides with a global false discovery rate (FDR) < 1% were used for further protein annotation. To minimize the incidence of false positives, a strict cutoff of unused ProtScore > 1.3 was applied for protein identification. Furthermore, at least one peptide with the 95% confidence was required for inclusion. The accuracy of each protein ratio is given by a calculated "error factor" in the software. The error factor expresses the 95% uncertainty range (95% confidence error) for a reported ratio, where this

95% confidence error is the weighted standard deviation of the weighted average of log ratios multiplied by the Student's t factor for n − 1 degrees of freedom, where n is the number of peptides contributing to relative protein quantification. In addition, we performed ANOVA with the biological and technical replicates data individual, and two-way analysis of variance (ANOVA) and LSD analysis (SPSS 18.0) were used to determine if the protein was significantly regulated over time. To control the FDR, we also performed Benjamini–Hochberg correction for multiple testing, and $p < 0.05$ was accepted. When identifying protein species abundance as significantly different, data were considered reliable when the p value was less than 0.05, the error factor < 2 and a ratio fold change > 1.5 (up-regulated) or < 0.67 (down-regulated) in two biological replicates and two technical replicates to designate significant changes in protein expression.

Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) analysis

Differentially expressed proteins were classified according to GO (http://www.geneontology.org). GO annotation was used to predict biological processes, cellular component, molecular function for differentially expressed proteins. KEGG (http://www.genome.jp/kegg/ or http://www.kegg.jp/) was used to predict significant pathways of proteins involved in response to *R. solani* infection. STRING (http://string.embl.de) was used to obtain protein–protein interaction information with the default score setting. Potential paths between differentially expressed proteins and metabolic compounds were used to query the knowledge-driven database with a shortest-path algorithm. Cytoscape (http://cytoscape.org/) (Version 3.1) was used to construct regulatory networks.

RNA extraction and qRT-PCR analysis of gene expression

To analyze the expression of the target genes, total RNA was extracted from ten rice stems at 0, 24 and 48 hpi following *R.* solani infection using TRIzol. A NanoDrop-1000 was used to detect the quality and quantity of RNA. Reverse transcription to cDNA was conducted by using the SuperScript first-strand synthesis system (Invitrogen). SYBR Green mix was used in qRT-PCR to determine the expression levels of the target genes. RT-PCR was performed in 10 µL reaction mixture consisting of 5 µL SYBR Green mix (Vazyme), 0.5 µL of 10 mM each primer, and 2 mL of appropriate diluted cDNA. The conditions for RT-qPCR were as follows: 95 °C for 5 min, then 40 cycles at 95 °C for 30 s and 60 °C for 34 s, followed by 72 °C for 35 s for PCR amplification. Transcript levels of each gene were measured used the Applied Biosystems 7500 system according to the manufacturer's instructions. We used $2^{\Delta\Delta CT}$ method analysis the RT-PCR data. The 18S RNA was used as a housekeeping gene in the qRT-PCR analysis. Primers used in this study are listed in Supplementary Table S8.

Functional analysis of proteins in *N. benthamiana*

Overexpression constructs of candidate genes were generated by cloning the full-length coding sequences (CDS) into a Gateway destination vector pEG100. The constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 by electric transfer. The agrobactrium harboring constructs were inoculated in liquid LB medium containing 50 µg mL^{-1} rifampicin and 50 µg mL^{-1} kanamycin at 28 °C overnight. Cultured cells were pelleted by centrifugation, washed once with and resuspended in 10 mL infiltration buffer (10 mM MgCl, 100 µM acetosyringone). Transient assays in *N. benthamiana* were performed by infiltrating whole leaf of 3-week-old *N. benthamiana* plants with Agrobacterium harboring constructs containing the proteins (OD600 = 1). Two days after infiltration, the leaves were inoculated with *R. solani* using a mycelial plug (5 mm in diameter) containing the actively growing mycelium from a culture. Lesions were measured and photos taken three days later. We used Photoshop CS6 calculate the lesions areas.

Statistical analysis

All data obtained were subjected to two-way analyses of variance (ANOVA) and mean differences were compared by the least significant difference (L.S.D.) test, and comparisons with $p < 0.05$ were considered significantly different. Principal component analysis (PCA) was performed with the program SIMCA-P Version 13.0 (Umetrics, Umea, Sweden).

Results

Proteomic changes in rice seedling sheath induced by *R. solani* infection

Four-leaf stage rice cultivars (resistant: Teqing, susceptible: Lemont) were infected with *R. solani*. At 24 h post inoculation (hpi), a large numbers of hyphae were produced at the inoculation site the rice stalks gradually became yellow and lodging was observed in Lemont. In contrast, Teqing exhibited fewer disease symptoms. At 192 hpi, the leaves of Lemont showed obvious drooping and the stalk was significantly thinner. In contrast, the leaf drooping of Teqing was not obvious and the stalk was not significantly thinner (Fig. 1). To quantitatively profile the protein abundance pattern in rice seedlings in response to *R. solani*, we employed the iTRAQ. Before analyzing differentially expressed proteins (DEPs), we made a PCA analysis raw data of iTRAQ

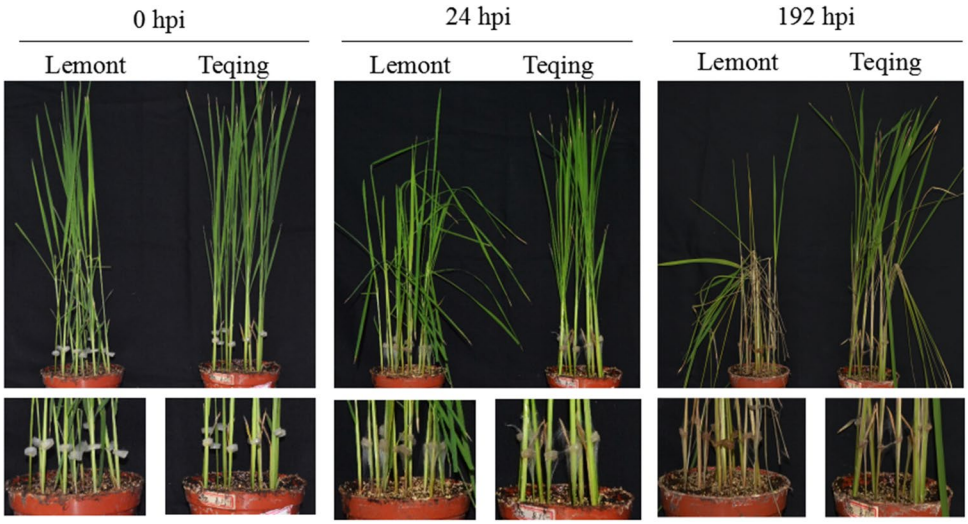

Fig. 1 The phenotype of two rice cultivars infected with *R. solani*. The stems of four-leaf stage seedlings were covered by a mycilia plug with actively growing *R. solani* mycelium onto the stem. The pictures were taken at 24 and 192 h post inoculation (hpi)

