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Identification of NaCl Stress-Responsive Apoplastic Proteins in Rice Shoot Stems by 2D-DIGE

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Abstract

Plants have evolved sophisticated systems to cope with adverse environmental conditions such as cold, drought, and salinity. Although a number of stress response networks have been proposed, the role of plant apoplast in plant stress response has been ignored. To investigate the role of apoplastic proteins in the salt-stress response, 10-day-old rice plants were treated with 200 mM NaCl for 1, 6 or 12 hours, and the soluble apoplast proteins of rice shoot stems were extracted for differential analysis, compared with untreated controls, by 2-D DIGE saturation labeling techniques. One hundred twenty-two significantly changed spots were identified by LC-MS/MS, and 117 spots representing 69 proteins have been identified. Of these proteins, 37 are apoplastic proteins according to the bioinformatic analysis. These proteins are mainly involved in the processes of carbohydrate metabolism, oxido-reduction, and protein processing and degradation. According to their functional categories and cluster analysis, a stress response model of apoplastic proteins has been proposed. These data indicate that the apoplast is important in plant stress signal reception and response.

Keywords

rice; salt stress; apoplast protein; 2-D DIGE; LC-MS/MS

Introduction

Soil salinity is one of the most important abiotic stresses that have adverse effects on agricultural productivity worldwide. Excessive salt ions can cause numerous changes in cellular activities such as inhibition of plant growth, photosynthesis and respiration, cell wall alterations, changes in protein synthesis and modifications of enzyme activity [1, 2]. To maintain growth in the presence of salinity, plants have developed a series of sophisticated response networks, including ion compartmentalization in vacuoles to reduce cellular

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damage alterations in the gene expression of specific proteins to establish cellular ionic and osmotic homeostasis and repair and detoxification processes [3–7]. Recently, more and more evidences proved that cell secretion is a critical biological system involved in plant stress response processes. Intracellular products will release into the extracellular space presumably to sense the environment change[8].

Plant extracellular space also refers to plant apoplast. The plant cell apoplast, which consists of all compartments beyond the plasmalemma, has been implicated in a variety of functions during plant growth and development as well as in plant defense responses to stress conditions [9, 10]. Because cells transport ions, assimilates and other metabolites from the apoplast during signal-transduction processes, a signal must cross the apoplast and plasmalemma for the cell to produce a response [11]. It has been reported that stress conditions greatly affect the composition of apoplastic proteins both qualitatively and quantitatively [12]. There is strong evidence that apoplast proteins are greatly altered in response to salt [13], chilling [14], salicylic acid [15], metal toxicity [16] and pathogen invasion [17]. When compared to the intracellular signaling pathway components and effectors, the roles of plant apoplastic proteins have been obviously ignored in analyzing the plant stress response. The lack of plant apoplast research is due to an out-dated concept of apoplast function as well as difficulties specific to the extraction and analysis of apoplastic proteins. Some efforts have been made to identify apoplastic proteins at bioinformatic level [18, 19], and 33,809 open reading frames (ORFs) were deduced that code for putative secreted peptides, but bioinformatic analysis has the disadvantages that the results are only putative proteins and that their functions cannot be directly assigned to any specific physiological process. Proteomics is emerging as a powerful tool for studying protein dynamics in specific physiological processes. Recently, apoplast proteomics has emerged as an active research field in spite of the difficulties in apoplastic protein extraction and identification [8, 20]. A proteomic approach has been employed to study apoplastic proteins from the leaves of *Arabidopsis thaliana*, *Triticum aestivum* and *Oryza sativa* [21]. Changes in the tobacco leaf apoplast proteome in response to salt have been identified [12]. Moreover, apoplastic proteins regulated by oligogalacturonides in *Arabidopsis thaliana* were characterized by 2-D DIGE [22]. Rice is considered as a model for monocot plants, and it is most important food crop specie in the world. Rice is a salt-sensitive monocot. In particular, seedlings and the reproductive stages are very sensitive to salinity[23]. Salinity is one of the major environmental factors limiting growth and productivity of rice plants. The rice root apoplastic proteins involved in the initial phase of salt stress were investigated [13]. However, information about the changes in rice shoot stem apoplastic proteins induced by salt has not been described as of yet.

In the present work, a sub-cellular, comparative proteomic approach coupled with mass spectrometry was used to study the changes in the apoplastic proteins of rice shoot stems under different exposure time of salt stress. The CyDye™ DIGE fluorescent saturation labeling method, which is more sensitive than its minimal-labeling counterparts [24], was used for apoplastic protein analysis. Finally, we successfully identified 117 protein spots. Sixty-four of these protein spots representing 37 proteins, were putative apoplastic proteins involved in different biological processes. Our findings revealed that the apoplast plays a role as a significant site of response to salt stress. Further functional characterization of these identified apoplastic proteins is needed to verify their role in the salt stress response.

Materials and Methods

Plant Materials and Salt Treatment

Rice seeds (*Oryza sativa* L. ssp. *japonica*) were soaked at 30°C chamber for approximately 48 h, and germinated seeds were transplanted into Hoagland solution in growth tanks. Plants

were grown at a relative humidity of 50% under long-day conditions (16 h light/8 h dark cycle) at 28°C/25°C [25]. After 10 days, seedlings were treated with 200 mM NaCl for 1 h, 6 h and 12 h, untreated plants were used as a control. Shoot stems were harvested between the first leaf and the second leaf from 10-day rice seedlings from control and salt-treated plants for apoplastic protein extraction. Three independent biological replicates were prepared at each time point.

Preparation of Apoplastic Protein Samples

A vacuum infiltration procedure [12, 26] was used for apoplast extraction. Two to three centimeter strips of shoot stems were cut and washed several times in deionized water, and then the bundles of stems were submerged in deionized water in a vacuum desiccator. A vacuum was applied at 80 kPa for 15 minutes. The pressure was gradually released back to atmospheric pressure over a period of 5 minutes. Stem strips were then blotted dry, carefully placed in centrifuge tubes with perforations (2 mm diam) in the middle, and centrifuged immediately at 800 g, for 15 min at 4°C. The apoplast fluid was collected at the bottom of the tubes. The solutions were lyophilized and stored at -80°C. Before use, the powder was resuspended in 100 µl cell lysis buffer and then Clean-Up Kit (GE Healthcare) was used to eliminate impurities and concentrate proteins in the samples [27].

Total Protein Extraction

Fresh leaf tissue was ground to powder in liquid nitrogen using a mortar and pestle. The powder continued to be ground for about 2 minutes in extraction buffer (50 mM Tris-HCl pH 7.4, 5 mM EDTA, 5 mM DTT, 250 mM sucrose, 0.25 mM PMSF), and then homogenized in a homogenizer for another 5 minutes. Finally the slurry was centrifuged at 15000 g, for 30min at 4°C. The supernatant was transferred to new tubes and stored in -80°C.

For immunoblots against tubulin, apoplastic proteins and total soluble proteins were separated by 12.5% SDS-PAGE, and stained with coomassie brilliant blue R-250 or transferred to a PVDF membrane. The membrane was probed with anti- α -tubulin monoclonal antibody (sigma, 1:2000 dilution). Alkaline phosphatase conjugated secondary antibody (Sigma, 1:10000) was used to develop the blot.

Malate Dehydrogenase Assay

To evaluate intracellular contamination of apoplastic proteins, malate dehydrogenase activity was examined suggested by Husted *et al.* [28], with some modifications. 10 µl samples were added to 3ml reaction mixture containing 0.17 mM oxalacetic acid and 0.094 mM β -NADH disodium salt in 0.1 M Tris buffer, pH 7.5. the reaction was measured by the decrease in absorbance at 340 nm for 180s in a spectrophotometer (Hitachi U-2001 Japan), the same reaction system only with sample buffer added in was used as a blank.

Determinations of Na⁺/K⁺ Ratio

Shoot stems treated with 200 mM NaCl for 0 h, 1 h, 6 h, and 12 h were collected and dried in an oven at 65° for 4 days. Dry materials (about 100 mg/sample) were digested in concentrated HNO₃ [29, 30]. Na⁺ and K⁺ contents were determined using an atomic absorption spectrophotometer (Varian 240Z+240FS).

