Proteome Responses to Individual Pathogens and Abiotic Conditions in Rice Seedlings

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ABSTRACT

Rice plants under field conditions experience various biotic and abiotic stresses and are adapted to survive using a molecular cross-talk of genes and their protein products based on the severity of a given stress. Seedlings of cultivated variety ASD16 (resistant to fungal disease, blast; tolerant to abiotic stress, salinity) were subjected to salt, drought, high temperature and low temperature stress as well as infection by *Rhizoctonia solani* and *Xanthomonas oryzae* pv. *oryzae* (causing reemerging diseases such as sheath blight and leaf blight), respectively, the sheath blight and bacterial leaf blight pathogens. Leaf proteome was analyzed using two-dimensional electrophoresis and differentially expressed proteins were identified using mass spectrometry. In addition to many other differentially expressed proteins, acidic endochitinase was found to be upregulated during fungal infection and drought treatment, and a germin-like protein upregulated during fungal infection and high

temperature stress. These two proteins were further validated at the gene expression level using reverse transcription-PCR in dual stress experiments. Pot culture plants were subjected to fungal infection followed by drought and drought followed by fungal infection to validate chitinase gene expression. Similarly, plants subjected to fungal infections followed by high temperature stress and vice versa were used to validate the expression of germin-like protein-coding gene. The results of the present study indicate that chitinase and germin-like protein are potential targets for further exploration to develop rice plants resistant or tolerant to biotic and abiotic stresses.

Keywords: abiotic stress, biochemistry and cell biology, biotic stress, cross-talking proteins, dual stress, genetics and resistance, proteome analysis, rice

Under field conditions, agricultural crops experience multiple biotic and abiotic stresses and at a given point of time, possibly by more than one stress occurring either simultaneously or combined. There are numerous reports on the overlapping molecular responses to biotic and abiotic stresses in plants (Cheong et al. 2002; Mathioni et al. 2011; Ramegowda and Senthil-Kumar 2015; Varela et al. 2018). Rice is a crop which is grown under various environmental conditions and geographical locations throughout the world. Biotic and abiotic stress factors are considered to be major limiting factors in productivity of rice. Sheath blight caused by *Rhizoctonia solani* causes up to 45% loss in rice yield (Margani and Widadi 2018). *Xanthomonas oryzae* pv. *oryzae* causes bacterial leaf blight in rice and affects various varieties worldwide (Singh et al. 2015). Similar to other plant species, rice has evolved with molecular and cellular responses to encounter stresses and also prioritizes according to life threatening status using convergent gene/protein nodes (Sruthilaxmi and Babu 2018). Molecular signaling and stress response pathways are known to cross-talk, leading to regulation of genes and their protein products required for acclimating the stress, based on priority.

Several bioinformatic, proteomic and genome-wide transcriptomic studies have attempted to understand the complexity of the overlapping cellular and molecular responses of many plants including rice, under biotic and abiotic stress conditions (Berens et al. 2019; Gupta et al. 2016; Ku et al. 2018; Nejat and Mantri 2017; Pandey et al. 2017; Sinha et al. 2019). In addition, overexpression of genes has contributed to explore the roles of important genes which

play dual roles, either synergistically or antagonistically, in regulating the response of rice plants to biotic and abiotic stresses (Alam et al. 2014; Asano et al. 2012; Liu et al. 2008; Mellacheruvu et al. 2016; Kuang et al. 2017; Pillai et al. 2018; Wang et al. 2016).

Proteomic approaches to study the molecular mechanisms underlying abiotic and biotic stress tolerance or resistance are mostly confined to the profiling of rice plants and organelle proteins on individual stress basis. This approach has been used to study responses of rice plants to salinity (Damaris et al. 2016; Ghaffari et al. 2014; Hussain et al. 2019; Yan et al. 2005), high temperature (Inomata et al. 2018; Lin et al. 2005), low temperature (Ji et al. 2017; Yan et al. 2006), chilling (Cen et al. 2018), drought (Chintakovid et al. 2017; Cunhaa et al. 2019; Salekdeh et al. 2002), drought-flood alteration (Xiong et al. 2018), ozone (Agrawal et al. 2002), osmotic (Zang and Komatsu 2007), wound (Shen et al. 2003), cadmium (Zhong et al. 2017), blast disease (Kim et al. 2003; Konishi et al. 2001; Narula et al. 2018), bacterial leaf blight (Kumar et al. 2015; Li et al. 2012; Mahmood et al. 2006), and yellow mottle virus (Ventelon-Debout et al. 2004). Approximately 45 proteins including pathogenesis-related, cell wall metabolism, and cytoskeleton development were identified to be differentially regulated during the interaction of rice with sheath blight pathogen *R. solani* (Prathi et al. 2018). Upregulation of 14 genes encoding some of these proteins was observed in the disease-resistant genotype.

Comparative proteomic studies between different biotic and abiotic stress responses in rice have been reported to some extent. A study by Xu et al. (2013) revealed cross-talk between protein-level responses induced by H₂O₂ and black-streaked dwarf virus infection. The SbAP37 transcription factor was found to play a key role in regulating downstream pathways toward tolerance during combined salt and drought stress in rice. A comprehensive proteome and metabolome analysis of rice spikes during contrasting stress conditions such as drought and flooding (Xiong et al. 2019) showed overlapping differential expression of proteins involved in energy metabolism, photosynthesis, carbohydrate

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metabolism, and reactive oxygen species metabolic processes. This indicates that part of the response mechanism in plants, even under contrasting stress conditions, could be common due to the commonalities in cellular damage and physiological stress caused by these stress conditions.

Pandey et al. (2017) reviewed previous reports on the impact of combined drought and pathogen stress and strategies for crop improvement. Hormone responsive genes and their protein products play a role in cross-talking during abiotic and abiotic stress responses and jasmonic acid is a core component (Yang et al. 2019). Berens et al. (2019) reported the leaf age related balancing of hormone cross-talk during simultaneous biotic and abiotic stress in *Arabidopsis*. Stress conditions are known to be more destructive during concurrent occurrence than when they occur separately. Bai et al. (2018) observed overlapping responses to combined salinity and pathogen stress in tomato. Rice proteome responsive to *X. oryzae* pv. *oryzae*, the bacterial leaf blight pathogen exhibited differentially expressed abiotic stress responsive proteins as well. Based on the analysis of publicly available transcriptome data, Cohen and Leach (2019) found a core transcriptome of 913 genes in rice common to biotic and abiotic stress responses. In addition to the common downregulation of photosynthesis-related genes during biotic and abiotic stress, phytohormone pathway genes and some transcription factors including bZIP were proposed by them as potential candidates for improving multiple stress tolerance in rice. Therefore, developing crop varieties tolerant to more than one stress involves understanding the molecular cross-talk at the gene and protein levels and the relative importance of their function during single and dual stress conditions.

In the present study, we report the proteome-level responses of ASD16, a popularly cultivated rice variety in India, to multiple abiotic (salt, drought, low temperature, and high temperature) and biotic (sheath blight and bacterial blight diseases) stresses and the two key proteins identified in common during biotic and abiotic stress responses.

MATERIALS AND METHODS

Seeds, chemicals, and microbial cultures. Rice seeds (cultivar ASD16) were obtained from the Paddy Breeding Institute, Tamil Nadu Agricultural University, Coimbatore, India. All the chemicals used for two-dimensional electrophoresis were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Hoagland's nutrient solution was prepared manually by mixing the salts and nutrients as mentioned in the composition of the commercial solution (Sigma-Aldrich, St. Louis, MO, U.S.A.). Cultures of *R. solani* and *X. oryzae* pv. *oryzae* were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India and subcultured, respectively, in potato dextrose agar (PDA) and Luria Bertani agar (LBA), HiMedia, Mumbai, Maharashtra, India.

Plant growth conditions and stress treatments. The seeds were surface sterilized in 70% ethanol for 5 min, followed by using 3% sodium hypochlorite for 2 min and extensively washed with three rounds of sterile water. The seeds were placed on two layers of sterile Whatman No. 1 filter paper moistened with sterile water in Petri dishes at 25°C in darkness for 3 days. After sprouting, 25 seeds were placed in the top portion of sterile germination paper (30 × 22 cm, National Scientific Supplies, Chennai, Tamil Nadu, India). These seeds were covered with a small piece of wet germination paper. The rolled germination paper setup was placed in a beaker containing Hoagland's nutrient solution in the upright position for 15 days under laboratory conditions (12 h light per day) and at room temperature (25 ± 2°C). In the case of abiotic stress treatments, a set of seedlings were grown under normal conditions (control) without any stress. Another set was subjected to abiotic stress as described below, followed by a recovery period from stress. The recovery period was used as per the followed model for abiotic stress

treatments (Osmolovskaya et al. 2018; Xu et al. 2010) to nullify the stress escape and avoidance mechanism if any in the tested variety, and to focus on the mechanisms of stress tolerance that will be obvious during the recovery period. The stress conditions were determined based on preliminary experiments carried out (explained in Results section).

Salt stress. Germination paper rolls with 15-day-old seedlings were transferred to fresh beakers containing nutrient solution and different concentrations of NaCl (0, 10, 50, 100, and 150 mM). Salt stress was imposed for 48 h followed by recovery in fresh nutrient solution without NaCl for 24 h. Three replicates of 25 seedlings each were used for each treatment.