from two biological replicates and two technical replicates, and the results showed that there was a good separation of the conditions and no variation (Fig. 2). After then, the total proteins of both Teqing and Lemont rice inoculated with *R. solani* for 24 h were analyzed by iTRAQ. A total of 6560 proteins were identified with less than 1% false discovery rate in two biological replicates and two technical replicates, and 755 proteins were identified with an error factor (EF) < 2, p value < 0.05 and more than 1.5-fold or less than 0.67-fold quantitative differences in both biological and technical replicates, representing significant changes in protein species abundance (Supplementary Tables S1 and S2). The peptide sequences are listed in Supplementary Table S3. Among these proteins, the abundance of 389 proteins changed significantly in the disease-resistant rice cultivar when infected by *R. solani* for 24 h, while 448 proteins showed significant abundance changes in the susceptible rice cultivar (Supplementary Table S2). Only 43 proteins showed similar abundance changes in the two rice cultivars (Fig. 2a).

Compared to the untreated plants, in the resistant cultivar, 161 proteins were significantly more abundant, while in the susceptible cultivar, 354 proteins increased in abundance in response to *R. solani* infection for 24 h (Fig. 3a). Interestingly, 35 identified proteins increased significantly in both Teqing and Lemont at 24 hpi following *R. solani* infection (Fig. 3a). Among the proteins that were suppressed

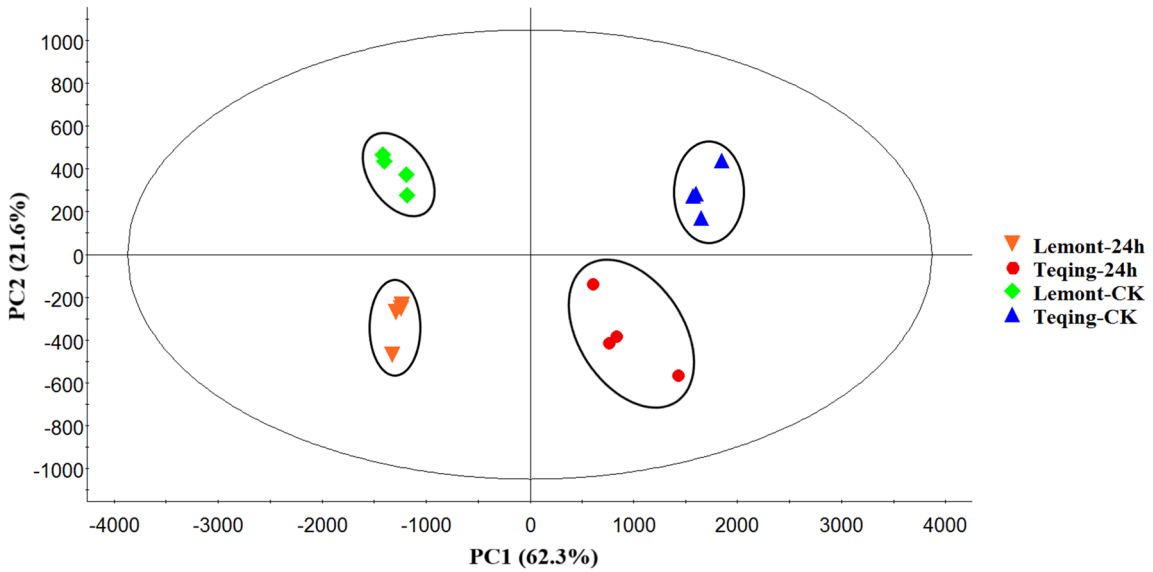

Fig. 2 Scores from principal component analysis (PCA) of differential abundance proteins using individual replicates from rice stem sheath in response to *R. solani* infection. The variation in the data explained by the first two PC scores (83.9%) is shown in brackets on the axes

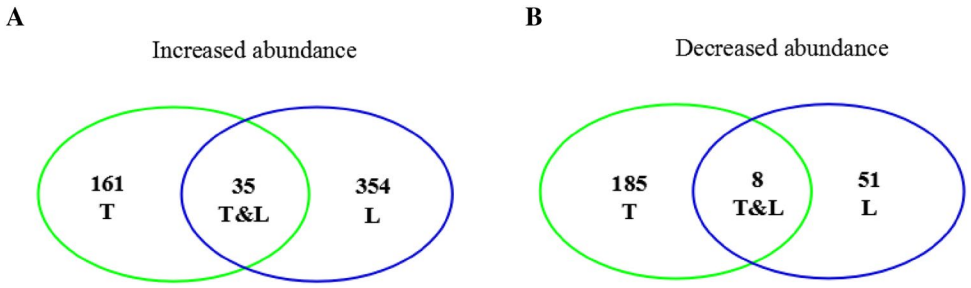

Fig. 3 Venn diagram of differentially abundant proteins. Group T represents Teqing and group L represents Lemont

by *R. solani*, 185 were identified in only Teqing, whereas 51 proteins were identified in only Lemont (Fig. 3b). Only 8 proteins decreased significantly in both Teqing and Lemont (Fig. 3b). These results show that more proteins were increased by infection in the disease-susceptible than in the resistant cultivar. In contrast, fewer proteins decreased in the disease-susceptible than in the disease-resistant cultivar. Thus, we chose to study the function of these differentially abundant proteins which have differentially relative levels between two cultivars.

Functional annotation and pathway analysis of differentially abundant proteins

The differentially abundant proteins from Teqing and Lemont were analyzed to extract information relevant to the pathways involved in *R. solani* infection. After KEGG pathway analysis, the top ten significantly enriched terms according to the GO hierarchy (level four) were depicted in Fig. 4. In the biological process analysis, cellular amino acid metabolism was the most significant term (p-value: 2.37e−09), followed by processes such as organonitrogen and alpha-amino acid metabolic (Supplementary Table S4) in Teqing. In contrast, plastid organization was the most representative term (p-value: 2.12e−08) in Lemont and was followed by other biological processes such as chloroplast organization and single-organism metabolic process (Supplementary Table S4). The cell component analysis showed that 21% of annotated proteins were located in the cytoplasm in Teqing (Supplementary Table S5), and 18% annotated proteins were located in the cytoplasm in Lemont (Supplementary Table S5). The GO analysis showed that oxidoreductase activity and GTP binding were the dominant molecular function in Teqing and Lemont, respectively (Supplementary Table S6).