Sample Preparation and Protein Labeling with Cydye

Apoplastic proteins from these preparations described as above were treated with 2-D Clean-Up Kit (GE Healthcare) following the manufacturer's instructions. The protein pellet was resolubilized in lysis buffer (30 mM Tris, 7 M urea, 2 M thiourea, and 4% (w/v)

CHAPS pH 8) and adjusted to pH8. Protein concentration was determined using the 2-D Quant Kit (GE Healthcare). Prior to labeling the proteins with saturation dye, we optimized the amounts of Tris (2-carboxyethyl) phosphine hydrochloride (TCEP; Invitrogen, USA) and dye according to the manufacturer's protocol. For an analytical gel, a 5- μ g sample of protein was labeled with Cy3 or Cy5. The labeling reaction was performed according to the manufacturer's protocol (GE Healthcare). A Cy3-labeled internal standard (a pool of aliquots from all samples within three biological replicates) and Cy5-labeled individual samples were mixed and separated on a single gel. Twelve gels were run for each mixture of Cy3-labeled internal standard and Cy5-labeled control or treated samples. Gel to gel variations could be eliminated by normalization of internal standard sample which was considered to be common in all gels. This process can be automatically achieved by Decyder 2D software, version 6.5 (GE Healthcare).

For a preparative gel, 300 μ g of internal standard sample was labeled with Cy3 saturation dye; the amounts of TCEP and dye were accordingly scaled up.

2-D Gel Electrophoresis

For the isoelectrofocusing (IEF) step, IPG strips (18 cm, pH 4–7, GE Healthcare) were rehydrated overnight in 350 μ l rehydration buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% v/v Pharmalyte pH 4–7, 13 mM DTT) in a reswelling tray. Prior to the first dimension, Cy3-labeled internal standard samples were mixed with Cy5-labeled control or treated samples, and then applied to the swollen strips using cathodic cup loading. IEF was carried out on an Ettan IPGphor™ (GE Healthcare) at 20°C with a maximum current of 50 μ A/strip and the settings were as follows: 500 V gradient for 1 h, and then 1000 V gradient for 3 h, gradient increase to 8000 V in 3 h, and remaining at 8000 V until reached at the desired total 36 kVh.

After IEF, the IPG strips were equilibrated in 10 ml of buffer (0.1 M Tris-HCl pH 8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.5% w/v DTT) for 10 min at room temperature. The equilibrated strips were then transferred to 12.5% acrylamide gels for the second dimension separation using the Ettan Dalt-six electrophoresis system (GE Healthcare) at a constant power of 2 W per gel overnight at 15°C, in the dark. For the preparative gels, 300 μ g of Cy3-labeled internal standard samples were loaded, and in-gel rehydration was performed overnight.

Image Acquisition and Biological Variation Analysis

Gels were scanned using a Typhoon 9410 imager (GE Healthcare). The excitation wavelengths for Cy3 and Cy5 are 532 nm and 633 nm, the emission wavelengths are 580 and 670 nm, respectively. Other related parameters were set according to the user manual. Gel images were analyzed using the DeCyder 2D Software V6.5 (GE Healthcare). In the DIA (differential in-gel analysis) module, the estimated number of spots was set at 2000. The borders and background intensities of the spots were determined to calculate spot volumes. The background compensated spot volumes between samples and internal standard were quantified as spot normalization. Then in BVA (biological variation analysis) module, the normalized protein spots were matched among different gels, and we consider spots with a statistically significantly change in abundance between control and treated samples to be changed in response to salt treatment (one-way ANOVA p value < 0.05). Gel images used for statistical analysis were from three independent biological repeats.

Protein Identification by LC-MS/MS

Protein spots of interest were identified by LC-MS/MS. First, they were excised from gels using an Ettan Spot Picker (GE Healthcare) and digested in-gel with trypsin as described

previously [31]. Briefly specific protein spots were washed twice with 50% acetonitrile in 25 mM ammonium bicarbonate (NH_4HCO_3) and vacuum-dried. Then the gel pieces rehydrated in 8 μl of digestion buffer (10 ng/ μl trypsin in 25 mM NH_4HCO_3), and covered with a 20 μl volume of 25mM NH_4HCO_3 . After overnight digestion at 37 °C, peptides were extracted twice with a solution containing 50% acetonitrile and 5% formic acid. The extracted digests were vacuum-dried and resuspended in 10 μl of 0.1% formic acid in water. The digests were separated by nanoflow liquid chromatography using a 75- μm ×150-mm reversed-phase C18 PepMap column (Dionex-LC Packings, San Francisco, CA) at a flow rate of 300 nl/min in a NanoLC-1D Proteomics high performance liquid chromatography system (Eksigent Technologies, Livermore, CA) equipped with a FAMOS autosampler (Dionex-LC Packings). Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. Following equilibration of the column in 5% solvent B, approximately 5 μl was injected, and then the organic content of the mobile phase was increased linearly to 40% over 30min and then to 50% in 1 min. The column effluent was directed to a microionspray source attached either to a QSTAR Elite (Applied Biosystems/MDS Sciex, South San Francisco, CA). Peptides were analyzed in positive ion mode. MS spectra were acquired for 1 s in the m/z range between 310 and 1400. MS acquisitions were followed by 3-s CID experiments in information-dependent acquisition mode. For each MS spectrum, the most intense multiple charged peaks over a threshold of 30 counts were selected for generation of CID mass spectra. The CID collision energy was automatically set according to m/z ratio and charge state of the precursor ion. A dynamic exclusion window was applied that prevented the same m/z from being selected for 1 min after its acquisition.

QSTAR Elite data were analyzed with Analyst QS 1.1 software (Applied Biosystems/MDS Sciex), and peak lists were generated using the mascot.dll script (Mascot.dll 1.6b18, Applied Biosystems). Precursor mass tolerance for grouping was set to 0.2 amu, and MS centroiding parameters were 50% peak height and 0.02 amu merge distance. MS/MS centroiding parameters were 50% peak height and 0.05 amu merge distance. The peak lists were searched in in-house software (Protein Prospector version 5.5.1). Enzyme specificity was set to trypsin, and the maximum number of missed enzyme cleavages per peptide was set at 2. Mass tolerance for QSTAR data was 150 ppm for precursor and 0.2 Da for fragment ions. The peak lists were searched against the *Oryza sativa* L. ssp. *Japonica* subset of the NCBI database as of Jan 1, 2009 (134,762 entries searched). Control searches of some of the files against the whole database (7,614,762 entries searched) confirmed the absence of contamination from human keratins or other non-rice proteins, therefore searches were routinely performed on the *Oryza sativa* subset. For all protein identifications, a minimal protein score of 22, a peptide score of 15, and a minimal discriminant score threshold of 0.0 were used in the initial identification criteria. Maximum expectation values for accepting individual spectra were set to 0.01. When several accession numbers in the database matched the same set of identified peptides, the entries with the most descriptive name were reported. Individual isoforms of proteins were reported according to the detection of peptides unique to their sequences. For identifications based on one or two peptide sequences with high scores, the MS/MS spectrum (Table S1, Figure S1) was reinterpreted manually by matching all the observed fragment ions to a theoretical fragmentation obtained using software of MS Product (Protein Prospector) [32].

RNA Extraction and Real-Time Q-PCR

Total RNA was extracted from the shoot stems of seedlings using the Trizol reagent (Invitrogen). Five hundred nanograms of total RNA was used to synthesize the first-strand cDNA which was diluted 20 fold; 5 μl of the diluted cDNA was used as template for real-time Q-PCR. SYBR Green-monitored real-time Q-PCR was performed on an ABI PRISM

7000 real-time PCR system (Applied Biosystems). The primer sequences used for real-time Q-PCR are listed in Table S2.

Results and Discussion

Response of Rice Shoot Stems to NaCl Stress

To determine whether the shoots of 10-day rice seedlings can sense NaCl stress under the selected time points of treatment, Na^+ and K^+ concentrations were quantified in shoots of control and 200 mM NaCl treated plants (Figure S2), and the ratios of Na^+/K^+ were calculated (Figure 1). It is known that salt stress causes Na^+ toxicity in plants; the influx of Na^+ into cells inhibits K^+ uptake and transport which leads to an increase in Na^+/K^+ ratio and thereby suppresses plant growth [33]. In our experiments, the Na^+/K^+ ratio in shoots changed within 1 h and then increased gradually as the NaCl treatment progressed (Figure 1), indicating that the accumulation of stress continues throughout the 12 h salt treatment. The results indicated that rice shoots might have a swift response during initial phase of salt stress.