Drought stress. To impose drought stress, PEG 6000 (polyethylene glycol, HiMedia, Mumbai, Maharashtra, India) was added to the nutrient solution to a concentration of 25% (Lawlor 1970). Drought stress was imposed for 0, 2, 5, 7, and 10 days followed by recovery in fresh nutrient solution. Three replicates of 25 seedlings each were used for each treatment.

Temperature stress. Seedlings were exposed to different temperatures by placing the germination paper setup in different plant growth chambers set at 15, 37, and 45°C. For 4°C exposure, seedlings in germination paper were kept in a cold room. Seedlings at room temperature served as control. Seedlings were collected after 48 h of exposure to different temperatures followed by a 24 h recovery period at room temperature.

Biotic stress-infection with *X. oryzae* pv. *oryzae*. *X. oryzae* pv. *oryzae* isolate was grown overnight at 30°C in LB broth. Seeds were germinated in germination paper for 10 days in nutrient solution as described earlier. Leaves of each plant were inoculated with *X. oryzae* pv. *oryzae* by leaf clipping method, in which the leaf was cut with a pair of sterile scissors predipped in the bacterial suspension (Kauffman et al. 1973). After inoculation, an artificial humidity chamber was created using polythene bags and sprayed with sterile water left undisturbed for 24 h. Seedling samples were collected at 24, 48, and 72 h. The seedlings clipped with scissors predipped in LB broth without bacteria served as control.

Biotic stress-infection with *R. solani*. *R. solani* was grown in PDA plates for 2 weeks. Rice seedlings in the germination paper setup were covered using polythene bags sprayed with sterile water 24 h before and after inoculation. Pin pricks were made on the leaves using sterile needle, and 5 mm mycelial discs were placed and tied using a piece of moist cotton and parafilm. Sterile water was used to wet the cotton every day. Seedlings inoculated with plain PDA discs served as control. Samples were collected at 5, 7, and 10 days postinoculation.

Growth measurements. In salt, drought and temperature stress experiments, root and shoot lengths of the seedlings were measured after stress treatments and recovery. All the seedlings were grown in germination paper rolls for 15 days maintained under open conditions (30 to 32°C) outside the lab in the corridor during day time (9 h direct sunlight) and inside the lab (25 ± 2°C) during rest of the period in a day (15 h) until stress treatments were done. The seedlings were removed from germination paper rolls, stretched straight individually near a ruler, and the length was measured. All the stress experiments were repeated twice.

Solubilization of leaf proteome. Frozen leaf tissues from stress treated and untreated seedlings were homogenized in pestle and mortar using liquid nitrogen. Two hundred milligrams of the sample was dissolved in a solution containing 10% trichloroacetic acid and 0.07% dithiothreitol (DTT, Bio-Rad). After incubation for an hour at -20°C, the suspension was centrifuged (12,000 rpm; 4°C; 10 min). The pellet was washed four times with a washing solution (ice-cold acetone containing 0.07% DTT). Air-dried pellets were suspended in Bio-Rad sample solubilization buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% carrier ampholytes, and 0.01% bromophenol blue). The samples were incubated overnight at 4°C and centrifuged at 12,000 rpm for 20 min at 4°C to get a clear supernatant. The supernatant was transferred to a fresh tube and the

protein content was quantified by the Bradford assay (Bradford 1976).

Two-dimensional electrophoresis. All the chemicals, reagents, IPG strips, and instrumentation used for two-dimensional gel electrophoresis were from Bio-Rad Laboratories. Protein samples of control (untreated) and stress-treated leaf tissues were used for two-dimensional electrophoretic analysis. The volume of samples equal to 100 µg of protein was mixed with the required volume of rehydration buffer to a total working volume of 125 µl. The samples were applied to a rehydration tray and 4 to 7 pH IPG strips were rehydrated overnight at room temperature. After application of sample to the strips and allowing for 45 min absorbing, the wells of the rehydration tray were filled with sterile mineral oil to avoid evaporation. First-dimension electrophoresis was carried out in a Protean IEF unit. Focusing of strips were programmed as follows: 50 µA limit per strip; 250 V for 15 min; 4,000 V for 2 h; 4,000 V for 5 h (20,000 Vh); 500 V hold step. The strips were equilibrated using equilibration buffers I and II as described (Babu et al. 2005). The strips were placed on polyacrylamide separating gels with a small piece of filter paper absorbed with protein molecular weight marker. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out in a 12% separating gel at a constant voltage of 50 V. When the dye front reached the bottom of the gel, electrophoresis was suspended and the gels were stained overnight with Coomassie brilliant blue R-250. After destaining, the gels were visualized using Gel documentation (Gel Doc EZ System, Bio-Rad Laboratories) and the images were recorded. Two replicated two-dimensional gels were tested for samples from each stress treatment and each experiment.

Image analysis and mass spectrometry. Gel images were further analyzed using PDQuest software. Gel images from replicated samples and repeated experiments were compared and differentially expressed as well as unique spots compared with untreated control samples were marked for mass spectrometry analysis. The spot intensities were quantified using GelQuant.NET software (<http://biochemlabsolutions.com/GelQuantNET.html>). The protein spots marked on the images were excised from the respective gels using sterile scalpel and stored in microcentrifuge tubes at -20°C. Mass spectrometry analyses were carried out at Sandor Life Sciences Pvt. Ltd., Hyderabad, India using a Bruker daltonics ULTRAFLEX III Mass Spectrometer with spectra internally calibrated using trypsin auto-digestion products. The excised gel pieces containing protein spots were destained and the proteins were alkylated, reduced, eluted, and trypsin-digested. The peptides obtained were mixed with HCCA matrix in 1:1 ratio and the resulted 2 µl was spotted onto the MALDI plate. After air drying the sample, it was analyzed on the MALDI TOF/TOF ULTRAFLEX III instrument and further analysis was done with FLEX analysis software to obtain the peptide mass fingerprint. The obtained peptide masses were searched on the Mascot™ Peptide Mass Finger print database (Matrix Science). Data were used to identify the protein using the MASCOT search tool (<http://www.matrixscience.com/>).

Dual stress experiments. Rice seeds (cultivar ASD16) were sown in soil mix (clay loam soil containing farmyard manure in 10:1 ratio) in pots (Silverspring, Coimbatore, India) of 30 cm diameter × 26 cm height and allowed to grow in greenhouse (30°C day and 24°C night temperature). Plants were watered at regular intervals. After 15 days, the seedlings were transplanted (four seedlings per pot) in fresh pots with the same soil mix. After 45 days, the plants were subjected to drought, high temperature, and sheath blight fungal stress.

Drought stress. Artificial drought was induced in a set of plants using PEG 6000 (25 g in 100 ml of water used for irrigating the pot soil mix). After 48 h, the plants were excessively watered to recover the drought stress draining PEG-treated water. Leaves were collected 24 h after recovery and stored at -20°C for further use.

Plants watered without PEG served as control. Three replicates of four plants each was used in the experiment.

Temperature stress. Rice plants were grown in pot culture as described. Forty-five-day-old plants were subjected to high temperature stress by placing the pots with plants in a 37°C growth chamber (Lark Innovative Fine Teknowledge, Chennai, India). After 48 h of temperature stress, the plants were recovered by placing them in greenhouse temperature for 24 h, and leaf samples were collected and stored at -20°C. Plants that continued to grow in greenhouse without temperature stress served as control.

Fungal stress. Rice plants were grown in pot culture as described and 45-day-old plants were inoculated with sheath blight pathogen (*R. solani*). Plants were covered using polythene bags sprayed with sterile water 24 h before and after inoculation. Sheath portions were pin pricked using a sterile needle and 5 mm mycelial discs were placed and tied using wet cotton and parafilm. The cotton was moistened with sterile water on a daily basis. Leaf sheath samples 5 cm below and above the inoculation site were cut after 10 days and stored at -20°C. Plants inoculated with plain PDA discs served as control. Three replicates with four plants each and three sheaths per plant were used in the experiment.

Drought stress followed by fungal infection. Pot culture-grown plants (45-day-old) were subjected to drought stress using PEG-treated water for 48 h, and *R. solani* mycelial discs were used to inoculate the same plants after drought stress. Leaf samples as well as leaf sheath samples (5 cm below and above the point of inoculation) were collected 10 days postinoculation.

Fungal infection followed by drought stress. Pot culture-grown plants (45-day-old) were inoculated with mycelial discs of *R. solani*. After 10 days, the plants were subjected to drought stress using PEG-treated water for 48 h followed by 24 h of recovery using normal water.

Temperature stress followed by fungal infection. Pot culture-grown plants (45-day-old) were exposed to 37°C in a growth chamber as described earlier for 48 h and taken to greenhouse. The plants were inoculated with *R. solani* and samples were collected 10 days postinoculation.

Fungal infection followed by temperature stress. Pot culture-grown plants (45-day-old) were inoculated with *R. solani* and after 10 days were exposed to 37°C in a growth chamber for 48 h. After a 24 h recovery period by keeping the plants in greenhouse conditions, samples were collected.

In all dual stress experiments, leaf samples as well as leaf sheath samples (5 cm below and above the point of inoculation of *R. solani* mycelia disc or plain PDA disc, as the case may be) were collected and pooled.