The KEGG pathway enrichment analysis revealed that the differentially abundant proteins at $p < 0.01$ in the resistant cultivar were mainly involved in glyoxylate and dicarboxylate metabolism; glycine, serine and threonine metabolism; and unsaturated fatty acid biosynthesis pathways (Fig. 5a, Supplementary Table S7). However, the differentially abundant proteins in susceptible plants were mainly involved in the glycolysis/gluconeogenesis regulation (Fig. 5b, Supplementary Table S7). We further focused on 78 significantly differentially abundant proteins based on an overall evaluation of their KEGG analyses and functions. The first 64 proteins are involved in the pathways identified by KEGG analyses, and the last 14 common proteins participate in the signal transduction which have an important role in the course of rice disease resistance (Supplementary Table S9). These candidate proteins were classified into 12 functional categories: pyruvate metabolism; proteasome; photosynthesis; oxidative phosphorylation; metabolic pathways; glyoxylate and dicarboxylate metabolism; glycolysis/gluconeogenesis; glycine, serine and threonine metabolism; carbon fixation in photosynthetic organisms; biosynthesis of unsaturated fatty acids; alpha-Linolenic acid metabolism; and signal transduction. We linked these candidate proteins using a protein interaction network. Functional protein association networks generated with STRING revealed the functional links between different proteins. Three major clusters of interacting proteins in the resistant cultivar are highlighted with circles in Fig. 6a. These proteins are involved in glyoxylate and dicarboxylate metabolism; glycine, serine and threonine metabolism; and unsaturated fatty acid biosynthesis. Ferredoxin-dependent glutamate synthase (Q69RJ0) is the central core protein of this interaction network, due to its interactions with many other proteins involved in glyoxylate/dicarboxylate and glycine/serine/threonine metabolism. A glycolysis/gluconeogenesis protein interaction network was constructed in the susceptible cultivar (Fig. 6b). Enolase 2 (Q10P35), which interacted with other proteins directly or indirectly, is the central core protein of this interaction network.

Validation of protein profiles by qRT-PCR

To investigate whether changes in the expression of proteins were regulated at the transcriptional level, the expression pattern of 12 selected genes which have roles in disease resistance was further validated experimentally by qRT-PCR. As shown in Fig. 7, *PR1* (*Os07g0129300*), *PBZ1* (*Os12g36880*), *LOX* (*rci-1*), *BAP* (*OJ1509C06.18*) and *PAL (PAL)* were up-regulated in both disease-resistant and disease-susceptible cultivars after 24 h and 48 h of infection. These genes may represent immune regulators acting

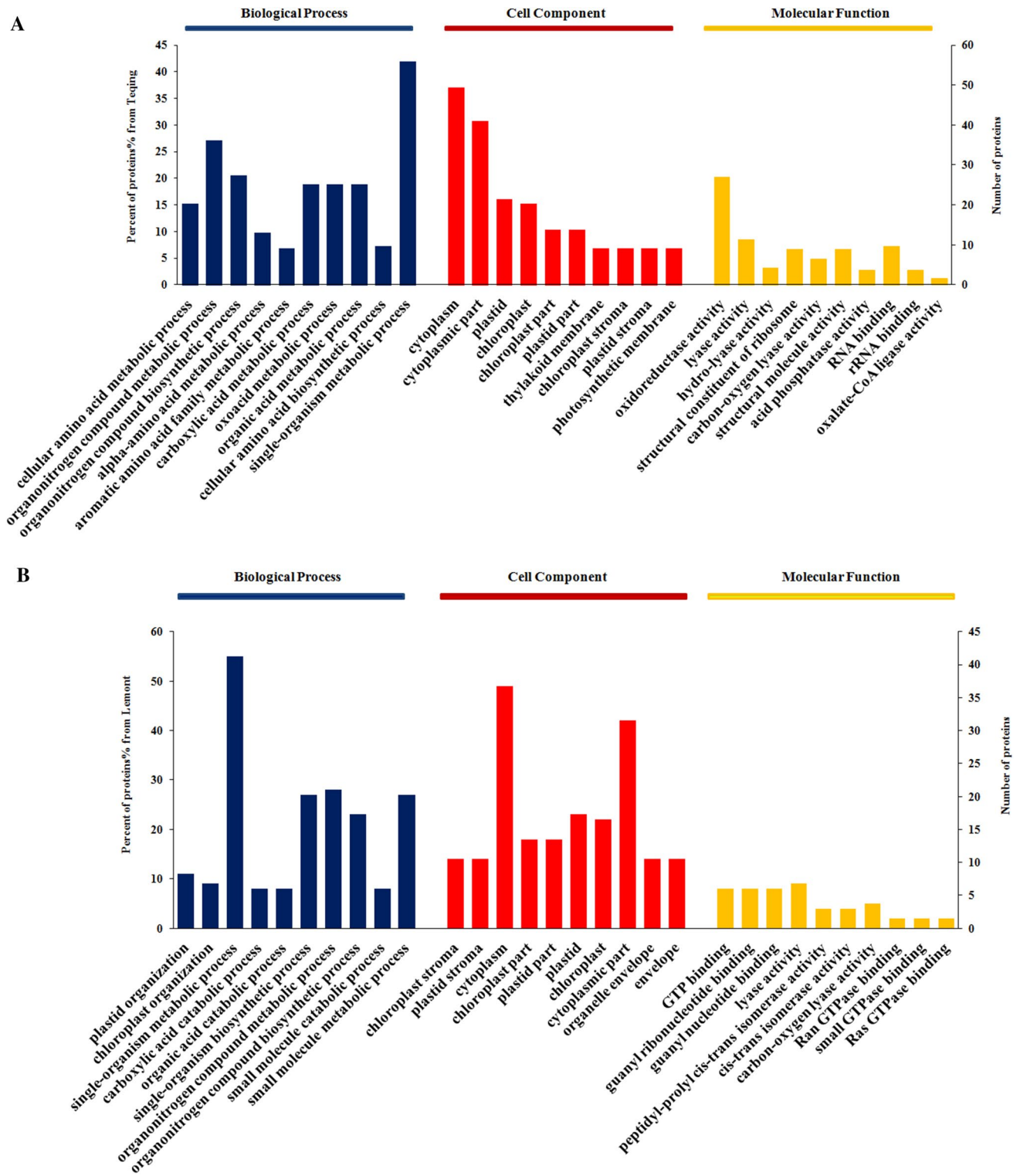

Fig. 4 Bioinformatic analysis of the identified proteins from the two cultivars. The ten most significantly enriched terms in the level four Gene Ontology hierarchy are shown, and the percentage and count of the proteins in each term are shown on the left and right y-axes. **a** Teqing, **b** Lemont