Assessment of Apoplast Extracts from Rice Shoot Stems

To evaluate the level of cytoplasmic contamination, the Malate Dehydrogenase (MDH) activity assay was conducted. Table 1. lists the data analysis of three independent experiments. These data show that at similar protein concentrations, the MDH activity of apoplastic proteins is 7.8%, 9.1%, 7.4% and 6.9% of that of the total protein sample at different salt treated time points, respectively. Therefore, our results show that contamination of the apoplast extracts with intracellular proteins or organelle components cannot be totally excluded, but we can limit the contamination to a reasonable level; only when the percentage of relative enzyme activity in salt-treated apoplast protein compared with the activity in the total protein shows values was below 10%, were the apoplast proteins considered acceptable for subsequent analysis. Immunoblotting against tubulin also used for probe intracellular contamination. Apoplastic extracts from independent extraction procedures and soluble cell total extracts were separated and probed with the anti-tubulin monoclonal antibody. With an apparent molecular mass of 49 kDa, tubulin was detectable in whole cell extracts, but not in any of our apoplastic extracts (Figure S3).

Identification of NaCl-Responsive Apoplastic Proteins in Rice Shoot Stems

Apoplastic proteins from rice shoot stems were analyzed using the 2-D DIGE saturation labeling method (Figure S4). Shoot stem refers to the shoots between the first leaf and the second leaf of 10-day rice seedlings, shoot stem is the main part of ten day old rice seedlings. This part includes the rolled-up leaf blade being enclosed by an older leaf sheath but not includes the older leaf blade. Up to 1000 spots were detected in our gels (Table S3), while previous apoplast proteome research detect fewer than 500 spots by DIGE minimal labeling, coomassie blue or silver staining for rice, Arabidopsis and tobacco [12, 13, 20, 22]. One hundred twenty-two spots with an 1-ANOVA p -value < 0.05 were considered to be statistically significant and excised from a preparative gel for identification by LC-MS/MS (Figure 2). Finally, 117 spots were successfully identified (Table 2). For the identified proteins, we first examined whether they have a signal peptide (<http://www.cbs.dtu.dk/services/SignalP/>; Table 2) and transmembrane domains (<http://www.cbs.dtu.dk/services/TMHMM/>). Proteins possessing a signal peptide and lacking transmembrane (TM) domains were further analyzed to determine whether they have cellular organelle retention signal sequences using programs such as <http://www.expasy.org/prosite/> (ER-resident proteins) or http://ccb.imb.uq.edu.au/golgi/golgi_predictor.shtml (Golgi-resident proteins), <http://www.cbs.dtu.dk/services/TargetP/> (Table 2) [8, 34]. For proteins without a signal

peptide, we attempted to predict whether they were involved in the non-classical secretory pathway using <http://www.cbs.dtu.dk/services/SecretomeP-1.0/> [20]. In summary, we consider proteins to be putative apoplastic proteins if they had a signal peptide, but lacked TM domains and cellular organelle retention signals or they were involved in non-classical secretory pathway. Ultimately, 64 spots representing 37 proteins were predicted to be apoplastic proteins, including 56 spots representing proteins with a signal peptide, and 8 spots representing proteins lacking signal peptides (Table 2) [20, 35]. Some apoplastic proteins were identified from multiple spots on the gel (Figure. 2) such as α -L-Arabinofuranosidase (spots 43, 40, 49, and 51), β -1, 3-glucanase (spots 137, 138, and 140), and subtilisin-like protease (spots 39, 44, 48, 57, and 50), which implying that these proteins may undergo posttranslational modifications (PTMs).

Eight spots involved in secretory pathways were found to lack signal peptide. A wealth of evidence reveals that these leaderless proteins (LSPs) can be secreted into the extracellular space through non-classical secretory pathways. On average, LSPs can account for more than 50% of the total identified secretome in plant [8]. Bendtsen et al. developed a program for the prediction of non-classical secretory proteins in mammals and bacteria [36] (<http://www.cbs.dtu.dk/services/SecretomeP-1.0/>). Recently, Jamet et al. used this tool in *Arabidopsis* cell wall proteomics and found several leaderless secretory proteins, most of which are associated with lipid metabolism [20]. In mammals, it is reported that the unconventional protein secretion pathway may be triggered by a variety of stressors such as heat shock, infection and lipopolysaccharides [37]. In plants, superoxide dismutase (SOD) has been reported to have extracellular function in response to salt stress and pathogen infection [38, 39]. Cheng et al. also found a number of nonclassically secreted proteins in suspension culture of *Arabidopsis thaliana* in response to salicylic acid [15]. These results indicate that some cytosolic proteins can be recruited to perform special tasks outside the cell through ER/Golgi-independent pathways under stress conditions.

To determine apoplastic proteins vary under specific salt-treated time points, a Venn diagram analysis of the number of differentially expressed apoplastic protein spots after different times of salt stress was conducted (Figure 3). Of the 64 apoplastic protein spots, 10 spots were upregulated and 15 spots were downregulated at all three time points of salt treatment, 39 spots were either upregulated or downregulated at different time points during salt stress. The large number of changes in apoplastic proteins suggests that these proteins may play important roles in the rice shoot stem salt stress response.

Twelve proteins were randomly selected to verify their salt response profile at the mRNA level by real-time quantitative RT-PCR. The results showed that the expression pattern of the four proteins (29, 105, 140, and 130) strongly correlation with the corresponding mRNA level, whereas there was low correlation between protein abundance and the mRNA amount for the other eight protein spots (42, 114, 7, 56, 101, 2, 75, and 139) under salt stress treatment (Figure S5). The low correlation between protein abundance and mRNA amount for some protein spots may be due to the dependence of protein expression levels not only depended on the corresponding mRNA levels but also on the regulation by translation and protein degradation systems [40, 41]. Furthermore, protein abundance in the apoplastic compartment of plant cells was also affected by secretory pathways and transport systems [42, 43].

Functional Categories of Abundance Changed Salt-Responsive Apoplastic Proteins

According to the biological processes proteins are involved in and gene ontology tools (<http://www.agbase.msstate.edu/>) and UniProtKB (<http://www.uniprot.org/>), all identified apoplastic proteins fall into eight main functional categories including carbohydrate metabolism, oxidoreductases, protein processing and degradation, cell wall biogenesis and

degradation, lipid metabolism, protein folding and assembly, and energy metabolism and other mechanisms (Figure 5). Proteins involved in carbohydrate metabolic process make up the largest proportion (28%), followed by the second largest group (25%) of oxidoreductases, and the third largest group (14%) of protein processing and degradation.

Meanwhile, we performed a cluster analysis of the identified apoplastic proteins to investigate the relationship between their profile in abundance changed at different salt-treated time points and their functions. According to the functional categories mentioned above, we placed them in three main groups: cell wall-related metabolism, oxidoreduction, and protein metabolism. Cell wall-related metabolism contains the following functional categories: carbohydrate metabolic processes, cell wall biogenesis and degradation. Protein metabolism contains protein processing and degradation, protein folding and assembly. We found that proteins that cluster together belong to the same gene families or have the same functions (Figure 4). These results indicated that the apoplast may act as site of response to stress signals during the initial phase of salt stress.

Abundance Changed Proteins are Mainly Implicated in Cell wall-Related Metabolism

Most of the apoplastic proteins identified in our assay were found to be involved in cell wall metabolism and categorized into two groups (Figure 5). The first group is composed of 18 spots representing 9 proteins related to carbohydrate metabolism including 5 proteins shown to increase under salt stress; among them are putative α -galactosidase, β -N-acetylhexosaminidase, heparanase-like protein, β -1,3-glucanase, β -1,3-glucanase precursor (Figure 4A, Table 2). Notably, β -1, 3-glucanase has been previously reported to play roles in plant antifungal defenses in many kinds of plants, such as rice, wheat, and tobacco [44–47]. In the leaf apoplast of *Brassica napus*, β -glucanase was also induced by pathogens [48]. However, β -1, 3-glucanase (OsGlu) has many gene isoforms, and they respond differently to salt stress in rice [45]. In our experiment, three spots (137, 138, and 140), the products of OsGlu27, display more abundant during salt stress. Spot 124, the β -1, 3-glucanase precursor that is the product of OsGlu24, also displays a tendency to increase. In contrast, another five spots (117, 110, 107, 112, and 113), the product of OsGlu2, display a tendency to decrease. Among the four downregulated proteins, α -L-Arabinofuranosidase/ β -D-xylosidase appear as 4 spots (43, 40, 49, and 51), and they display a tendency to decrease during salt stress. This enzyme hydrolyses the carbohydrate moieties of arabinogalactan, arabinoxylan, and other polysaccharides, which are the main components of hemicellulose. Thus, the less abundant of this protein may help prevent the degradation of cell wall hemicellulose thereby stabilizing the cell wall.