RNA extraction and RT-PCR analysis. Total RNA was extracted from frozen leaf tissue using Plant RNA isolation kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. After quantification using a nano-drop facility (Vellore Institute of Technology, Vellore, India), 200 ng of RNA was used for first-strand cDNA synthesis. The reaction mixture contained RNase-free water, 2× reverse transcription buffer, 5 mM dNTPs, 0.5 mM dithiothreitol, 0.5 µM oligo dT primer, RNase inhibitor, and 2 units of AMV reverse transcription enzyme. cDNA synthesis was carried out at 42°C for 1 h followed by stopping the reaction at 70°C for 10 min and chilling on ice. The rice actin gene was used as an internal control. The synthesized cDNA was amplified using gene-specific primers for the rice germin-like protein gene, chitinase gene and actin. The reaction mixture contained 100 ng of cDNA, 0.5 µM respective forward and reverse primers, 2× PCR master mix in a final volume of 25 µl. PCR was carried out in a thermal cycler (Eppendorf, Germany) with initial denaturation at 94°C for 2 min and 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C (germin-like protein), 61°C (chitinase), 49°C (rice actin) for 1 min, extension at 72°C for 1 min followed by final extension at 72°C for 10 min. The experiment was repeated twice to check the reproducibility. The primers used for

amplification: germin-like protein gene (forward, 5'CACTGC CAACAAGCTCTTCA3'; reverse, 5'CTTGAAGTTGGCCTT GATGG3'), chitinase (forward, 5'CGGGGTGAAGTCTCTGCTCT3'; reverse, 5'GGCGAGGTCGTCCCAGAACT3'), and rice action (forward, 5'GTATCCATGAGACTACATACT3'; reverse, 5'TACT CAGCCTTGGCAATCCACA3'). Amplified cDNAs were analyzed in 1.5% agarose gel stained with ethidium bromide. Images of the gels were obtained using the Gel Doc EZ System, Bio-Rad Laboratories. The band intensities were calculated by analyzing the images using ImageJ software (Schneider et al. 2012).

Statistical analysis. Statistical analyses were performed using GraphPad prism software v.7. Growth data (root and shoot length) were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Two-dimensional gel spot intensity quantification data were analyzed using two-way ANOVA with spot intensity values obtained from images of two replicated experiments per sample. Mean values were compared using Tukey's multiple comparisons test. Out of the cross comparisons, for every spot intensity quantification value, the mean $\pm$ standard error (SE) difference between control and stress treatment were individually used to represent in the bar chart. Band intensity quantification data of RT-PCR experiments were analyzed using one-way ANOVA with intensity values obtained from two replicated experiments. Mean values were compared using Tukey's multiple comparisons test. For the chitinase gene, in the case of individual stress treatments, mean $\pm$ SE difference was used to compare fungal and drought stress with their corresponding control. For dual stress treatments, comparison was done as follows: (i) data of drought control was used as the control to compare with data on drought followed by fungal infection and (ii) data of fungal control was used as the control to compare with data on fungal infection followed by drought. For the germin gene, in the case of individual stress treatments, mean $\pm$ SE difference was used to compare fungal and high-temperature stress with their corresponding control. For dual stress treatments, comparison was done as follows: (i) data of high-temperature control was used as the control to compare with high temperature followed by fungal infection and (ii) data of fungal control was used as the control to compare with fungal infection followed by high temperature stress. In all analyses, $P < 0.05$ implied a significant difference.

RESULTS

Growth responses of stressed rice plants. The effect of an increase in the concentration of salt (NaCl) on the length of root and shoot of 20-day-old seedlings is shown in Supplementary Figure S1. There was a significant reduction in root and shoot length under salt stress with higher concentrations such as 50, 100, and 150 mM NaCl compared with the control. Root and shoot length measurements of seedlings treated under drought conditions are shown in Supplementary Figure S2. There were no significant differences in the root and shoot lengths of seedlings under drought conditions compared with the untreated control. In the temperature stress treatment, the short length of seedlings was reduced significantly compared with the control at 37 and 45°C (Supplementary Fig. S3). In the case of root growth, seedlings subjected to 15°C showed significantly higher root length and those subjected to 45°C showed significantly lower root length compared with the control. After the inoculation of *R. solani*, the inoculated spot in the seedlings showed small lesions on the 5th day, which were later found to spread on 7 and 10 days postinoculation. Similarly, *X. oryzae* pv. *oryzae*-inoculated seedlings showed yellowing symptoms on the inoculated leaves at 48 h and were observed to spread at 72 h postinoculation.

Proteome-level responses of stressed rice plants. In order to understand the molecular mechanisms of stress response, a proteomics approach was applied to analyze the changes in protein profile during different stress conditions in rice seedlings. When the plants were subjected to salt stress conditions, we observed that all

the higher concentrations of NaCl tested (above 10 mM) had a significant effect on root and shoot length. Although significant root and shoot length differences were not observed among the 50, 100, and 150 mM NaCl treatments, seedlings were visually slender with thin leaves under 150 mM NaCl treatment. To have sufficient tissue for proteome analysis, seedlings treated with 100 mM NaCl water were used. Since seedlings treated with different drought periods had no significant difference in root and shoot length, a middle value of 5 days drought was used for proteome analysis. For temperature stress, seedlings treated with low temperature (4°C) as well as high temperature (37°C) stress were used for proteome analysis irrespective of the root and shoot length differences observed, since the effect of temperature stress on root growth of the seedlings was different from that on the shoot growth. In *R. solani*-inoculated seedlings, although lesions started to appear from 48 h post-inoculation, typical blight symptoms were observed on the 10th day. In *X. oryzae* pv. *oryzae*-inoculated seedlings, typical blight symptoms appeared at 48 h postinoculation. Hence, samples from these two time periods were included in the proteome analysis.

The two-dimensional gel images of proteome analysis in abiotic and biotic stress-treated rice seedlings compared with the respective control are shown in Figures 1 and 2. Comparison of proteome gels of control and stress-treated samples were done using normalization, spot matching, spot warping, expression analysis, and statistical steps in the image analysis software. Background subtraction and normalization were done both automatically and manually to match the spots and also eliminate artifacts. There was more than 85% correlation between biological repeats. Differentially expressed proteins (upregulated and downregulated compared with control) and unique proteins (downregulated below detectable limit in the compared sample) in each stress treatment were compared. The initial comparisons were between the control and treated samples of each stress. Data on the quantification of spot intensities based on image analysis (across replicated gels) are presented in Figures 1 and 2. Spot intensities of all the proteins were found significantly different when compared between control and treated samples. Based on statistical significance in differential expression, 31 protein spots covering all the comparisons were selected for mass spectrometry. Based on spot intensity quantification data, five protein spots upregulated in the salt-treated samples were selected. In drought treatment, three spots upregulated in the treated sample and one spot downregulated in the stressed sample were selected. In low-temperature stress, two spots each upregulated and unique to treated sample and one spot unique to the control sample were selected. Among the spots analyzed in high-temperature stress experiments, five spots upregulated in treated samples and two spots unique to treated samples were chosen. All five spots chosen from the fungal stress-treated sample were upregulated proteins. In the bacterial inoculation experiment, four proteins that were upregulated in the inoculated sample and one protein spot downregulated in the control sample were chosen. For the control downregulated spot, the corresponding matching spot from the treated sample gel was excised. The protein spots were labeled as SBSL1 to SBSL31.

The results of mass spectrometry and the identification of protein spots are presented in Table 1. Details of spot labels, accession number of the corresponding genes, protein name, list of matching peptides, expression level under stress condition (unique, upregulated or downregulated compared with control), calculated and observed molecular mass, fold change in spot intensities compared with control are shown in Table 1. For the proteins that were unique in a given gel image, spot intensities were compared with matching region (with no visible spot) in the corresponding control or treated sample image. Hence, the intensities of these protein spots are high, showing hundreds to thousands of fold increase according to data obtained using the image analysis software (Table 1; Figs. 1 and 2).

Ribosomal proteins, photosystem II stability/assembly factor, and barley-B recombinant like protein were found to be upregulated

in salt-stressed rice seedlings. Translation factor GUF1, acidic endochitinase, and ribosomal protein were upregulated and chloroplastic glutamate synthase 1 was downregulated under drought stress. Oxygen-evolving enhancer protein and germin-like protein were upregulated under low-temperature stress. While coleoptiles phototropism protein 1 was downregulated below the detectable limit, superoxide dismutase (Cu-Zn) and a 50S ribosomal protein L12 were found to be unique in low-temperature stress. Upregulated proteins during high-temperature stress include 30S ribosomal protein 2, germin-like protein, chloroplast 23 kDa polypeptide of photosystem II, L-ascorbate peroxidase 1, and triosephosphate isomerase. Oxygen-evolving enhancer protein 1 and histone H3 were uniquely expressed in high temperature stress compared with seedlings grown at room temperature. All the protein spots selected from the *R. solani* infection experiment for mass spectrometry analysis were upregulated proteins in inoculated tissues (acidic

endochitinase, germin-like protein, cellulose synthase A, kinesin-like protein, and peroxiredoxin). Superoxide dismutase (Cu-Zn), fructose biphosphate aldolase, arginine decarboxylase 1, and calcium transporting ATPase 7 were the proteins upregulated upon inoculation with *X. oryzae* pv. *oryzae*.

Of the 31 proteins analyzed, other than the house-keeping proteins, two proteins were found to be overlapping across different abiotic and biotic stresses. Acidic endochitinase (SBSL 8 and SBSL 22) was observed upregulated in drought and fungal stress. Germin-like protein (SBSL 15 and SBSL 23) was upregulated in high temperature and fungal stress.