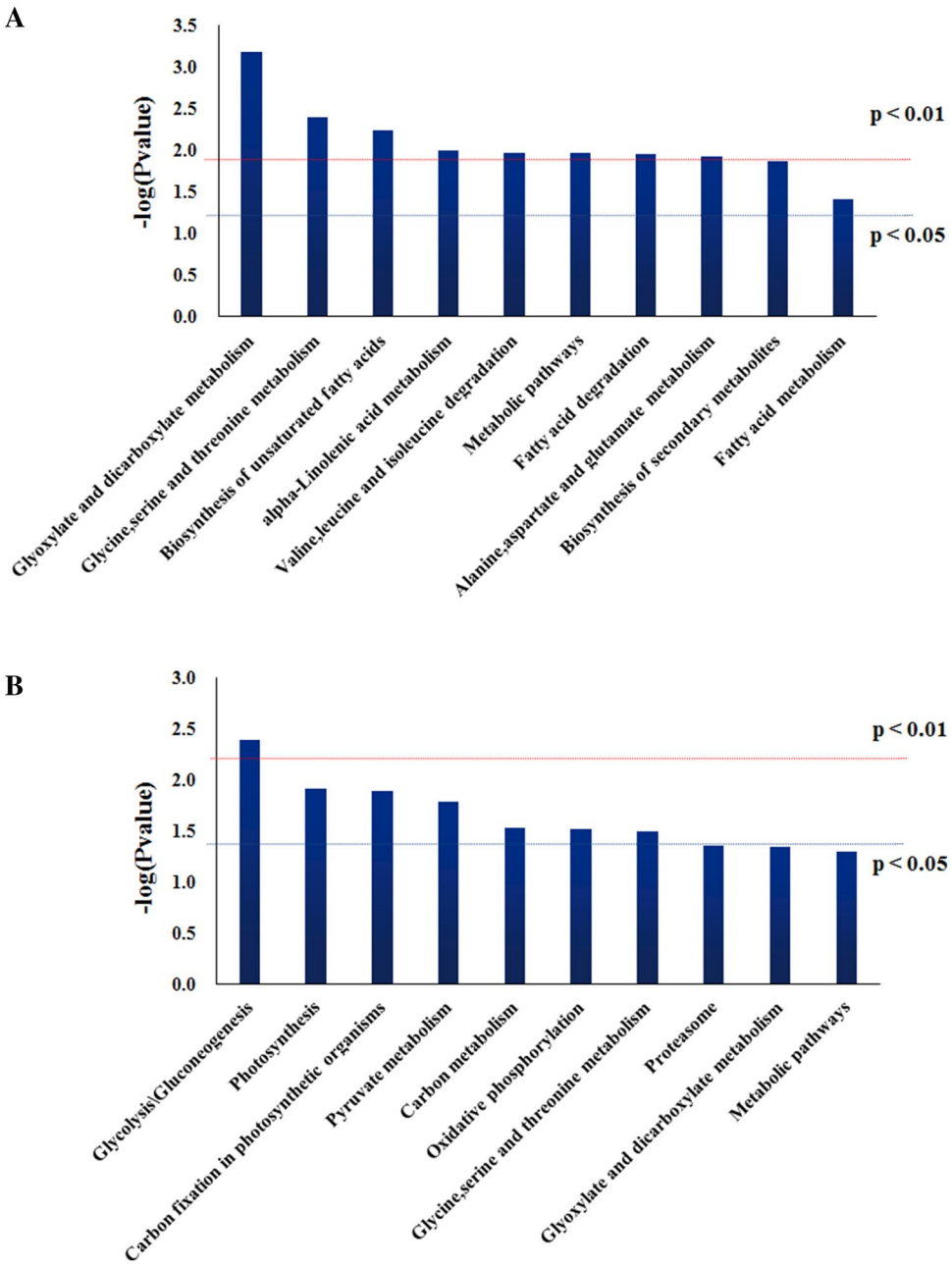

Fig. 5 Enriched KEGG pathways in the two cultivars. **a** Teqing, **b** Lemont

in basal responses, since their expression was increased in both cultivars upon infection. However, their expression levels were different in the two cultivars. For example, *PBZ1* showed a higher expression level in Teqing than in Lemont after pathogen stress. *PR2* (*Gns6*), *MDAR* (*Os08g0557600*) and *POD* (*APX1*) were induced in only the disease-resistant cultivar. In contrast, *MDH* (*OsI29345*) was induced in the susceptible cultivar but down-regulated in the resistant cultivar after *R. solani* infection. Therefore, it may negatively regulate rice immunity. However, *PR1* expression was induced approximately fourfold and sevenfold in Teqing and Lemont, respectively, at 48 hpi following *R. solani* infection. The expression level of *CS* (*OsI06215*) was similar in the two cultivars against *R. solani* infection (data not shown). In the proteomic analysis, this protein was unchanged in Lemont but increased in abundance in Teqing.

Assessment of protein function

To further understand the role of the differently abundant proteins in plant immunity, a total of four proteins were chosen for function assessment based on their protein expression during infection and the putative functions. PR1 which involved in SA signal pathway, was induced in both cultivars