The second group consists of seven spots representing four proteins involved in cell wall biogenesis and degradation (Figure 5). Among them, three spots (134, 132, and 128) corresponding to xyloglucan endotransglycosylase (XET) revealed different expression patterns. Spot 132 shows continued upregulation under saline conditions, while spots 134 and 128 undergo complex changes. Spot 120 corresponds to endo-xyloglucan transferase (EXT) and plays a similar role to XET in cell wall modification by catalyzing the cleavage and re-joining of xyloglucan, a major component of hemicellulose in plant cell walls [49–51]. The observed differences in the expression patterns of XET and EXT in response to salt stress may be due to the function of XET in either creating cell wall components or in breaking down the cell wall depending on the availability of substrate and other wall modifying proteins [52]. Over-expression of hot pepper XTH (xyloglucan endotransglycosylase/hydrolase) in *Arabidopsis* led to improved tolerance to salt stress and drought [53]. Additionally, chitinase, which was previously shown to be involved in pathogen defense and induced by heavy metals, osmotic stress, low temperature, and wounding [54–58], was also observed in this study. The *Arabidopsis* hot2 mutant, encoding an endochitinase-like protein, was found to have a tolerance to salt and drought stresses

[59]. Overexpressing chitinases in tobacco has conferred resistance to salinity, pathogens and heavy metals [60]. There are three spots (142, 135, 136) corresponding to chitinase in our study. They exhibit different responses to salt stress. Spots 142 and 136 show increased abundance pattern, which may contribute to the synthesis of cell wall cellulose, thereby reinforcing the plant cell wall against stress conditions. In contrast, the intensity of spot 135 decreased under saline conditions; this phenomenon indicates the possibility that posttranslational modifications of the protein may result in different responses to salt stress.

Our results suggest that when plants are faced with salt stress, the outermost cell wall, which is the main component of apoplast was greatly disturbed. The abundant changes of cell wall related proteins in response to salt stress suggest that many cell wall metabolic processes such as carbohydrate degradation, glycoprotein processing and restructure of cellulose and hemicellulose ultimately lead to the reinforcement of the cell wall, thereby enhancing the physical barrier against deleterious salt stress.

Many Abundance Changed Proteins are Involved in ROS Detoxification

A total of 16 spots representing eight proteins (25% in total) were related to oxidoreduction (Figure 5), six of which belong to the peroxidase protein family. Various kinds of stress conditions such as pathogen, drought, high salinity and chilling can lead to the production of reactive oxygen species (ROS), which oxidize cellular components causing cytotoxicity. They may also participate in lignification, suberization and cross-link of cell wall structural proteins [61]. In our experiments, peroxidase 6 (spot 95) and peroxidase (spots 105, 98, and 100) show a trend of augmented expressions (Figure 4B, Table 2) indicating that these peroxidases may be recruited to modify cell wall proteins to confer structural strength to plants against salt stress or scavenge excessive ROS. In contrast, class III peroxidase 12 (spot 86), peroxidase 52 precursor (spot 92), and peroxidase 43 (spot 115) show less abundant, different expression patterns of peroxidase in response to salt treatment may result from the fact that peroxidase in plants is a large multigene family, and an equilibrium exists between scavenging excessive ROS and producing ROS within the cell [62].

6-phosphogluconate dehydrogenase (6PGDH, spot 73) was upregulated by salt treatment. This protein was present in plastid and cytosol in prior studies[63] and is involved in the oxidative pentose phosphate pathway, in which NADPH is generated and used to prevent oxidative stress. 6PGDH was upregulated in response to both chilling stress in rice roots[64] and salt stress in rice shoots[65]. The annotated hypothetical protein (spot 69, 66, 78, 67, 60) shares 86.69% identity with putative CPRD2, they all contain the FAD binding domain and their changes in abundance in response to salt stress may regulate oxidoreductase activities by acting as co-factors of oxidoreductase.

Many Abundance Changed Proteins Were Associated with Protein Metabolism

Many apoplastic proteins (17% in total) are expressed differentially in response to salt stress and involved in protein metabolism including protein processing, degradation and folding (Figure 5). In our study, six proteins were found to participate in these processes, including subtilisin-like protease, serine carboxypeptidase, β subunit of the 20S proteasome, subtilisin proteinase, aspartyl protease nepenthesin precursor, and heat shock protein 81-1. All were downregulated other than aspartyl protease nepenthesin precursor (Figure 4C, Table 2). Plant subtilisin-like protease (subtilases) functions in the modification of plant morphology and the cleavage of cell wall structure proteins [66, 67]. There is also evidence that subtilases in *Arabidopsis* are involved in responses to pathogens and salicylic acid and in activating the expression of salt stress-responsive genes [50, 51, 68, 69]. In the present study, the subtilases (spot 39, 44, 48, 57, and 50), present in multiple spots, show similar trends of less abundant patterns following salt treatment suggesting that short-term salt

stress may inhibit the degradation of cell wall proteins. Plant serine carboxypeptidase has been proposed to function in protein turnover, C-terminal processing, wound response, oxidative stress and secondary metabolism [49, 70], but its role in salt stress was not well-documented. Aspartyl protease nepenthesin (NAP) has been characterized as an extracellular endopeptidase that mediates a peptide signal system in the activation of inducible resistance mechanisms in *Arabidopsis* [71, 72].

Notably, hsp81-1 and the β subunit of the 20S proteasome, which were previously believed to function within plant cells, appeared in our apoplastic extract of rice shoot stems in response to salt stress. Oh, et al. also found heat shock proteins (HSPs) in *Arabidopsis* secretome studies [17]. HSPs acting as intracellular molecular chaperones were reported to assist in repair or degradation of misfolded proteins within the cell under stress conditions [73]. In recent years, there was much evidence to indicate that extracellular HSPs play roles in immunity response of mammals and protect cells from cytotoxicity [74, 75]. Similarly, the 26S proteasome is responsible for the degradation of damaged and misfolded proteins. In mammalian cells, the 20S proteasome, the proteolytic core of the 26S proteasome, can be released into the extracellular space and has been implicated in the degradation of oxidized proteins [76, 77]. There were no related reports about the extracellular distribution of the 20S proteasome in a plant species before now. HSPs and the 20S proteasome were not secreted through the classical ER-Golgi pathway because they lack a signal peptide, but instead may be secreted by way of cell lysis, secretory vesicles, exosomes or secretory lysosomal endosomes [74, 78]. Under salt stress, the secretory pathways of the proteins may be inhibited and thereby attenuate the protein abundance in the extracellular milieu, which may account for the decreased level of several proteins observed in our data set.

A Predicted NaCl Stress-Responsive Apoplast Protein Network in the Shoot Stems of Rice Seedlings

The apoplast is the first subcellular compartment confronted with stress conditions when plants are subjected to NaCl stress. However, there was no systematic research about how the rice shoot stem apoplast functions in initial perception of the salt stress signal until now. Based on proteomic and physical data from the present study, we propose a NaCl stress-responsive apoplastic protein network containing most of the identified apoplast proteins in rice shoot stems (Figure 6).

When rice seedlings were grown under saline conditions, the main component of the apoplast, the cell wall, was significantly altered. Several cell wall-related proteins were involved in cell wall remodeling, which occurred following salt treatment. This remodeling would strengthen the cell wall and assist in plant resistance against stress-induced damages.

Meanwhile, our physical experiments show that the Na^+/K^+ ratio in shoots increased with increasing duration of salt stress treatment. This increase could cause ion toxicity and concomitant osmotic stress. Osmoprotectant can be synthesized through lipid metabolism to help cells maintain osmotic homeostasis. Additionally, high levels of salinity can stimulate the production of ROS, which would cause the subsequent oxidative damage of cells. The extracellular peroxidases, together with hsp 81-1 and the 20s proteasome, were responsible for the scavenging of oxidized cellular components for the purpose of damage repair. However they can produce ROS as a signal to trigger the expression of downstream salt-responsive genes.

Conclusions

In the present work, we investigate the global changes in the apoplast proteome of rice shoot stems exposed to salinity using 2-D DIGE via liquid chromatography-tandem mass

spectrometry (LC-MS/MS). The DIGE gels revealed a total of 122 significantly changed in abundancesalt-responsive spots, from which 117 spots were successfully identified. Sixty-four spots were apoplastic proteins according to bioinformatic analysis. The discovery of these novel apoplast proteins of rice shoot stems in response to salt provides us evidence for the hypothesis that apoplast may be an important site for the plant cell response to stress signals during the initial phase of salt stress.