Expression analysis of acidic endochitinase and germin genes under dual stress conditions. Proteome level differences induced by different biotic and abiotic stress treatments were studied at the seedling stage of rice plants, since the crop is expected to be affected by these stresses (except *R. solani* infection) at the

Fig. 1. Two-dimensional gel electrophoresis patterns of leaf proteins from rice seedlings grown under salt, drought, and low temperature stress treatments. Proteome of **A**, control seedlings (salt stress); **B**, salt-stressed seedlings; **D**, control seedlings (drought stress); **E**, drought-stressed seedlings; **G**, control seedlings (low temperature stress); and **H**, low temperature stressed seedlings. Quantification of spot intensities of differentially expressed proteins in **C**, salt stress; **F**, drought stress; and **I**, low temperature stress. Arrows and circles indicate the protein spots chosen for mass spectrometry analysis. SBSL represents the label of protein spots. Spot intensities were calculated using GelQuantNET software in the replicated gel images. Numerical values assigned to the spot intensities were compared between spots in control and treated sample gels and statistically analyzed, and the mean values were compared using Tukey's multiple comparisons test. The mean of spot intensity numerical values are represented with $\pm$ standard error values. * indicates a significant difference ($P < 0.05$).

seedling stage. Inoculation of *R. solani* was done on leaves of the 20-day-old seedlings due to the lack of fully formed sheath tissues for handy inoculation. However, based on the proteome analysis, the biotic stress response-related proteins (chitinase and germin) that were found to overlap with abiotic stress response were observed in *R. solani* infection. There were no overlapping proteins observed between *X. oryzae* pv. *oryzae* infection and abiotic stress. Thus, further studies focused on *R. solani* infection as a common biotic stress in dual stress conditions with high temperature and drought. Objective was to find how rice plants prioritize molecular responses to biotic (sheath blight infection) and abiotic stress (drought or high temperature) when occurring one after the other on the same set of plants, thus referred to dual stress conditions. Under natural field conditions, sheath blight infection occurs during the active tillering stage of rice crop. Hence, for the dual stress

experiments, potted rice plants in the active tillering stage (45 days) were used.

Expression of the acidic endochitinase gene (corresponding protein of which was observed to be upregulated in two-dimensional proteome gels of fungal infection and drought-treated samples) was significantly higher in fungal-inoculated and drought-treated plants than in the respective control plants (Fig. 3A and C). Quantification of band intensities was done and the data are presented (Fig. 3B and D). In the dual stress treatments, comparison of band intensities was performed between dual stressed sample with corresponding control sample of the first among the two stresses imposed. The expression level of chitinase in drought stress followed by fungal inoculation was compared with drought control and the expression level in fungal inoculation followed by drought was compared with fungal control. Similar comparison was done

Fig. 2. Two-dimensional gel electrophoresis patterns of leaf proteins from rice seedlings grown under high-temperature stress, fungal and bacterial infections. Proteome of **A**, control seedlings (high temperature stress); **B**, high temperature stressed seedlings; **D**, control seedlings (*Rhizoctonia solani* infection); **E**, *R. solani*-infected seedlings; **G**, control seedlings (*Xanthomonas oryzae* pv. *oryzae* infection); and **H**, *X. oryzae* pv. *oryzae*-infected seedlings. Quantification of spot intensities of differentially expressed proteins in **C**, high temperature stress; **F**, *R. solani* infection; and **I**, *X. oryzae* pv. *oryzae* infection. Arrows and circles indicate the protein spots chosen for mass spectrometry analysis. SBSL represents the label of protein spots. Spot intensities were calculated using GelQuantNET software in the replicated gel images. Numerical values assigned to the spot intensities were compared between spots in control and treated sample gels and statistically analyzed, and the mean values were compared using Tukey's multiple comparisons test. The mean of spot intensity numerical values are represented with $\pm$ standard error values. * indicates a significant difference ($P < 0.05$).

TABLE 1. Differentially expressed proteins in rice seedlings subjected to biotic and abiotic stress^a

Spot label	Accession number	Protein ID	Peptides matched	Number of peptides matched	Score	Stress	Gel and expression level	Molecular function	Experimental molecular mass	Calculated molecular mass	pI	Spot intensity-fold change
SBSL 1	AAB66886.1	Ribosomal protein L12 homolog, partial	R.IASIKVVR.A R.GLVVDHLQER.L R.ALTNLAKEAK.D K.TEFDVVIEVPSSAR.I K.VLELGDAIAGLTLEEARG	5	193	Salt	Treated-upregulating	Structural constituent of ribosome	32.4 kDa	16.3 kDa	5.5	1.84 ↑
SBSL 2	XP_015610679.1	30S ribosomal protein 2, chloroplastic	K.LYVGNIPR.T R.AEVMFDKYTGR.S + Oxidation (M) K.VNVTESFLPNIDR.S R.SAPEPEPVFVDSQYK.V K.IKVVNVTESFLPNIDR.S K.NFFSEKGEVLSATVSR.I R.TVTNDELAAMFADHGTVER.A + Oxidation (M)	8	241	Salt	Treated-upregulating	Structural constituent of ribosome	39.7 kDa	26.7 kDa	5.2	1.55 ↑
SBSL 3	XP_015610679.1	30S ribosomal protein 2, chloroplastic	K.LYVGNIPR.T R.KLYVGNIPR.T R.AEVMFDKYTGR.S R.AEVMFDKYTGR.S + Oxidation (M) K.VNVTESFLPNIDR.S R.SAPEPEPVFVDSQYK.V K.IKVVNVTESFLPNIDR.S K.NFFSEKGEVLSATVSR.I R.TVTNDELAAMFADHGTVER.A R.TVTNDELAAMFADHGTVER.A + Oxidation (M) R.SAPEPEPVFVDSQ YKVVYVGNLAK.S R.FGFVTMSTPEEANAIESLN ETEVGGR.K R.FGFVTMSTPEEANAIESLN EVGGR.K + Oxidation (M) R.FGFVTMSTPEEANAIESLN TEVGGRK.I R.FGFVTMSTPEEANAIESLN EVGGRK.I + Oxidation (M)	15	358	Salt	Treated-upregulating	Structural constituent of ribosome	41 kDa	26.7 kDa	5.8	1.65 ↑
SBSL 4	BAD62115.1	Photosystem II stability/assembly factor HCF136, chloroplastic	K.NGGNTWFP.R.S R.GFGILDVGYR.S R.ADGGLLWLLVR.G K.GYVLGNDGVLLR.Y K.AAVQETVSATLNR.T R.SIPSAEDEDFNRY.F K.GSGITEDFEEASYQSR.G R.TPLARADQPPSLSEWER.V R.TVSSGISGASYTGTFTVNR.S K.ATGEGQSAEMVTDEGAIVTSNR.G	10	243	Salt	Treated-upregulating	Essential for photosystem II (PSII) biogenesis	29.2 kDa	42 kDa	6.0	2.30 ↑
SBSL 5	XP_015641153.1	Barley-B recombinant like protein	R.QRPDQYK.G R.KMSGGAFTKLLSR.L K.DHQSMNLLALMND.R.D K.NVGMSGCGDDSAHASVMK.N + Oxidation (M)	4	65	Salt	Treated-downregulating	DNA binding transcription factor regulates activity sequence—specifically DNA binds to GA elements in regulating sequences of genes involved in developmental process	24 kDa	36 kDa	5.0	0.74 ↓
SBSL 6	EEE56339.1	Translation factor GUF1 homolog, chloroplastic	K.GDKICFMASGK.E R.NFSIIAHIDHGK.S K.VDGTLPSTMNLR.N K.VDGTLPSTMNLR.N + Oxidation (M) K.GYASMEYSLIGYR.E K.GYASMEYSLIGYR.E + Oxidation (M) R.SVADARVGDITTHSSK.R K.EYIGPIMELGQERR.G K.EYIGPIMELGQERR.G + Oxidation (M) R.SKGYASMEYSLIGYR.E + Oxidation (M) R.SIEEPYVKIDMLTPK.E R.NFSIIAHIDHGKSTLADK.L R.CGFLGLLHMEIVQERLER.E K.VDGTLPSTMNLRNFSIIAHIDHGK.S K.TCNLRGMLK.F + Oxidation (M)	14	55	Drought	Treated-upregulating	Promotes mitochondrial protein synthesis, binds to mitochondrial ribosomes in GTP dependent manner	37.1 kDa	71 kDa	5.6	2.23 ↑
SBSL 7	XP_015649242.1	Glutamate synthase 1, (NADH), chloroplastic	R.VLGHNGEINTLK.G R.VAPGISGLTQKNEK.K K.MGHFVTVFERADR.I R.YHMKGLPAGTIHVK.L + Oxidation (M) R.KNGEVHLNDPLAMAK.L R.KNGEVHLNDPLAMAK.L + Oxidation (M)	25	72	Drought	Treated-upregulating	Involved in glutamate biosynthesis Plays a major role in primary ammonium ions assimilation in seedling roots involved in re-utilization of glutamine in developing organism Plays a role in development of tillers	36.7 kDa	238 kDa	5.9	1.68 ↑

(Continued on next page)

^a Protein accession numbers are from RefSeq or GenBank annotation obtained from Uniprot database. Up and down arrows in last column indicate relative fold increase and decrease of spot intensity compared with control.