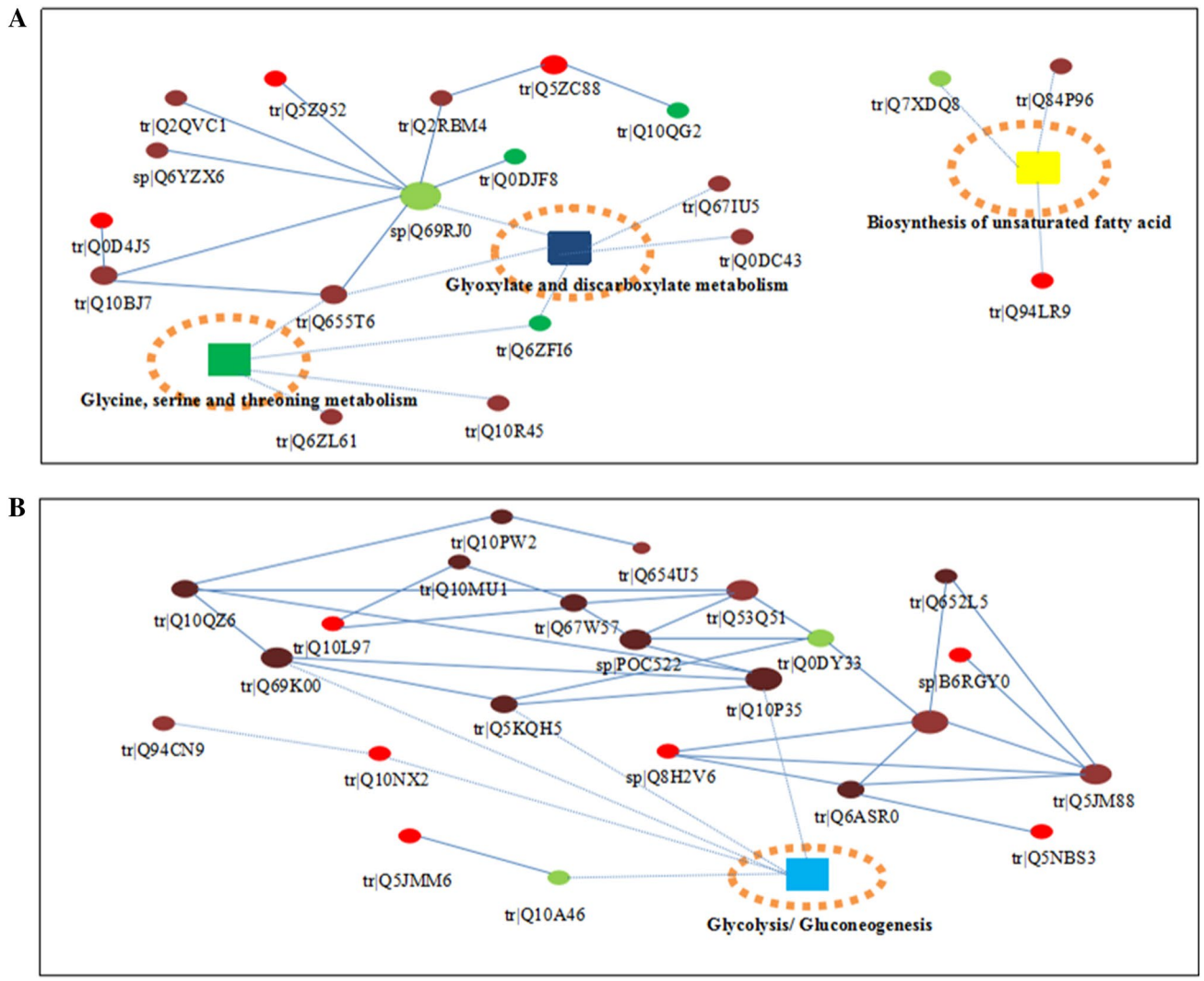

Fig. 6 Protein interaction network generated with STRING based on fold change and KEGG pathway enrichment in the two cultivars. **a** Teqing, **b** Lemont

(Fig. 7). PBZ1, a JA signal pathway inducible protein, was also induced in both cultivars (Fig. 7). Monodehydroascorbate reductase (MDAR) is crucial for Ascorbate (AsA) regeneration and essential for maintaining a reduced pool of AsA. Overexpression of MDAR confers enhanced resistance against ozone, salt and polyethylene glycol (PEG) stress (Eltayeb et al. 2007). Blight-associated proteins (BAPs) are found throughout the plant, most abundantly in the xylem and vascular cambial zone of roots and stems. One BAP that was analyzed and selected for further characterization was found to resemble β-1,3-glucanase (Derrick et al. 1993). In this study, both MDAR (Q6ZJ08) and BAP proteins (Q6K4C4) were increased in the resistant cultivar. We introduced the open reading frames of candidate protein under the control of the CaMV 35S promoter into *Nicotiana benthamiana* and inoculated the leaves with *R. solani* at 48 h after agroinfiltration. Compared with the empty vector (EV), overexpression of these proteins in *N. benthamiana* increased resistance to *R. solani* (Fig. 8a). In addition, small lesions were observed on leaves expressing each individual protein at 48 hpi (Fig. 8b).

Discussion

R. solani is an important soil-borne pathogen that causes significant damage to rice globally (Zheng et al. 2013). To date, little is known about the mechanisms of host defense in response to infection. In this study, an iTRAQ-based comparative proteomic analysis between the resistant (Teqing) and susceptible (Lemont) rice cultivars was employed to investigate the potential functional proteins related to