According to the possible functions of the identified apoplast protein, we suggest an extracellular salt stress responsive protein network which reflects the possible mechanisms involved in the initial perception of salt stress for the rice shoot stem apoplast. We expect that these results will help us to better understand the roles of rice shoot stem apoplast under saline conditions and provide candidate proteins for further functional research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Chaves MM, Flexas J, Pinheiro C. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Ann Bot (Lond)*. 2009; 103:551–60.
- Parida AK, Das AB. Salt tolerance and salinity effects on plants: a review. *Ecotoxicol Environ Saf*. 2005; 60:324–49. [PubMed: 15590011]
- Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ. Plant Cellular and Molecular Responses to High Salinity. *Annu Rev Plant Physiol Plant Mol Biol*. 2000; 51:463–99. [PubMed: 15012199]
- Zhu JK. Regulation of ion homeostasis under salt stress. *Curr Opin Plant Biol*. 2003; 6:441–5. [PubMed: 12972044]
- Zhu JK. Salt and drought stress signal transduction in plants. *Annu Rev Plant Biol*. 2002; 53:247–73. [PubMed: 12221975]
- Kreps JA, Wu Y, Chang HS, Zhu T, Wang X, Harper JF. Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. *Plant Physiol*. 2002; 130:2129–41. [PubMed: 12481097]
- Munns R, Tester M. Mechanisms of salinity tolerance. *Annu Rev Plant Biol*. 2008; 59:651–81. [PubMed: 18444910]
- Agrawal GK, Jwa NS, Lebrun MH, Job D, Rakwal R. Plant secretome: unlocking secrets of the secreted proteins. *Proteomics*. 2010; 10:799–827. [PubMed: 19953550]
- Tian L, Zhang L, Zhang J, Song Y, Guo Y. Differential proteomic analysis of soluble extracellular proteins reveals the cysteine protease and cystatin involved in suspension-cultured cell proliferation in rice. *Biochim Biophys Acta*. 2009; 1794:459–67. [PubMed: 19118651]
- Pennell R. Cell walls: structures and signals. *Curr Opin Plant Biol*. 1998; 1:504–10. [PubMed: 10066626]
- Sakurai N. Dynamic function and regulation of apoplast in the plant body. *Journal of Plant Research*. 1998; 111:133–48.
- Dani V, Simon WJ, Duranti M, Croy RR. Changes in the tobacco leaf apoplast proteome in response to salt stress. *Proteomics*. 2005; 5:737–45. [PubMed: 15682462]

13. Zhang L, Tian LH, Zhao JF, Song Y, Zhang CJ, Guo Y. Identification of an apoplastic protein involved in the initial phase of salt stress response in rice root by two-dimensional electrophoresis. *Plant Physiol.* 2009; 149:916–28. [PubMed: 19036832]
14. Marentes E, Griffith M, Mlynarz A, Brush RA. Proteins Accumulate in the Apoplast of Winter Rye Leaves during Cold-Acclimation. *Physiologia Plantarum.* 1993; 87:499–507.
15. Cheng FY, Blackburn K, Lin YM, Goshe MB, Williamson JD. Absolute protein quantification by LC/MS(E) for global analysis of salicylic acid-induced plant protein secretion responses. *J Proteome Res.* 2009; 8:82–93. [PubMed: 18998720]
16. Fecht-Christoffers MM, Braun HP, Lemaitre-Guillier C, VanDorselaer A, Horst WJ. Effect of manganese toxicity on the proteome of the leaf apoplast in cowpea. *Plant Physiol.* 2003; 133:1935–46. [PubMed: 14605229]
17. Oh IS, Park AR, Bae MS, Kwon SJ, Kim YS, Lee JE, et al. Secretome analysis reveals an Arabidopsis lipase involved in defense against *Alternaria brassicicola*. *Plant Cell.* 2005; 17:2832–47. [PubMed: 16126835]
18. Lease KA, Walker JC. The Arabidopsis unannotated secreted peptide database, a resource for plant peptidomics. *Plant Physiol.* 2006; 142:831–8. [PubMed: 16998087]
19. Liu H, Guo Y, Sun DY. The study and application about amino acid composition of extracellular polypeptide. *Acta Biophysica Sinica.* 2006; 22:117–24.
20. Jamet E, Albenne C, Boudart G, Irshad M, Canut H, Pont-Lezica R. Recent advances in plant cell wall proteomics. *Proteomics.* 2008; 8:893–908. [PubMed: 18210371]
21. Haslam RP, Downie AL, Raveton M, Gallardo K, Job D, Pallett KE, et al. The assessment of enriched apoplastic extracts using proteomic approaches. *Annals of Applied Biology.* 2003; 143:81–91.
22. Casasoli M, Spadoni S, Lilley KS, Cervone F, De Lorenzo G, Mattei B. Identification by 2-D DIGE of apoplastic proteins regulated by oligogalacturonides in *Arabidopsis thaliana*. *Proteomics.* 2008; 8:1042–54. [PubMed: 18324730]
23. Darwish E, Testerink C, Khalil M, El-Shihy O, Munnik T. Phospholipid signaling responses in salt-stressed rice leaves. *Plant & cell physiology.* 2009; 50:986–97. [PubMed: 19369274]
24. Shaw J, Rowlinson R, Nickson J, Stone T, Sweet A, Williams K, et al. Evaluation of saturation labelling two-dimensional difference gel electrophoresis fluorescent dyes. *Proteomics.* 2003; 3:1181–95. [PubMed: 12872219]
25. Kawasaki S, Borchert C, Deyholos M, Wang H, Brazille S, Kawai K, et al. Gene expression profiles during the initial phase of salt stress in rice. *Plant Cell.* 2001; 13:889–905. [PubMed: 11283343]
26. Lohaus G, Pennewiss K, Sattelmacher B, Hussmann M, Muehling KH. Is the infiltration-centrifugation technique appropriate for the isolation of apoplastic fluid? A critical evaluation with different plant species. *Physiologia Plantarum.* 2001; 111:457–65. [PubMed: 11299010]
27. Marcellin E, Gruber CW, Archer C, Craik DJ, Nielsen LK. Proteome analysis of the hyaluronic acid-producing bacterium, *Streptococcus zooepidemicus*. *Proteome Sci.* 2009; 7:13. [PubMed: 19327162]
28. Husted S, Schjoerring JK. Apoplastic pH and Ammonium Concentration in Leaves of *Brassica napus* L. *Plant Physiol.* 1995; 109:1453–60. [PubMed: 12228682]
29. Rus A, Lee BH, Munoz-Mayor A, Sharkhuu A, Miura K, Zhu JK, et al. AtHKT1 facilitates Na⁺ homeostasis and K⁺ nutrition in planta. *Plant Physiol.* 2004; 136:2500–11. [PubMed: 15347798]
30. Garg AK, Kim JK, Owens TG, Ranwala AP, Choi YD, Kochian LV, et al. Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *Proc Natl Acad Sci U S A.* 2002; 99:15898–903. [PubMed: 12456878]
31. Rosenfeld J, Capdevielle J, Guillemot JC, Ferrara P. In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. *Anal Biochem.* 1992; 203:173–9. [PubMed: 1524213]
32. Clauser KR, Baker P, Burlingame AL. Role of accurate mass measurement (± 10 ppm) in protein identification strategies employing MS or MS/MS and database searching. *Anal Chem.* 1999; 71:2871–82. [PubMed: 10424174]