TABLE 1. (Continued from previous page)

Spot label	Accession number	Protein ID	Peptides matched	Number of peptides matched	Score	Stress	Gel and expression level	Molecular function	Experimental molecular mass	Calculated molecular mass	pI	Spot intensity-fold change
			K.GLEETLDRICTEAR.G K.VAIIIGSGPAGLAAADQLNK.M K.SLLDSNLEDGRYISAK.G K.SLLDSNLEDGRYISAK.G K.GFEEGWMVPRPPLQR.T R.ADRIGGLMMYGVPMNK.T + 3 Oxidation (M) R.NFAAGMSGGIAYVYDIDGK.F R.FSTNTFPSWDRAQPMR.V + Oxidation (M) R.HGCTSIYNLELLTKPPSK.R K.IVTSMECMIGPEGDLLETTEK.Q + 2 Oxidation (M) R.FYVTHSGRVVMGSEVGVVDVPSK.D R.FYVTHSGRVVMGSEVGVVDVPSK.D + Oxidation (M) K.ILPVIVDATSSDSGAFDGVLELLIR.G R.CMDCGTPFCHQESSGAGCPLGNK.I + Oxidation (M) K.YLSRNEQDAAEITPSGAGFVQPVA R.FYDVQNNFSNQV.K K.VILSIGGAGSYGLSSTQDAQ.R K.VADYLVNWNFLGGSSGK.L R.YDELATFLSRYSAQGGGKYGGIM..K R.VWNRFYDVQNNFSNQV.K									
SBSL 8	NP_001044872.1	Acidic endochitinase	K.YLSRNEQDAAEITPSGAGFVQPVA R.FYDVQNNFSNQV.K K.VILSIGGAGSYGLSSTQDAQ.R K.VADYLVNWNFLGGSSGK.L R.YDELATFLSRYSAQGGGKYGGIM..K R.VWNRFYDVQNNFSNQV.K	5	215	Drought	Treated-upregulating		34 kDa	37 kDa	5.1	1.60 ↑
SBSL 9	XP_015610679.1	30S ribosomal protein 2, chloroplastic	K.LYVGNIPR.T R.KLYVGNIPR.T R.AEVMFDKYTGR.S R.AEVMFDKYTGR.S + Oxidation(M) K.VNVTESFLPNIDR.S R.SAPEPEPVFVDSQYK.V K.IKVVNVTESFLPNIDR.S K.NFFSEKGEVLSATVSR.I R.TVTNDELAAMFADHGTVER.A R.TVTNDELAAMFADHGTVER.A + Oxidation (M) R.FGFVTMSTPEEANAIESLNE TEVGGR.K R.FGFVTMSTPEEANAIESLNE TEVGGR.K + Oxidation (M) R.FGFVTMSTPEEANAIESLNE TEVGGRK.I R.FGFVTMSTPEEANAIESL NETEVGGRK.I + Oxidation (M)	14	307	Drought	Treated-upregulating	Structural constituent of ribosome	38 kDa	26 kDa	5.5	1.20 ↑
SBSL 10	XP_015618593.1	Oxygen-evolving enhancer protein 1, chloroplastic	R.RLTDEIQSK.T R.GGSTGYDNAVALPAGGR.G K.DGIDYAAVTQPLPGER.V K.GRGSTGYDNAVALPAGGR.G K.GTGTAQCPTVEGGVDSFAFK.A K.NLVATGKPESEFGGPFVPSYR.G K.FEEKDGIDYAAVTQPLPGER.V K.SKPETGEVIGVFESVQPSDSDLGAK.V K.QIPLSGPNSVVGR.A K.GGHELSLSTGNAGGR.L R.AFVVHELEDDLKGK.G R.HAGDLGNIVANAEGVAEATIVDK.Q K.GTSQVEGVVTLTQDDQGPPTVNV.R.V R.AFVVHELEDDLKGKGGHELSL STGNAGGR.L K.AVAVLKGTSQVEGVVTLTQDD QGPTTVNV.R.V	6	261	Low temperature	Treated-upregulating	Calcium ion binding	40.8 kDa	35 kDa	6.2	6.94 ↑
SBSL 11	XP_015632608.1	Putative superoxide dismutase [Cu-Zn], chloroplast precursor	K.QIPLSGPNSVVGR.A K.GGHELSLSTGNAGGR.L R.AFVVHELEDDLKGK.G R.HAGDLGNIVANAEGVAEATIVDK.Q K.GTSQVEGVVTLTQDDQGPPTVNV.R.V R.AFVVHELEDDLKGKGGHELSL STGNAGGR.L K.AVAVLKGTSQVEGVVTLTQDD QGPTTVNV.R.V	7	159	Low temperature	Treated-unique	Copper ion binding superoxide dismutase activity	35.6 kDa	20.6 kDa	6.0	82.61 ↑
SBSL 12	XP_015638705.1	50S ribosomal protein L12, chloroplastic-like	R.GLVDLHQLER.L K.EAVSKEEAEAEAKK.Q K.TEFDVVIEEVPSSAR.I K.VLELGDAIAGLTLEEAR.G	4	301	Low temperature	Treated-unique	Structural constituent of ribosome	20 kDa	18 kDa	6.8	19.60 ↑
SBSL 13	XP_015619657.1	Germin-like protein 8-14	K.KLKSVLGGSG.- K.AAVTPAFVGFQFPGVNLGISAAR.L	2	62	Low temperature	Treated-upregulating	Manganese ion binding nutrient reservoir activity	33.4 kDa	22 kDa	6.3	19.40 ↑
SBSL 14	XP_015624758.1	Coleoptile phototropism protein 1	K.YPMISRSGRMSR.A + 2 Oxidation (M) K.TEAFLSYVVLSSWR.D R.WNVGGGGGESKESSPSR.Q K.DINTLKFELESMAK.Y K.DINTLKFELESMAK.Y R.LAIMLRAAPALVTELEK.R K.VKGMRFDLIGAITHYASK.W R.AVDSYLKAHPTLTEHERK.R R.GVRWAYTGAGAGSGGARGGPAAIR.G R.DSVSCGFLLRLRLAIML.R.A R.LRLRLAIMLRAAPALVTELEK.R + Oxidation (M) R.DSVSCGFLLRLRLAIML.R.A + Oxidation (M) R.LVEHFLVQEQTMAVASSPGR.G + Oxidation (M) K.TEAFLSYVVLSSWRDSIAVLK.S K.AKYLELQHEMDALQKQVDGR.G + Oxidation (M) R.CAAEYLEMTEEMEENLIFK.T + Oxidation (M) R.LCRVMDQCQLSFDACMHAAQNER.L	20	85	Low temperature	Control-unique	Phototropism protein ubiquitination	21 kDa	81 kDa	5.2	0.03 ↓

(Continued on next page)

TABLE 1. (Continued from previous page)