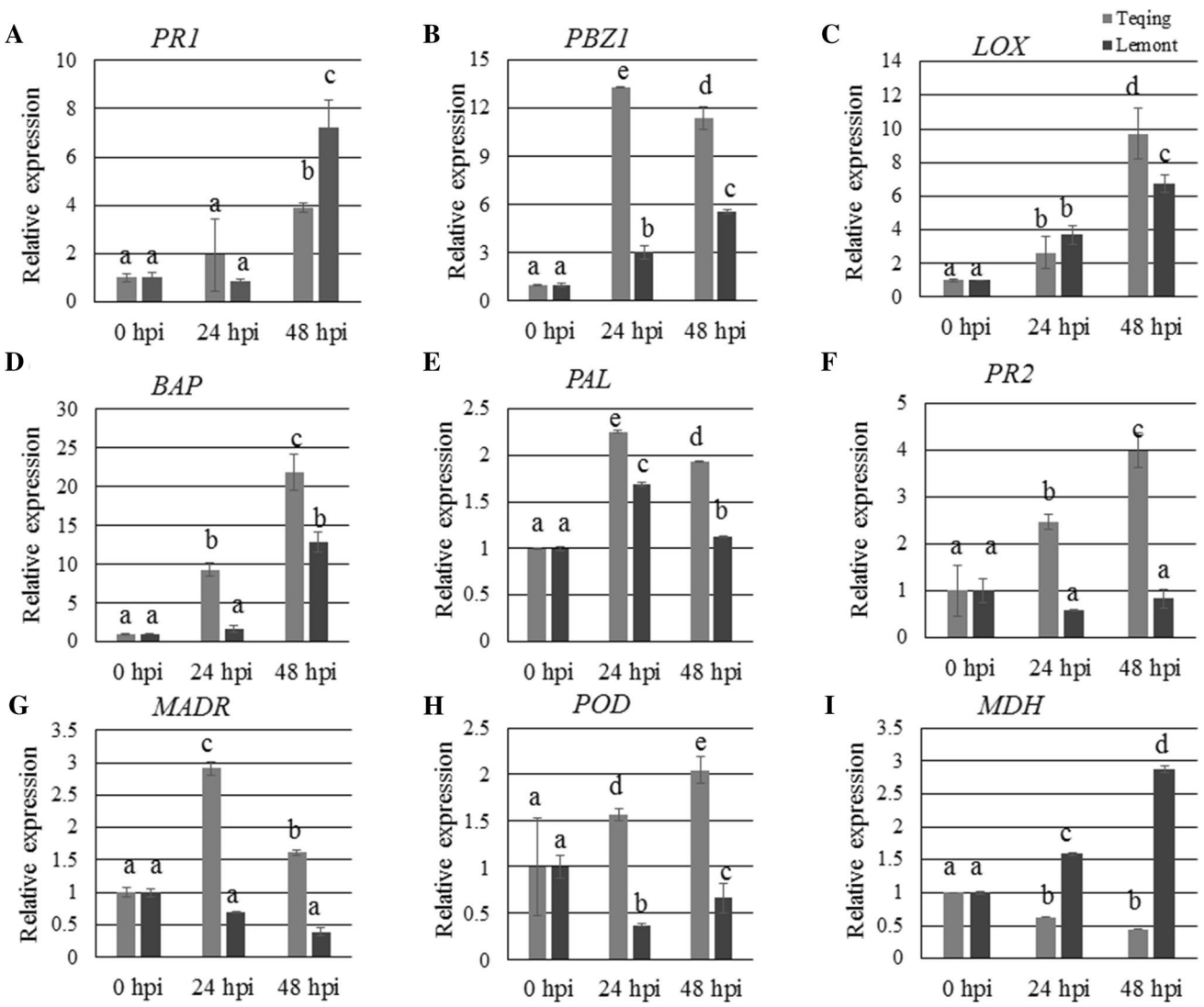

Fig. 7 Expression of representative genes encoding differentially expressed proteins as shown qRT-PCR in disease-resistant and disease-susceptible rice cultivars at 0, 24 and 48 h after *R. solani* infection. Different letters indicate significant differences based on two-way ANOVA ($p < 0.05$)

challenge with the fungal pathogen *R. solani*. A total of 755 differentially expressed proteins were identified in the resistant and susceptible rice cultivars after inoculation with *R. solani* (Table S2).

Influence of *R. solani* infection on glyoxylate and dicarboxylate metabolism

In plants, the glyoxylate cycle has a key role in conversion of acetyl coenzyme A (CoA) into oxaloacetate via fatty acid β-oxidation and in the subsequent conversion into sugar (Beevers 1961). In this proteomic study, five proteins, including ferredoxin-dependent glutamate synthase (Q69RJ0), glycine decarboxylase complex H-protein (Q655T6), formate dehydrogenase (Q0DC43), ribulose bisphosphate carboxylase small chain (Q67IU5) and pyridoxal phosphate-dependent transferase (Q6ZFI6) were identified (Supplementary Table S9). Formate dehydrogenase (FDH) is used to oxidize formate to CO_2 (Garrett and Grisham 2000), which is involved in abiotic and biotic stress responses in a variety of higher plants (Hourton-Cabassa et al. 1998; Suzuki et al. 1998; David et al. 2010). Our results showed that FDH was up-regulated in the resistant cultivar (Supplementary Table S9), suggesting putative role in immunity to *R. solani*. Ribulose bisphosphate carboxylase small chain (RBCS) is known to be one of the most important enzymes involved in glyoxylate and dicarboxylate metabolism (Yang et al. 2015). RBCS increased in both the susceptible and resistant rice cultivars after pathogen challenge (Supplementary Table S9). This result may indicate

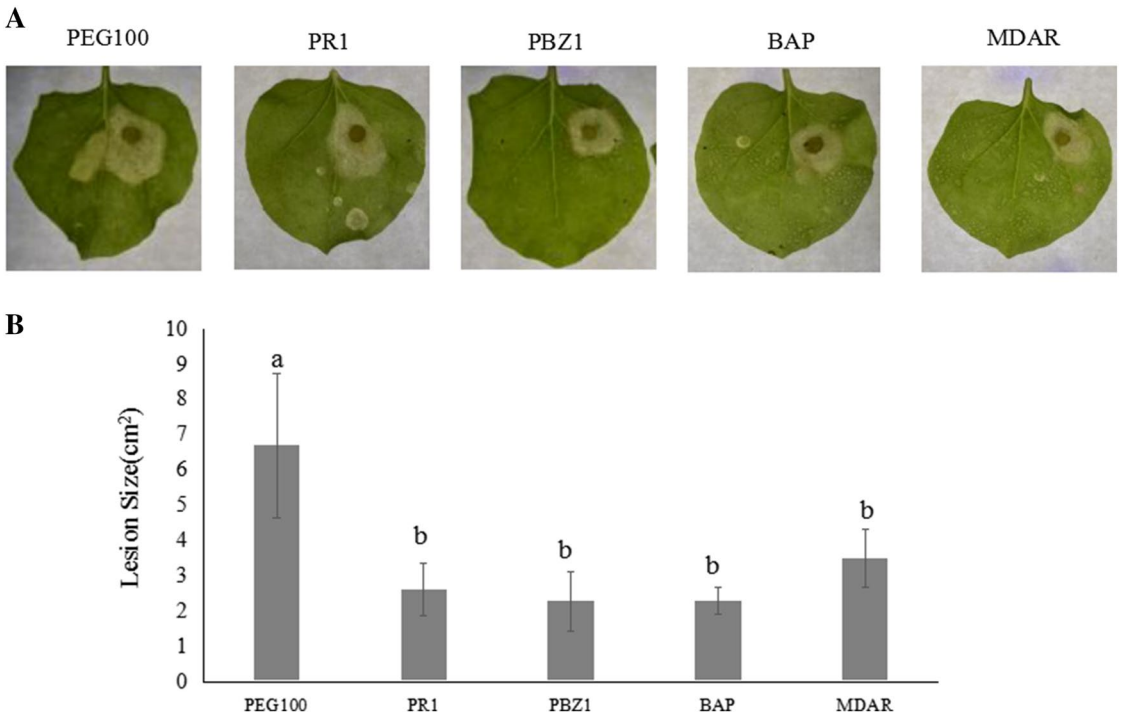

Fig. 8 Functional tests of proteins in promoting resistance to *R. solani* infection. **a** Photos of agroinfiltrated *N. benthamiana* leaves expressing recombinant proteins and subsequently inoculated with *R. solani*. **b** Lesion sizes on *R. solani*-inoculated *N. benthamiana* leaves expressing recombinant proteins. Different letters indicate significant differences based on one-way ANOVA ($p < 0.05$). Similar results were obtained from three biological replicates

that increased glyoxylate and dicarboxylate metabolism is needed to support basic defense during rice immunity to *R. solani*. Ferredoxin-dependent glutamate synthase (Fd-GOGAT) is a chloroplastic enzyme responsible for the reassimilation of photorespiratory ammonia as well as for primary nitrogen assimilation, with concomitant consumption of both ATP and reducing power (Leegood et al. 1995; Douce et al. 2001). In our study, Fd-GOGAT decreased in the resistant cultivar and increased in the susceptible cultivar. It is likely that decreasing of Fd-GOGAT reduced energy consumption in resistant cultivar than in susceptible cultivar response to *R. solani* infection, which may be an important reason why Teqing is more resistance than Lemont.