33. Botella MA, Martinez V, Pardines J, Cerda A. Salinity induced potassium deficiency in maize plants. *Journal of Plant Physiology*. 1997; 150:200–5.
34. Emanuelsson O, Brunak S, von Heijne G, Nielsen H. Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Protoc*. 2007; 2:953–71. [PubMed: 17446895]
35. Agrawal GK, Jwa NS, Lebrun MH, Job D, Rakwal R. Plant secretome: Unlocking secrets of the secreted proteins. *Proteomics*. 2009
36. Bendtsen JD, Jensen LJ, Blom N, Von Heijne G, Brunak S. Feature-based prediction of non-classical and leaderless protein secretion. *Protein Eng Des Sel*. 2004; 17:349–56. [PubMed: 15115854]
37. Schafer T, Zentgraf H, Zehe C, Brugger B, Bernhagen J, Nickel W. Unconventional secretion of fibroblast growth factor 2 is mediated by direct translocation across the plasma membrane of mammalian cells. *J Biol Chem*. 2004; 279:6244–51. [PubMed: 14645213]
38. Garc-a-Limones C, Dorado G, Navas-Cortes JA, Jimenez-Diaz RM, Tena M. Changes in the redox status of chickpea roots in response to infection by *Fusarium oxysporum* f. sp. *ciceris*: apoplastic antioxidant enzyme activities and expression of oxidative stress-related genes. *Plant Biology*. 2009; 11:194–203. [PubMed: 19228326]
39. Hernandez JA, Ferrer MA, Jimenez A, Barcelo AR, Sevilla F. Antioxidant systems and O(2)(-)/H(2)O(2) production in the apoplast of pea leaves. Its relation with salt-induced necrotic lesions in minor veins. *Plant Physiol*. 2001; 127:817–31. [PubMed: 11706165]
40. Cox J, Mann M. Is proteomics the new genomics? *Cell*. 2007; 130:395–8. [PubMed: 17693247]
41. Lu P, Vogel C, Wang R, Yao X, Marcotte EM. Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation. *Nat Biotechnol*. 2007; 25:117–24. [PubMed: 17187058]
42. Hadlington JL, Denecke J. Sorting of soluble proteins in the secretory pathway of plants. *Curr Opin Plant Biol*. 2000; 3:461–8. [PubMed: 11074376]
43. Neumann U, Brandizzi F, Hawes C. Protein transport in plant cells: in and out of the Golgi. *Ann Bot*. 2003; 92:167–80. [PubMed: 12876187]
44. Epel BL. Plant viruses spread by diffusion on ER-associated movement-protein-rafts through plasmodesmata gated by viral induced host beta-1,3-glucanases. *Semin Cell Dev Biol*. 2009
45. Hwang du H, Kim ST, Kim SG, Kang KY. Comprehensive analysis of the expression of twenty-seven beta-1, 3-glucanase genes in rice (*Oryza sativa* L.). *Mol Cells*. 2007; 23:207–14. [PubMed: 17464198]
46. Kong L, Anderson JM, Ohm HW. Induction of wheat defense and stress-related genes in response to *Fusarium graminearum*. *Genome*. 2005; 48:29–40. [PubMed: 15729394]
47. Linthorst HJM, Melchers LS, Mayer A, Vanroekel JSC, Cornelissen BJC, Bol JF. Analysis of Gene Families Encoding Acidic and Basic Beta-1,3-Glucanases of Tobacco. *Proceedings of the National Academy of Sciences of the United States of America*. 1990; 87:8756–60. [PubMed: 2247445]
48. Floerl S, Druebert C, Majcherczyk A, Karlovsky P, Kues U, Polle A. Defence reactions in the apoplastic proteome of oilseed rape (*Brassica napus* var. *napus*) attenuate *Verticillium longisporum* growth but not disease symptoms. *BMC Plant Biol*. 2008; 8:129. [PubMed: 19094241]
49. Stehle F, Brandt W, Milkowski C, Strack D. Structure determinants and substrate recognition of serine carboxypeptidase-like acyltransferases from plant secondary metabolism. *FEBS Lett*. 2006; 580:6366–74. [PubMed: 17094968]
50. Tanaka H, Onouchi H, Kondo M, Hara-Nishimura I, Nishimura M, Machida C, et al. A subtilisin-like serine protease is required for epidermal surface formation in *Arabidopsis* embryos and juvenile plants. *Development*. 2001; 128:4681–9. [PubMed: 11731449]
51. Berger D, Altmann T. A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in *Arabidopsis thaliana*. *Genes Dev*. 2000; 14:1119–31. [PubMed: 10809670]
52. Campbell P, Braam J. Xyloglucan endotransglycosylases: diversity of genes, enzymes and potential wall-modifying functions. *Trends Plant Sci*. 1999; 4:361–6. [PubMed: 10462769]

53. Cho SK, Kim JE, Park JA, Eom TJ, Kim WT. Constitutive expression of abiotic stress-inducible hot pepper CaXTH3, which encodes a xyloglucan endotransglucosylase/hydrolase homolog, improves drought and salt tolerance in transgenic Arabidopsis plants. *FEBS Lett.* 2006; 580:3136–44. [PubMed: 16684525]
54. Bekesiova B, Hraska S, Libantova J, Moravcikova J, Matusikova I. Heavy-metal stress induced accumulation of chitinase isoforms in plants. *Mol Biol Rep.* 2008; 35:579–88. [PubMed: 17701287]
55. Stressmann M, Kitao S, Griffith M, Moresoli C, Bravo LA, Marangoni AG. Calcium interacts with antifreeze proteins and chitinase from cold-acclimated winter rye. *Plant Physiol.* 2004; 135:364–76. [PubMed: 15122015]
56. Wu CT, Bradford KJ. Class I chitinase and beta-1,3-glucanase are differentially regulated by wounding, methyl jasmonate, ethylene, and gibberellin in tomato seeds and leaves. *Plant Physiol.* 2003; 133:263–73. [PubMed: 12970492]
57. Metwally A, Finkemeier I, Georgi M, Dietz KJ. Salicylic acid alleviates the cadmium toxicity in barley seedlings. *Plant Physiol.* 2003; 132:272–81. [PubMed: 12746532]
58. Kasprzewska A. Plant chitinases--regulation and function. *Cell Mol Biol Lett.* 2003; 8:809–24. [PubMed: 12949620]
59. Kwon Y, Kim SH, Jung MS, Kim MS, Oh JE, Ju HW, et al. Arabidopsis hot2 encodes an endochitinase-like protein that is essential for tolerance to heat, salt and drought stresses. *Plant J.* 2007; 49:184–93. [PubMed: 17156413]
60. de las Mercedes Dana M, Pintor-Toro JA, Cubero B. Transgenic tobacco plants overexpressing chitinases of fungal origin show enhanced resistance to biotic and abiotic stress agents. *Plant Physiol.* 2006; 142:722–30. [PubMed: 16891545]
61. Hiraga S, Sasaki K, Ito H, Ohashi Y, Matsui H. A large family of class III plant peroxidases. *Plant Cell Physiol.* 2001; 42:462–8. [PubMed: 11382811]
62. Passardi F, Penel C, Dunand C. Performing the paradoxical: how plant peroxidases modify the cell wall. *Trends Plant Sci.* 2004; 9:534–40. [PubMed: 15501178]
63. Hauschild R, von Schaewen A. Differential regulation of glucose-6-phosphate dehydrogenase isoenzyme activities in potato. *Plant Physiol.* 2003; 133:47–62. [PubMed: 12970474]
64. Lee DG, Ahsan N, Lee SH, Lee JJ, Bahk JD, Kang KY, et al. Chilling stress-induced proteomic changes in rice roots. *J Plant Physiol.* 2009; 166:1–11. [PubMed: 18433929]
65. Huang J, Zhang H, Wang J, Yang J. Molecular cloning and characterization of rice 6-phosphogluconate dehydrogenase gene that is up-regulated by salt stress. *Mol Biol Rep.* 2003; 30:223–7. [PubMed: 14672408]
66. Neuteboom LW, Ng JM, Kuyper M, Clijdesdale OR, Hooykaas PJ, van der Zaal BJ. Isolation and characterization of cDNA clones corresponding with mRNAs that accumulate during auxin-induced lateral root formation. *Plant Mol Biol.* 1999; 39:273–87. [PubMed: 10080694]
67. Knox JP. The extracellular matrix in higher plants. 4. Developmentally regulated proteoglycans and glycoproteins of the plant cell surface. *FASEB J.* 1995; 9:1004–12. [PubMed: 7544308]
68. Liu JX, Srivastava R, Che P, Howell SH. Salt stress responses in Arabidopsis utilize a signal transduction pathway related to endoplasmic reticulum stress signaling. *Plant J.* 2007; 51:897–909. [PubMed: 17662035]
69. Zhao C, Johnson BJ, Kositsup B, Beers EP. Exploiting secondary growth in Arabidopsis. Construction of xylem and bark cDNA libraries and cloning of three xylem endopeptidases. *Plant Physiol.* 2000; 123:1185–96. [PubMed: 10889267]
70. Moura DS, Bergery DR, Ryan CA. Characterization and localization of a wound-inducible type I serine-carboxypeptidase from leaves of tomato plants (*Lycopersicon esculentum* Mill.). *Planta.* 2001; 212:222–30. [PubMed: 11216843]
71. Takahashi K, Athauda SB, Matsumoto K, Rajapakshe S, Kuribayashi M, Kojima M, et al. Nepenthesin, a unique member of a novel subfamily of aspartic proteinases: enzymatic and structural characteristics. *Curr Protein Pept Sci.* 2005; 6:513–25. [PubMed: 16381601]
72. Xia Y, Suzuki H, Borevitz J, Blount J, Guo Z, Patel K, et al. An extracellular aspartic protease functions in Arabidopsis disease resistance signaling. *EMBO J.* 2004; 23:980–8. [PubMed: 14765119]