Spot label	Accession number	Protein ID	Peptides matched	Number of peptides matched	Score	Stress	Gel and expression level	Molecular function	Experimental molecular mass	Calculated molecular mass	pI	Spot intensity-fold change
SBSL 15	XP_015648639.1	Germin-like protein 8-14	R.MVVESIIITPPQRDSVSC GFLRLRL.R.L K.ISNALASSAALRSSSSAPGADAA PAMPTTRR.Q + Oxidation (M) R.MVVESIIITPPQRDSVSCGF LLRLRL.L + Oxidation (M) K.AAVTPAFVGQFPGVNGLGISAAR.L	1	98	High temperature	Treated-upregulating	Manganese ion binding nutrient reservoir activity	29.8 kDa	22 kDa	6.0	5.85 ↑
SBSL 16	XP_015618593.1	Oxygen-evolving enhancer protein 1, chloroplastic	K.VPKDVK.I K.YNMKK.F + Oxidation (M) R.VPFLFTIK.N R.GGSTGYDNAVALPAGGR.G K.DGIDYAAVTVQLPGER.V K.FCLEPTSFTVKAEGVAK.N K.GTGTANQCPTVEGGVDSFAFK.A K.NLVATGKPESFGGPFVPSYR.G K.FEEKDGIDYAAVTVQLPGER.V K.GTGTANQCPTVEGGVDSFAFKAGK.Y K.SKPETGEVIGVFESVQPSDTDLGAK.V K.SKPETGEVIGVFESVQPSDT DLGAKVPK.D	12	234	High temperature	Treated-unique	Calcium ion binding	43.6 kDa	35 kDa	5.4	916.32 ↑
SBSL 17	XP_015610679.1	30S ribosomal protein 2, chloroplastic	R.KLYVGNIPR.T R.AEVMFDKYTGR.S R.AEVMFDKYTGR.S + Oxidation (M) K.VNVTESFLPNIDR.S R.SAPEPEPVFVDSQYK.V K.IKVNVTESFLPNIDR.S K.NFFSEKGEVLSATVSR.I R.TVTNDELAAMFADHGTVER.A R.TVTNDELAAMFADHGTVER.A + Oxidation (M) R.SAPEPEPVFVDSQYKVYVGNLAK.S R.FGFVTMTSTPEEANAIESLNET EVGGR.K + Oxidation (M) R.FGFVTMTSTPEEANAIESLN ETEVGGRK.I + Oxidation (M)	12	125	High temperature	Treated-upregulating	Structural constituent of ribosome	38.3 kDa	26 kDa	5.3	2.81 ↑
SBSL 18	XP_015630498.1	L- ascorbate peroxidase 1, cytosolic	R.SGFEGPWTR.N R.LPDATKGSDDL.R.Q K.ERSGFEGPWTR.N R.LAWHSAGTFDVSSK.T K.ALLSDPAFRPLVEK.Y K.TPAELSHAANAGLDIAVR.M K.NYPVVSAAEYQEAVERKAR.Q R.QVFGAQMGLSDQDIVALSG GHTLGR.C	8	199	High temperature	Treated-upregulating	Cytochrome-c peroxidase activity metal ion binding	37.6 kDa	27 kDa	6.0	5.08 ↑
SBSL 19	XP_015621507.1	Triosephosphate isomerase, cytosolic	K.VIACVGTELEQR.E K.WLAANVSAEVAESTR.I K.VATPDQAQEVHDGLRK.W K.SQLRPEIQVAAQNCWVK.K K.SQLRPEIQVAAQNCWVK.G K.VIACVGTELEQREGSTMD VVAAQTK.A K.VIACVGTELEQREGSTMDV VAAQTK.A + Oxidation (M) K.ELAAKPDVDGFLVGGASLKPEFID IINSATVK.S	8	90	High temperature	Treated-upregulating	Triose-phosphate isomerase activity	37.2 kDa	27 kDa	6.2	6.02 ↑
SBSL 20	ABY52939.1	Chloroplast 23 kDa polypeptide of photosystem II	R.EFPGQVLR.Y K.QYYSVTVLTR.T K.LLIPSKWNPSE R.KFVESAASSFSVA.- K.TNTEFIAYSGEGF.L R.TADGDEGGKHQLITATVNDGK.L R.YEDNFDANSNVSVIINPTTK.K R.YEDNFDANSNVSVIINPTTK.T R.EFPGQVLR.YEDNFDANSNVSVIINPTTK.K	10	195	High temperature	Treated-upregulating	Electron transfer activity metal ion binding	37 kDa	20 kDa	5.6	3.92 ↑
SBSL21	XP_015630537.1	Histone, H3	R.KLPFQR.L K.STELLIR.K K.QLATKAAR.K K.STELLIRK.L R.FRPGTVALR.E R.EIAQDFKTDLR.F	6	57	High temperature	Treated-unique	DNA binding protein heterodimerization activity	25.4 kDa	15 kDa	4.8	5,394.04 ↑
SBSL 22	NP_001044872.1	Acidic endochitinase	K.VILSIGGGAGSYGLSSTQDAQ.R K.VADYLWNNFLGGSSGK.L R.SRPLGDAVLGDGDFDIETGNPA.K R.YDELATFLSRYSAQGGGKYGGIM..K R.VWNRFYDVQNNFSNQV.K K.SFYVGVGPAEAAAGSGYVAPG.V K.VTFLDDAQVK.K K.AAVTPAFVGQFPGVNGLGISAAR.L			Fungal	Treated-upregulating		34 kDa	37 kDa	5.1	15.07 ↑
SBSL 23	XP_015619657.1	Germin-like protein 8-14	K.VTFLDDAQVK.K K.AAVTPAFVGQFPGVNGLGISAAR.L	2	61	Fungal	Treated-upregulating	Manganese ion binding nutrient reservoir activity	43.1 kDa	22 kDa	5.4	8.25 ↑
SBSL 24	EAY96732.1	Cellulose synthase A catalytic subunit 1	R.EAMCFMMDPALGR.K + Oxidation (M) R.EAMCFMMDPALGRK.T + 2 Oxidation (M) K.HNIEPRAPEFYFAQK.I K.ALREAMCFMMDPALGR.K R.HSIRSGTSSYVDPSVPVPR.I K.VPEEGWTMADGTAWPGNNPR.D + Oxidation (M)	11	62	Fungal	Treated-upregulating	Cellulose synthase activity	28.9 kDa	122 kDa	5.5	7.50 ↑

(Continued on next page)

TABLE 1. (Continued from previous page)

Spot label	Accession number	Protein ID	Peptides matched	Number of peptides matched	Score	Stress	Gel and expression level	Molecular function	Experimental molecular mass	Calculated molecular mass	pI	Spot intensity-fold change
SBSL 25	XP_015639990.1	Kinesin like protein	R.IPRLTSGQQISGEIPDASPDR.H R.SGTSSYVDPSVPVVRIVDPK.D R.NRNEFVMIRPDGDAPPAKPGK.S + Oxidation (M) R.QALYGYDPVLTEADLEPNIVVK.S K.LFFSIWVILHLYPFLKGLMGR.Q + Oxidation (M) K.RLIEQVR.M MAGHGAGGRR.A R.KWIYESR.S K.EICMLTSR.L K.EICMLTSR.L + Oxidation (M) R.QKSSGAEGER.L R.SANSEFSRR.L R.EKSAETEIR.K R.EKSAETEIR.K K.FEKPSGRTR.G R.CELKQYQK.C K.LIIMAEESTK.L K.LIIMAEESTK.L + Oxidation (M) K.AMKVSIEKEK.I + Oxidation (M) R.EDKLKICK.C K.NMEAQGDVTIK.E + Oxidation (M) R.DSMTHLRFGR.L + Oxidation (M) K.LECEIKSLNR.L K.YQEMEHEMISK.Q K.LEDVLHLVEDK.S R.MLEKFSKQGER.E K.MYGHDEISVYR.Q K.DCTVQELNDNVK.Q R.QAAEISKVYAEKE.E K.DVMMLLLQGVANR.K + Oxidation (M) R.KIECLTMEASCK.D + Oxidation (M) K.LLQESTSQAKDMK.D + Oxidation (M) R.CISSIVQQMNSEK.D K.ANEELKLEKDSR.Q R.ELEKKTSAMESILK.Q + Oxidation (M) K.VMLEEEAMMISSETK.A + Oxidation (M) R.WERDSMTHLRFGR.L + Oxidation (M) K.FEKPSGRTRGSGSPFR.C R.EQMLEAQNGILQMEK.D + 2 Oxidation (M) K.VLEEEEAENGAALLILR.S K.YARPPKNMEAQGDVTIK.E + Oxidation (M) R.HECHLPDNLVGTVAEISHNR.D R.SLSTLGLVIMTLVDVANGKNR.H R.GSGSPFRCISSIVQQMNSEK.D R.AAARRVEAETNENDDLAAAAAR.D K.ENNNLKVMLEEEAMMISSETK.A + 3 Oxidation (M) R.SYLVNMGALLEEQGNSYAEQNR.R + Oxidation (M) R.IEELEGLVCNKQKEICMLTSR.L + Oxidation (M) R.CISSIVQQMNSEKDQEISVARQR.I K.TYTMMGELSKLDNELSKDSGLTPR.I + 2 Oxidation (M) K.LPGVVGLPMVENCMMSGYNGCLFAYGQTGSGK.T K.TVTVAELTAGR.K K.AVLFAVPGAFTPTCSQK.H K.GVDAIACVSVNDAFVMR.A K.GVDAIACVSVNDAFVMR.A + Oxidation (M) K.LPDATLSYFDPADGELK.T R.ALGVEMDLSDKPMGLGVR.S R.ALGVEMDLSDKPMGLGVR.S + Oxidation (M) R.ALGVEMDLSDKPMGLGVR.S + 2 Oxidation (M) R.KAVLFAVPGAFTPTCSQK.H K.VLNLEEGGAFTTSSAEEMLK.A K.VLNLEEGGAFTTSSAEEMLK.A + Oxidation (M) K.ESLGLGDADVLLSDGNLELTR.A K.LYVGNIPR.T R.KLYVGNIPR.T R.AEVMFDKYTGR.S R.AEVMFDKYTGR.S + Oxidation (M) K.VNVTESFLPNIDR.S R.SAPEPEPVFVDSQYK.V K.IKVNVTESFLPNIDR.S K.NFFSEKGEVLSATVSR.I R.TVTNDELAAMFADHGTVER.A R.TVTNDELAAMFADHGTVER.A + Oxidation (M) R.FGFVTMSTPEANAAIESLNETEVGGR.K + Oxidation (M)	46	66	Fungal	Treated-upregulating	Microtubule motor activity, actin binding ATPase activity	40.8 kDa	216 kDa	5.0	11.5 ↑
SBSL 26	XP_015623954.1	Peroxioredoxin-2E-2, chloroplastic	K.TVTVAELTAGR.K K.AVLFAVPGAFTPTCSQK.H K.GVDAIACVSVNDAFVMR.A K.GVDAIACVSVNDAFVMR.A + Oxidation (M) K.LPDATLSYFDPADGELK.T R.ALGVEMDLSDKPMGLGVR.S R.ALGVEMDLSDKPMGLGVR.S + Oxidation (M) R.ALGVEMDLSDKPMGLGVR.S + 2 Oxidation (M) R.KAVLFAVPGAFTPTCSQK.H K.VLNLEEGGAFTTSSAEEMLK.A K.VLNLEEGGAFTTSSAEEMLK.A + Oxidation (M) K.ESLGLGDADVLLSDGNLELTR.A K.LYVGNIPR.T R.KLYVGNIPR.T R.AEVMFDKYTGR.S R.AEVMFDKYTGR.S + Oxidation (M) K.VNVTESFLPNIDR.S R.SAPEPEPVFVDSQYK.V K.IKVNVTESFLPNIDR.S K.NFFSEKGEVLSATVSR.I R.TVTNDELAAMFADHGTVER.A R.TVTNDELAAMFADHGTVER.A + Oxidation (M) R.FGFVTMSTPEANAAIESLNETEVGGR.K + Oxidation (M)	12	208	Fungal	Treated-upregulating	Peroxioredoxin activity	34.2 kDa	23 kDa	5.1	9.17 ↑
SBSL 27	XP_015610679.1	30S ribosomal protein 2, chloroplastic	K.LYVGNIPR.T R.KLYVGNIPR.T R.AEVMFDKYTGR.S R.AEVMFDKYTGR.S + Oxidation (M) K.VNVTESFLPNIDR.S R.SAPEPEPVFVDSQYK.V K.IKVNVTESFLPNIDR.S K.NFFSEKGEVLSATVSR.I R.TVTNDELAAMFADHGTVER.A R.TVTNDELAAMFADHGTVER.A + Oxidation (M) R.FGFVTMSTPEANAAIESLNETEVGGR.K + Oxidation (M)	11	168	Bacterial	Treated-upregulated	Structural constituent of ribosome	42.8 kDa	26.7 kDa	5.6	2.09 ↑