Proteins involved in glycolysis/gluconeogenesis

Glycolysis/gluconeogenesis is a catabolic anaerobic pathway that oxidizes hexoses to generate ATP, reducing agents, and pyruvate and produces building blocks for anabolism (Plaxton 1996). In this study, dihydrolipoyl dehydrogenase (Q94CN9), triosephosphate isomerase (Q69K00), 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (Q5KQH5), enolase 2 (Q10P35), phosphoenolpyruvate carboxykinase (Q10NX2) and alcohol dehydrogenase 2 (Q10A46) were identified as significantly differentially expressed proteins (Supplementary Table S9), providing evidence that the glycolysis/gluconeogenesis pathway (Houde and Diallo 2008) is involved in response to infection. Alteration in glycolysis/gluconeogenesis metabolism has also been reported previously during *R. solani* infection (Mutuku and Nose 2012; Ghosh et al. 2017). The abundance of all identified proteins related to glycolysis (except alcohol dehydrogenase 2) increased in the susceptible cultivar, but no variation was observed in the resistant cultivar, indicating that the susceptible cultivar needs more ATP by strengthening the glycolysis/gluconeogenesis pathway under R. *solani* infection.

Glycine, serine and threonine metabolism-related proteins

A general symptom of photosynthetic plants under stress is energy deficiency (Baena-Gonzalez et al. 2007), glycine, serine and threonine metabolism is vital to the regulation of plant energy metabolism(Ma et al. 2017; Igamberdiev and Kleczkowski 2018; Matityahu et al. 2019). In response to high soil temperature, glycine, serine, and threonine accumulate in the roots of foxtail millet, which accumulates more protective metabolites and is more resistant to high soil

temperature than rice (Aidoo et al. 2016). By identification of genes underlying drought resistance, alanine-glyoxylate aminotransferase 2 (AGT) was found to have significant role in energy and carbon metabolism (Sehgal et al. 2012). In our study, tryptophan synthase alpha chain protein (Q6ZL61) abundance was increased in the resistant cultivar, and malate dehydrogenase (MDH) protein (Q6YYW3) was down-regulated in the susceptible cultivar. Moreover, AGT (Q10R45) displayed higher induction in the resistant cultivar than that in the susceptible cultivar (Supplementary Table S9). These results also suggest that the resistant cultivar perceived environmental stress and reduced energy consumption under infection.

Biosynthesis of unsaturated fatty acids

In this study, two 3-ketoacyl-CoA thiolase (KAT) proteins (Q94LR9 and Q84P96) showed increased abundance in the resistant cultivar (Supplementary Table S9). KAT catalysis is a key step in fatty acid β-oxidation, which has an important role in seed development, via thiolase activity (Germain et al. 2001). KAT catalysis is a key step in fatty acid β-oxidation, which is a multistep process to produce energy (Lopaschuk et al. 2010). However, the exact role of these proteins in rice immunity remains unknown.

Potential model of rice immunity under *R. solani* infection

Based on the functions of the differentially expressed proteins and the expression patterns found using comparative proteomics, qRT-PCR and protein function, a possible mechanism is proposed for rice resistance to *R. solani* (Fig. 9). This network consists of several functional components, such as the SA and JA signaling pathways, reactive oxygen species (ROS) production and the TCA cycle.

SA plays a role in rice resistance to pathogen infection (Li et al. 2012). Phenylalanine ammonia-lyase (PAL) is a crucial enzyme in phenylpropanoid metabolism, catalyzing the formation of trans-cinnamic acid via the l-deamination