73. Garrido C, Brunet M, Didelot C, Zermati Y, Schmitt E, Kroemer G. Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties. *Cell Cycle*. 2006; 5:2592–601. [PubMed: 17106261]
74. Calderwood SK, Mambula SS, Gray PJ Jr. Extracellular heat shock proteins in cell signaling and immunity. *Ann N Y Acad Sci*. 2007; 1113:28–39. [PubMed: 17978280]
75. Srivastava PK. Heat shock protein-based novel immunotherapies. *Drug News Perspect*. 2000; 13:517–22. [PubMed: 12879122]
76. Sixt SU, Dahlmann B. Extracellular, circulating proteasomes and ubiquitin - incidence and relevance. *Biochim Biophys Acta*. 2008; 1782:817–23. [PubMed: 18602990]
77. Shringarpure R, Grune T, Mehlhase J, Davies KJ. Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome. *J Biol Chem*. 2003; 278:311–8. [PubMed: 12401807]
78. Calderwood SK, Theriault J, Gray PJ, Gong J. Cell surface receptors for molecular chaperones. *Methods*. 2007; 43:199–206. [PubMed: 17920516]

Figure 1. Na⁺/K⁺ ratio determinations

Shoots of 10-day rice seedlings were treated with 200 mM NaCl during different time course. Na⁺ and K⁺ contents in the shoots were determined using an atomic absorption spectrophotometer. The ratio of Na⁺/K⁺ was calculated from Na⁺ and K⁺ content. Values are the mean ± SD (n=10).

Figure 2. A Preparative gel of apoplastic proteins obtained from shoot stems of rice seedlings
 An equal amount (75 μ g) of apoplast proteins from rice shoots treated for 0 h, 1 h, 6 h, 12 h were mixed and labeled with Cy3 for a preparative gel. The number of the 117 changed in abundance protein spots correspond to position numbers listed in Table 2.

Figure 3. Venn diagram analysis of abundance changed secretory protein spots under different time courses of salt stress

The number of up- or down-regulated protein spots for a specific salt-treated time points is shown in the different sections. A, up-regulated protein spots; B, down-regulated protein spots.

Figure 4. Hierarchical cluster analysis of salt-responsive apoplastic proteins associated with cell-wall related metabolism (A), oxido-reduction (B) and protein metabolism (C)
The cluster analysis was performed using software CLUSTER 2.11 (<http://rana.lbl.gov/EisenSoftware.htm>). Red represents upregulated protein spots; green represents downregulated protein spots; black represents protein spots that remain the same under different conditions. The log-transformed average ratios of the fold change are listed in Table 2.

Figure 5. Functional categories of abundance changed secretory proteins

The pie charts were generated according to information provided by gene ontology tools at <http://www.agbase.msstate.edu/> and UniProtKB (<http://www.uniprot.org/>).

Figure 6. A predicted NaCl stress-responsive apoplastic protein network in shoot stems of rice seedlings

All of the identified apoplastic proteins were categorized by function and the ratios of change in their abundance levels were subjected to cluster analysis. Based on these results, a putative model of a NaCl stress-responsive apoplastic proteins network in rice shoots was determined.

Table 1

Malate Dehydrogenase (MDH) Activity Assay

Protein samples	Protein content (mg/ml)	Enzyme activity (u/ml)	Relative enzyme activity (u/mg)	MDH activity ratio (apoplast/total, %)
Total protein	0.24±0.07	60.22±6.79	250.92±10.12	-
Salt-treated apoplast protein	0 h	0.24±0.07	4.73±0.05	19.71±2.51
	1 h	0.27±0.06	6.15±0.07	22.78±2.94
	6 h	0.22±0.08	4.08±0.07	18.55±1.57
	12 h	0.27±0.10	4.72±0.08	17.48±3.05

Three independent extraction was used for MDH analysis. One enzyme activity unit (u) was calculated by the decrease in absorbance at 340 nm/min.mg protein. Values are mean ± SD.

Table 2

Abundance Changed Proteins during the Salt Stress time course were Identified by LC-MS/MS

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e		1-ANOVA	Abundance graph/ graph/ graph/	
					theoretical	experimental			1 h/con	6 h/con			12 h/con
Carbohydrate metabolic process													
25	115453437	45/45.6	DTVGGY ESHM(Oxidation) AFTM(Oxidation)P GLYR	8.1e-10	5.9/92 908.9	5.9/92 908	Sucrose synthase 2	-	1.14	-1.05	-2.97		
21	115453437	4/5.8	TM(Oxidation)AST VPLAVE GEPSNK	3.2e-8	5.9/92 908.9	5.88/9 2283	Sucrose synthase 2	-	-1.46	-1.81	-2.84	0.047	
29	115453437	27/51	LTSLHPE IEELLYS EVDNNE HK	1.9e-6	5.9/92 908.9	6.02/8 9688	Sucrose synthase 2	-	1.14	-1.43	-2.63	8.8e-005	
22	115453437	10/12.7	IGDSLSA HPNELV AVFTR	1.5e-9	5.9/92 908.9	5.65/8 9957	Sucrose synthase 2	-	1.02	-1.65	-1.84	0.015	
30	115453437	40/45.8	LTSLHPE IEELLYS EVDNNE HKFM(Oxidation)L K	7.2e-10	5.9/92 908.9	6.09/8 9688	Sucrose synthase 2	-	1.19	-1.17	-1.93	0.0021	
31	115453437	28/33.2	AEEHLS GLSADT PYSEFH HR	4.1e-8	5.9/92 908.9	6.16/8 9688	Sucrose synthase 2	-	1.16	1.21	-1.37	0.006	