(Continued on next page)

TABLE 1. (Continued from previous page)

Spot label	Accession number	Protein ID	Peptides matched	Number of peptides matched	Score	Stress	Gel and expression level	Molecular function	Experimental molecular mass	Calculated molecular mass	pI	Spot intensity-fold change
SBSL 28	XP_015649518.1	Putative superoxide dismutase [Cu-Zn], chloroplast precursor	K.QIPLSGPNSVVGR.A R.HAGDGLGNIVANAEGVAEATIVDK.Q K.GTSQVEGVVTLTQDDQGPTTVNVR.V R.AFVVHEEDDLGKGGHELSLS TGNAGGR.L K.AVAVLKGTQSGVEGVVTLTQDDQG PTTVNVR.V K.KAVAVLKGTQSGVEGVVTLTQDDQG PTTVNVR.V	6	143	Bacterial	Treated-upregulating	Copper ion binding superoxide dismutase activity	32.3 kDa	20.6 kDa	6.2	2.71 ↑
SBSL 29	XP_015616895.1	Fructose-bisphosphate aldolase, chloroplast precursor	K.EAAWGLAR.Y K.AAQDALLR.A K.TWGGQPENVK.A R.EAAYYQGAR.F R.ATPEQVSDYTLK.L R.LASIGLENTEANR.Q R.TVVSIPNGPSELAVK.E K.RLASIGLENTEANR.Q K.DRATPEQVSDYTLK.L R.GILAMDESNTACGKR.L + Oxidation (M) R.LASIGLENTEANRQAYR.T K.TWGGQPENVKAAQDALLR.A R.TVVSIPNGPSELAVKEAAWGLAR.Y K.GLVPLAGSNNESWCQGLDGLASR.E K.VDKGLVPLAGSNNESWCQGLDGLASR.E R.YAASIQDNGLVPIVEPEILLD GEHIDR.T	16	323	Bacterial	Treated-upregulating	Fructose-bisphosphate aldolase activity	64.7 kDa	41 kDa	6.1	9.69 ↑
SBSL30	XP_015643038.1	Arginine decarboxylase 1	R.LGAAMR.V + Oxidation (M) R.GNPDALLICNGYK.D R.LGVRPVVGMRAKL.R.T + Oxidation (M) K.LRTKHAGHFGSTSGEK.G K.DGVLGLEHLAAVDSLCEIVAR.G R.FGLEAGSKPELLAMSCLAAR.G + Oxidation (M) R.NLMAAAVRGDFDTCALYADQLKR.R + Oxidation (M) R.AAAGPSCADVLRSMQHEPEVM FEVLK.Q + Oxidation (M) K.DGVLGLEHLAAVDSLCEIVARG MGAAEPPR.T K.DGVLGLEHLAAVDSLCEIVARGMG AAEPPR.T + Oxidation (M)	10	64	Bacteria	Treated-upregulating	arginine decarboxylase activity	78 kDa	74 kDa	5.9	1.79 ↑
SBSL 31	XP_015613068.1	Calcium transporting ATPase 7, plasma membrane type	K.REDCFR.L R.GCHRLRL.L K.LVLVQRLK.Q R.RCSPSTSTSR.E R.RCSPSTSTSR.E K.SAIEACTKAGIAVK.M R.LLLGVLSAAAGIMR.R + Oxidation (M) 1623.5052 K.SPFLASGVKVVVDGYGK.M K.MVVTAAGTDTAWGEMMR.T + Oxidation (M) R.LLGGGAGVAAVLASGAERGIR.G R.CSPSTSTSTSR.EAWRPEK.Q K.CIPVPERPFHEITARR.R R.ENTDPTPLQERLEGLTSSIGK.V R.MFLGIVAVTVALQVVMVELLTK FAGTER.L 1 R.MFLGIVAVTVALQVVMVEL LTKFAGTER.L + 2 Oxidation (M) K.DGWYDGVSIPLAVFLVAASAV SNHSQGR.F R.GFGGAGAGERANGTMIFNAFVL CQVFNEFNAR.E + Oxidation (M)	17	59	Bacteria	Treated-upregulating	ATP binding calmodulin binding calcium-transporting ATPase activity	62.9 kDa	112 kDa	6.5	1.68 ↑

for expression of germin gene. Expression levels of the acidic endochitinase gene were higher in dual stress (both drought followed by fungal inoculation and fungal inoculation followed by drought). Expression of the germin gene (corresponding protein of which was observed to be upregulated in two-dimensional proteome gels of high temperature stress and fungal inoculated samples) was higher in the high temperature and fungal inoculated plants than in the respective controls. Germin gene expression under dual stress indicated higher expression both at high temperature followed by fungal inoculation and fungal inoculation followed by high temperature stress.

DISCUSSION

Most of the studies to unravel the cross-talking molecular mechanisms in biotic and abiotic stress responses in plants are based

on comparative gene expression profiling, bioinformatics analysis of microarray data and overexpression and in transgenic plant conditions (Alam et al. 2014; Asano et al. 2012; Kuang et al. 2017; Liu et al. 2008; Mellacheruvu et al. 2016; Pillai et al. 2018; Wang et al. 2016). Bioinformation from transcriptome analysis has contributed to the identification of candidate genes for molecular breeding or transgenics toward multiple stress tolerance.

Many of the NAC transcription factors have been shown to have differential expression patterns across various stresses including salt, drought, cold and blast disease (Sun et al. 2015). The expression patterns of some of these genes were similar under different biotic stresses also (blast and bacterial leaf blight disease) and overexpression of NAC 6 displayed tolerance to blast disease, dehydration and salt stress in rice (Nakashima et al. 2007). Similarly, WRKY67 has been reported to positively regulate blast and bacterial blight resistance (Liu et al. 2008). In a parallel work to

this current study, we have demonstrated the overlapping role of WRKY13 transcription factor in resistance against two different sheath infecting fungi, *R. solani* causing sheath blight and *Sarocladium oryzae* causing sheath rot in rice (Jimmy and Babu 2019). In an earlier report, Xiao et al. (2013) observed the antagonistic role of WRKY13 in cold, salt and drought stress when overexpressed in rice plants for resistance to *M. oryzae*. Experiments carried out by Ueno et al. (2015) indicated that upregulation and downregulation of OsPTP/2 could cause the switching of tolerance responses respectively to biotic and abiotic stress. Ethylene-responsive factors were found to be induced commonly in response to *M. oryzae* and *R. solani* infection as well as salt, cold,

drought, and wound stress (Cao et al. 2006; Helliwell et al. 2013). Although pathogenesis-related (PR) proteins are well studied during incompatible interactions of host plants with pathogens, some of them are known to play important roles in defense responses to abiotic stress as well, including PR-10 (Kim et al. 2008), PR-4, PAL, and Cht-1 in drought-fungal infection dual stress and PR-1a, PBZ1, Gns1, and Cht-1 in salt-fungal infection dual stress (Zhang et al. 2015). Several glutathione S-transferases that play a key role in cellular detoxification also mediate cross-talk between various biotic and abiotic stress response pathways (Jain et al. 2010). Calcium-dependent protein kinase CPK12 has been reported to oppositely modulate salt tolerance and blast disease

Fig. 3. RT-PCR analysis of rice chitinase and germin genes in individual and dual stress conditions. **A**, Acidic endochitinase gene. Lane 1, negative control; lane 2, drought control; lane 3, drought treated; lane 4, fungal control; lane 5, fungal infected; lane 6, drought followed by fungal infection; and lane 7, fungal infection followed by drought. Expected size of amplified DNA: rice actin (internal control gene), 400 bp; acidic endochitinase, 205 bp. **B**, Quantification of band intensities of the amplified acidic endochitinase gene. Data from two replicated experiments were analyzed statistically, and the mean values were compared using Tukey's multiple comparisons test. For individual stress treatments, corresponding controls were used for comparison. For dual stress treatments, mean data of control, corresponding to the first among the two stresses imposed, were used for comparison. * indicates a significant difference ($P < 0.05$). **C**, Germin gene. Lane 1, negative control; lane 2, high-temperature control; lane 3, high temperature treated; lane 4, fungal control; lane 5, fungal infected; lane 6, high temperature followed by fungal infection; and lane 7, fungal infection followed by high temperature. Expected size of amplified DNA: rice actin (internal control gene), 400 bp; germin, 265 bp. **D**, Quantification of band intensities of the amplified germin gene. Data from two replicated experiments were analyzed statistically, and the mean values were compared using Tukey's multiple comparisons test. For individual stress treatments, corresponding controls were used for comparison. For dual stress treatments, mean data of control, corresponding to the first among the two stresses imposed, were used for comparison. * indicates a significant difference ($P < 0.05$).

resistance (Asano et al. 2012). In addition to the overexpression of rice native genes, ectopic expression of genes from other sources have also been reported to enhance tolerance to abiotic and biotic stresses.