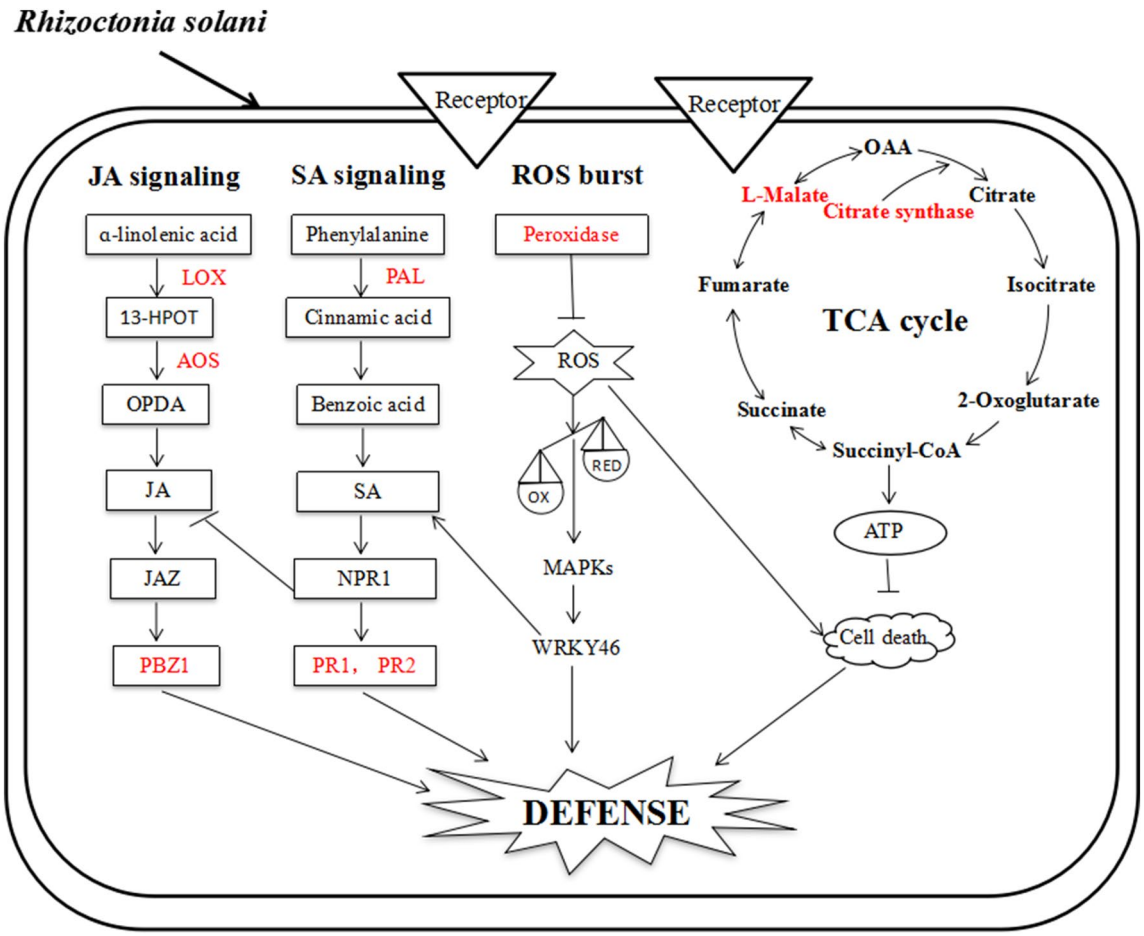

Fig. 9 A putative model of rice defense response to *R. solani* infection. This network consists of the SA and JA signaling pathways, ROS production and the TCA cycle. Red color represents significantly differentially expressed proteins identified in the study

of phenylalanine, which is a key regulator of SA accumulation (Sendon et al. 2011). The abundance of PAL proteins was up-regulated in both the resistant (A0A0E0K2L5 and Q7X720) and susceptible rice cultivars (Q75HQ7 and I1P2D6) (Supplementary Table S9), but the expression levels were higher in Teqing than in Lemont (Fig. 8). The marker genes PR1 and PR2 in the SA signal pathway were induced strongly in the resistant cultivar, but not in the susceptible cultivar (Supplementary Table S9). However, in the transcriptional analysis, the expression of PR1 was more highly up-regulated in Lemont than in Teqing (Fig. 8). The difference between the qRT-PCR and proteomic results may be due to mRNA stability, splicing, and translational regulation (Fabian et al. 2010; Brown et al. 2015). So, we assume that resistance to *R. solani* mediated by SA is due to higher levels of PR1 and PR2, which in turn give higher resistance.

JA signaling also plays an important role in rice basal defense against pathogen infection (Mei et al. 2006; Yamada et al. 2012). LOX catalyzes the oxygenation of linoleic polyunsaturated fatty acids, resulting in the formation of hydroperoxides important for JA biosynthesis (Christensen et al. 2014). *AOS2* encodes an allene oxide synthase, a key enzyme in JA biosynthesis, which plays an important role in resistance to rice blast (Mei et al. 2006). Incereased expression of *LOX* and the defense gene, *PBZ1*, can enhance resistance to *R. solani* and *M. oryzae* (Peng et al. 2012). In the present study, LOX protein abundance in the susceptible cultivar (Q9FSE5, A0A0E0QL12, and I1PFA2) was higher than in the resistant cultivar (Supplementary Table S9). However, *LOX* expression level was higher in the resistant than that in the susceptible cultivar at 48 hpi according to qRT-PCR (Fig. 8). The AOS protein (Q10EK5) was up-regulated in only the resistant cultivar (Supplementary Table S9). This result implies that the AOS protein is likely a key factor in JA biogenesis when rice is challenged with *R. solani*. Indeed, *PBZ1*, which is a JA-inducible gene (Mahmood et al. 2006), was more up-regulated in the resistant cultivar than in the susceptible cultivar according to qRT-PCR (Fig. 8).

The generation of ROS is one of the first universal reactions to abiotic or biotic challenges in plants (Torres 2010; Suzuki et al. 2012). ROS can be either beneficial or harmful to cells and tissues. Plants have evolved complex regulatory mechanisms to maintain steady-state levels of ROS and to respond to different environmental and developmental signals (Mittler et al. 2004; Circu and Aw 2010). The peroxidase family plays a major role in regulating the levels of ROS, and these detoxifying enzymes oxidize a wide variety of compounds in the presence of H_2O_2 (Teixeira et al. 2004). In this study, ten (APX1_ORYSI, Q5U1S3, Q5U1T0, Q9ST80, O22438, Q5U1Q2, Q9FYP0, Q5U1F5, Q6AVZ3 and Q6AVZ3) and seven peroxidase homologous proteins (Q7XSU7, A0A0P0VZ16, Q6K4J4, Q9SMG8, Q9FYP0, Q9LDL0 and A0A0E0H918) (Supplementary Table S9) with putative functions in ROS level regulation were detected in the resistant and susceptible cultivars, respectively. In the resistant cultivar, five proteins were up-regulated, and five were down-regulated. In the susceptible cultivar, six proteins were up-regulated, and one protein was down-regulated. Interestingly, one peroxidase protein (Q9FYP0) had different expression patterns in the susceptible and resistant cultivars, suggesting that plants have different strategies in response to pathogen infection depending on the plant variety. ROS have been reported to play a role in the interaction between *R.* solani and wheat (Foley et al. 2016). The accumulation of ROS may be essential for cell death in the infected tissues.

The TCA cycle is one of the iconic pathways in metabolism and is associated with energy metabolism or the oxidation of respiratory substrates (Sweetlove et al. 2010). In the present study, two identified proteins were involved in the TCA cycle. Citrate synthase (Q9FUJ7) was up-regulated in only the resistant cultivar (Supplementary Table S9). However, MD was down-regulated in the resistant cultivar (A0A0E0KJK7) and up-regulated in the susceptible cultivar (A2YVI5), which was consistent with the transcriptional data (Supplementary Table S9 and Fig. 8). The increase in the levels of metabolic proteins related to the TCA cycle indicates enhanced respiration in rice after infection. Increased respiration has previously been observed during the interaction between *R. solani* and rice (Suharti et al. 2016; Ghosh et al. 2017). Plants usually increase respiration to support the induction of defense programs upon pathogen infection (Berger et al. 2007). Our recent study found that SA and JA signal pathways are also involved in defense response to the blast disease (Liu et al. 2014). In contrast, ROS production and TCA cycle are only found in rice immunity against *R. solani* infection. So we inferred that SA and JA are the general defense machinery to broad-spectrum pathogen.

Conclusions

In conclusion, our findings provide a possible model by which multiple signal pathways are activated to regulate rice immunity following *R. solani* infection. These pathways do not function separately; instead, they work together as an integral network via multilateral cross talk. However, further research is needed to explore the importance of these proteins and their potential roles during the establishment of rice sheath blight disease.

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Authors' contributions DN conceived the idea and designed the project; HM, CS, and LQ performed the experiments; DN, HM, CS, and HZ analysed and interpreted the data; DN, HM and CS wrote the manuscript; all authors read and approved the final manuscript for publication.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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