Kuang et al. (2017) observed the positive regulatory role of class II small heat shock protein in mediating resistance to bacterial blight in rice caused by *X. oryzae* pv. *oryzae* and tolerance to salt and heat stress. On the other hand, overexpression of WRKY42, which increased salt tolerance in rice plants by enhancing callose deposition, failed to enhance tolerance to bacterial infection, since the gene is a negative regulator of jasmonic acid mediated responses (Pillai et al. 2018). WRKY11 has been reported to integrate responses of rice plants to defend against bacterial leaf blight infection and tolerate drought (Lee et al. 2018). Shaik and Ramakrishna (2013) developed a common coexpression network of stress-responsive genes in drought and bacterial leaf blight stress responses in rice and Arabidopsis.

Of late, translational and posttranslational machineries are known to play major roles in plant stress responses (Ghatak et al. 2017), particularly when there is an immediate need for the plant to prioritize the responses during simultaneous or combined stress encountered in the natural environment. Therefore, analysis and comparison at the proteome level is expected to provide more direct insight into protein products and alternate spliced variants of the stress-responsive cross-talking genes under biotic and abiotic stress conditions.

ASD16 is a short-duration dwarf rice variety developed by crossing ADT31 × CO39 by Tamil Nadu Agricultural University, Coimbatore, India. The variety is known to be resistant to blast disease, moderately resistant to sheath rot disease, and tolerant to salinity (http://www.agritech.tnau.ac.in/expert_system/paddy/TNvarieties.html). Since most of the varieties are developed with resistance or tolerance to single stress (biotic or abiotic), the choice of ASD16 for the current study is based on two criteria: (i) popularity of the variety under large scale cultivation, and (ii) resistant/tolerant to more than one stress, one at least in biotic and abiotic.

Among the differentially expressed proteins observed, most of them were upregulated and few were downregulated upon stress treatment. The ribosomal proteins and translation factors observed to be upregulated in stress treatments such as salt (SBSL1, SBSL2, and SBSL3), drought (SBSL6, SBSL9), high temperature (SBSL17), and bacterial infection (SBSL27) are indicative of the negative impact of stress on protein synthesis and the proteome-level response of the rice plants to compensate for the same.

Photosystem II stability/assembly factor HCF (high chlorophyll fluorescence) 136 (SBSL4) was observed to be upregulated in salt-treated plants and is a photo system II stability/assembly protein present in the stroma lamellae of chloroplasts (Lu 2016). The stability assembly functions of HCF136 include the formation of PS-II precomplex and PS-II reaction center complex. Photosystem II is known to be susceptible to salt, high temperature, and water-deficient stress (Jajoo 2013). Particularly, the effect of salt stress on PS-II is cultivar specific. Since the ASD16 variety used in this study is tolerant to salt stress, the observation of upregulated HCF136 indicates that the variety uses this protein to stabilize/assemble PS-II to tolerate salt stress. Plastidic glutamate synthase (GS-II) is involved in biosynthesis and thus in drought stress tolerance (Diaz et al. 2010). Upregulation of glutamate synthase I (SBSL7) in 5 days drought-treated seedlings indicates (i) that the seedlings have experienced the imposed drought and (ii) glutamate synthase is accumulated to enhance proline biosynthesis to alleviate drought stress. Many proteins in the photosynthesis pathway have been reported to upregulate under various abiotic stress conditions such as drought, salinity, high temperature, low temperature and flooding (reviewed by Nouri et al. 2015; Sang et al. 2017). We also observed upregulation of chloroplast oxygen-evolving enhancer protein 1 (SBSL10, low temperature stress; and SBSL16, high temperature

stress), triosephosphate isomerase (SBSL19, high temperature stress), chloroplast 23 kDa polypeptide of photosystem II (SBSL20, high temperature stress), and fructose biphosphate aldolase (SBSL29, bacterial infection) indicating their protective role in response to high temperature stress and bacterial infection, through possible increase in photosynthesis. Although the ASD16 rice variety is not resistant or tolerant to bacterial leaf blight disease, the plants responded in a similar way to abiotic stress. Similarly, the results of Huda et al. (2013) and Singh et al. (2014) suggested that calcium-binding ATPases have diverse roles as regulators of abiotic stress signaling pathways in rice plants. Upregulation of a calcium transporting ATPase (SBSL31) during the interaction of rice plants with *X. oryzae* pv. *oryzae* indicates their possible role in biotic stress signaling pathways also and the importance of cellular calcium homeostasis during bacterial infection. Antioxidant enzymes such as Cu-Zn superoxide dismutase (SBSL11, low temperature stress; SBSL28, bacterial infection), L-ascorbate peroxidase (SBSL18, high temperature stress), and peroxiredoxin (SBSL26, fungal infection) are common proteins that are induced during many biotic and abiotic stresses in plants.

In the compatible interaction, rice blast fungus is reported to produce effector proteins to suppress rice ubiquitin E3 ligase and make the host susceptible (Park et al. 2012). This ubiquitin ligase targets the transcription factors required to regulate drought susceptibility (Lyzena and Stone 2012). The coleoptile phototropism protein 1 (SBSL14) identified in the present study (known to serve as an adapter for ubiquitin E3 ligase to mediate ubiquitination of target proteins) was found to be suppressed in rice plants upon low-temperature treatment. This is also indicative of the susceptibility of ASD16, the variety under study, to low temperature stress. Therefore, the key to display tolerance to multiple abiotic and biotic stresses relies somewhere in maintaining the abundance of ubiquitin ligase.

The two important proteins that showed overlapping upregulation across biotic and abiotic stresses are acidic endochitinase (SBSL8, drought stress; SBSL22, *R. solani* infection) and germin-like protein (SBSL13, high temperature stress; SBSL23, *R. solani* infection). Although there are many family members of chitinase and germin-like proteins in rice, the genes chosen for further studies are the ones corresponding to the proteins observed in the two-dimensional electrophoretic analysis. Chitinases in plants offer protection against fungal pathogens and insects by degrading chitin in their cell wall (Schlumbaum et al. 1986). In addition to their role in resisting pathogenic growth (Brogue et al. 1991; Gijzen et al. 2001; Nakazaki et al. 2006; Park et al. 2004; Zhu and Lamb 1991), some of the chitinases play a role in response to abiotic stress (Guo et al. 2013). Genes coding for plant chitinases have been cloned, and transgenic plants expressing chitinase have been demonstrated in rice for resistance against *R. solani* causing sheath blight (Datta et al. 2001; Lin et al. 1995; Maruthasalam et al. 2007; Nandakumar et al. 2007; Richa et al. 2017). Chitinases not only hydrolyze chitin, but also complex carbohydrates containing at least one N-acetylglucosamine residue (Takenaka et al. 2009). Glycolipid desaturation has been proposed as one of the mechanisms in rice plants to stabilize cell membranes under drought stress, leading to less ion leakage and drought tolerance (Borah et al. 2017).

It is tempting to speculate a key role for chitinase in drought tolerance phenotype of ASD16, based on the upregulation of chitinase in drought-imposed ASD16 rice plants observed in the present study. Our RT-PCR experiment on dual stress also revealed that the chitinase protein, upregulated expression of which was found to overlap during fungal infection and drought treatment, also showed higher level expression in dual stress (fungal infection followed by drought stress and drought stress followed by fungal infection) indicating its dual role. Quantification data of gene expression based on band intensity of RT-PCR gels indicated higher expression levels of chitinase in drought followed by fungal infection compared with fungal infection followed by drought.

True germins are well-conserved (in terms of sequence and structural similarities) marker proteins for seed germination in cereals, while germin-like proteins belong to a heterogeneous group and reported in a wide range of plants (Li et al. 2016). Germin-like proteins have been reported to have enzyme activities such as superoxide dismutase, oxalate oxidase, and polyphenol oxidase. These enzymes participate in basal level defense or tolerance to both biotic and abiotic stresses in plants. Higher gene expression levels of germin-like proteins have been reported under salt, drought and wound stress (Wang et al. 2013), fungal (Knecht et al. 2010), bacterial (Godfrey et al. 2007), and viral infections (Guevara-Olvera et al. 2012) in many plants. Manosalva et al. (2009) reported the role of germin-like proteins in rice against blast and sheath blight diseases.

In our two-dimensional experiments, upregulation of germin-like protein was observed to be overlapping in *R. solani* infection and high temperature stress. This was supported by the observations made at the RNA level in dual stress experiments. The expression level was significantly higher in plants with fungal infection and high temperature stress, either individually or one after the other. Quantification data of gene expression based on band intensity of RT-PCR gels indicated higher expression level of germin gene in fungal infection followed by high temperature stress, compared with high temperature stress followed by fungal infection. However, it is too early to conclude and thus warrants further investigations on whether drought conditions predispose rice plants to become tolerant to sheath blight infection by induction of chitinase, and whether fungal infection predisposes rice plants to become tolerant to high temperature stress by induction of germin.

Our work has shown over-lapping proteins during biotic and abiotic stress responses in rice. In addition, the genes coding for these proteins have shown higher expression levels during dual stress involving biotic and abiotic. Based on the results of the present study, chitinase, and germin-like protein appear to be potential targets for further exploration to develop biotic and abiotic stress tolerance in rice plants.

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