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Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA		Abundance graph/ graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con			
4	115453437	4/4.8	YVSNLE R	0.018	5.9/92 908.9	5.94/1 00529	Sucrose synthase 2	-	1.07	-1.65	-1.85		0.0061	
28	115453437	7/9.9	IGDSLSA HPNELY AVFTR	9.6e-10	5.9/92 908.9	5.87/8 9688	Sucrose synthase 2	-	1.15	-1.94	-3.12		0.024	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph/ ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
24	115453437	17/19.6	TM(Oxidation)AST VPLAVE GEPSNK	2.6e-9	5.9/92 908.9	5.79/8 9822	Sucrose synthase 2	-	1.23	-1.84	-2.63	0.0026	
23	115453437	28/28.2	TM(Oxidation)AST VPLAVE GEPSNK	1.3e-7	5.9/92 908.9	5.71/8 9957	Sucrose synthase 2	-	1.23	-1.27	-1.78	0.00028	
43	115460876	12/17.9	YTEDAV VSQDL DDTFQP PFK	1.4e-9	6.4/80 921.5	5.88/8 3954	α -L-Arabinofuranosidase/ β -D-xylosidase	S	-1.21	1.07	-2.85	0.037	
40	115460876	11/11.9	AIGEVV STEAR	5.0e-5	6.4/80 921.5	6.35/8 3702	α -L-Arabinofuranosidase/ β -D-xylosidase	S	1.38	1.55	-1.04	0.016	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
49	115460876	9/11.1	ATTNFI VLM(Oxidation)R	2.8e-6	6.4/80 921.5	5.66/8 0618	<i>α</i> -L-Arabinofuranosidase/β-D-xylosidase	S	-1.27	1.40	-2.40	0.021	
51	115460876	6/8.4	GQETPG EDPLLA SK	8.7e-5	6.4/80 921.5	6.17/8 0256	<i>α</i> -L-Arabinofuranosidase/β-D-xylosidase	S	-1.48	1.21	-1.70	0.036	
7	115434328	2/2.0	EPAPVVT GGR	1.9e-5	6.1/10 2968.2	6.08/9 8290	<i>α</i> -glucosidase	S	1.08	-1.76	-1.85	0.0046	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
61	115481730	5/9.6	FAQAQL DVYGR	7.2e-6	5.4/56 399.8	4.68/6 7833	Putative glucan 1,3-β-glucosidase	M	-1.43	1.02	1.21	0.031	
64	115481730	8/14.3	YTSTAY VVM(Oxidation)SN R	8.5e-9	5.4/56 399.8	4.74/6 5432	Putative glucan 1,3-β-glucosidase	M	-1.12	1.09	-1.44	0.023	
118	115481730	1/2.2	FISTGSL NAVR	2.1e-4	5.4/56 399.8	5.86/4 1328	Putative glucan 1,3-β-glucosidase	M	-1.19	2.83	1.01	0.023	
90	115474081	11/27.1	ETMEILS NKEVIQ VNQDPL GVQGR	6.9e-6	5.9/47 049.0	5.06/5 0920	Putative α-galactosida	S	1.09	1.18	1.81	0.044	
85	115474081	1/2.4	TTDDIQ DTWK	0.0040	5.9/47 049.0	5.22/5 4479	Putative α-galactosida	S	1.21	2.31	1.81	0.043	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
53	115463915	4/8.5	GIAAGP VTGYGR	2.2e-5	5.7/58 964.8	5.74/7 3342	Putative β-N-acetylhexosaminidase	S	-1.22	1.25	1.23	0.033	
59	115473645	4/8.2	LAVSTG EGVEDK ELLEK	7.8e-6	5.4/1792.8	5.25/7 0006	Putative xylulose kinase	-	1.11	1.12	-2.73	0.0085	
79	115489270	1/1.5	DLQM(Oxidation)T IQR	0.0042	5.9/59 539.1	5.52/5 8200	Heparanase-like protein	S	1.22	1.22	1.90	0.031	
137	115463555	3/9.6	IYNQNLI NHVGR	1.5e-5	5.9/34 706.5	5.86/3 2649	Putative β-1,3-glucanase	S	-1.08	1.05	1.52	0.011	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph/ graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
138	115463555	4/16.8	IYNQNLI NHVGR	4.6e-6	5.9/34 706.5	6.22/3 2066	Putative β-1,3-glucanase	S	-1.97	-1.96	3.79	0.051	
140	115463555	3/9.6	IYNQNLI NHVGR	2.6e-5	5.9/34 706.5	5.18/2 8737	Putative β-1,3-glucanase	S	1.27	1.90	4.09	0.0016	
113	115439545	4/15.4	NVNSAL VAAAGLG NIK	1.9e-4	6.8/35 809.8	6.22/4 3038	β-1,3-glucanase	S	-1.85	-1.22	-4.72	0.047	
117	115439545	3/12.7	VGASVN NAQTYN QGLINH VR	4.6e-7	6.8/35 809.8	4.78/42460	β-1,3-glucanase	S	-1.01	1.33	-1.29	0.016	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph/ graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
110	115439545	2/9.5	VGASVN NAQTYN QGLINH VR	6.7e-7	6.8/35 809.8	5.85/4 4284	β-1,3-glucanase	S	-1.63	1.60	-1.23	0.019	
107	115439545	5/20.7	VGASVN NAQTYN QGLINH VR	2.1e-8	6.8/35 809.8	6.22/4 4954	β-1,3-glucanase	S	-1.14	1.16	-1.95	0.019	
112	115439545	3/12.7	VGASVN NAQTYN QGLINH VR	3.6e-9	6.8/35 809.8	6.69/43755	β-1,3-glucanase	S	-1.49	1.03	-3.90	0.024	
*139	115461390	2/9.3	SGM(Oxidation)LN VSPGR	3.2e-6	5.5/28 193.2	5.4/31 540	Phosphomannomutase	-	-1.08	-1.18	-2.10	0.0066	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
124	115442155	1/3.3	LISHVQ GGTPK	1.5e-4	4.7/34 798.5	3.89/3 9330	β-1,3-glucanase precursor	S	-1.15	1.28	1.89	0.0080	
Protein processing and degradation													
39	115454067	8/13.7	DAGGA GM(Oxidation)VLS NTATNG EELVAD AHLPPA AGVGA K	6.9e-9	5.977 992.6	5.12/8 3954	Subtilisin-like protease	S	-1.51	-2.10	-4.04	0.029	
44	115454067	3/3.3	GYEAA M(Oxidation)GPM(Oxidation)DTTR	7.6e-6	5.977 992.6	5.48/8 2331	Subtilisin-like protease	S	-1.19	1.05	-2.77	0.01	
48	115454067	3/3.1	AGPTGI AADTR	3.7e-5	5.977 992.6	4.63/8 1226	Subtilisin-like protease	S	-1.06	-1.61	-3.47	0.040	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
57	115454067	2/1.6	AGPTGI AADTR	4.3e-5	5.977 992.6	6.270 428	Subtilisin-like protease	S	-2.09	-2.02	-2.04	0.0054	
50	115454067	1/1.4	AGPTGI AADTR	5.1e-5	5.977 992.6	4.258 0618	Subtilisin-like protease	S	1.28	-1.34	-1.92	0.010	
42	115465395	2/2.7	TSEFEV VR	3.1e-4	5.767 413.3	5.883 201	Putative phenylalanyl-tRNA synthetase beta chain	-	-1.13	-1.39	-5.87	0.00049	
58	115474653	4/11.3	GDSIVL M(Oxidation)/GK	3.0e-4	5.434 377.9	6.070 111	60S acidic ribosomal protein P0	-	-1.21	-1.06	-2.02	0.00072	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
121	115474653	3/8.8	GDSIVL M(Oxidation)GK	7.4e-5	5.4/34 377.9	5.58/4 0408	60S acidic ribosomal protein P0	-	1.75	-1.22	1.18	0.026	
126	115474653	3/8.8	GDSIVL M(Oxidation)GK	2.3e-4	5.4/34 377.9	5.31/3 8397	60S acidic ribosomal protein P0	-	1.35	-1.08	2.24	0.045	

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Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA		Abundance graph/ graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con			
82	115488928	3/6.4	IGYQKP SLIESR	9.8e-4	5.6/46 274.2	5.58/5 6225	Tryptophanyl-tRNA synthetase, putative	-	1.20	-1.03	-1.70	0.050		
96	115451209	1/2.1	VVFDDA NSR	9.7e-5	6.6/45 077.4	6.52/4 8096	Aspartyl protease nepenthesin precursor	S	3.32	2.71	1.87	0.0030		

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f	
					theoretical	experimental			1 h/con	6 h/con	12 h/con			
129	115473357	5/17.7	YVDIGIP ANNK	1.4e-6	5.0/32 972.5	4.98/3 5513	Putative 40S ribosomal protein	-	-1.52	-1.06	-2.76	0.021		
133	115444057	1/6.3	VADHAG VALAGL TADGR	5.4e-6	5.4/29 630.6	5.23/3 4462	Proteasome subunit α type-1	-	-1.18	-1.04	2.01	0.017		

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127	115488046	1/2.4	SVPGFD GALPSK	0.0013	7.0/55 710.2	4.79/3 7711	Serine carboxypeptidase 1	S	1.17	1.32	-1.66	0.033		

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
143	115454751	3/18.4	TDEKVM(Oxidation)VLDSHK	1.1e-5	5.4/23478.0	5.23/25180	β subunit of 20S proteasome	S	-1.46	-2.77	-2.48	0.017	
33	115449043	8/17.4	VAGGAGM(Oxidation)ILVNT AESGEE LVADSH LVPATM(Oxidation)VGQK	3.2e-10	6.0/80840.8	4.66/87034	Subtilisin proteinase	S	-1.57	-1.1	-1.28	0.051	

Protein folding and assembly

2	115434904	6/9.6	FIGTAGAASSTM(Oxidation)NPK	1.4e-5	5.2/93108.5	4.7/102203	Putative heat shock protein	-	-1.06	-1.38	-1.78	0.00820	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
47	115486793	30/45.3	NINPDEA VAYGAA VQAAIL SGEGNE K	1.7e-7	5.1/71 185.3	4.56/8 1348	Heat shock cognate 70 kDa protein, putative	-	1.17	-1.34	-3.07	0.021	
147	115443875	1/5.2	VVEGM(Oxidation)DVVK	0.010	8.6/18 361.2	6.4/22 229	Peptidyl-prolyl cis-transisomerase	-	-1.87	-1.26	-2.60	0.019	

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Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
54	115477126	4/5.9	SDLVNN LGTIAR	6.2e-5	5.079 617.4	4.872 357	Heat shock protein 81-1	S	-1.03	-1.32	-3.49	0.041	
52	115477126	2/3.9	KPEEITK BEYAAF YK	3.7e-5	5.079 617.4	4.777 6376	Heat shock protein 81-1	S	1.22	-1.23	-1.53	0.048	

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Oxido-reductases													
73	115465974	6/15.8	GLLYLG M(Oxidation)GVSG GEEGAR	7.6e-7	5.9/52 721.7	5.78/6 2175	6-phosphogluconate dehydrogenase	S	1.23	-1.03	1.38	0.0093	
*69	115468462	7/12.1	AAM(Oxidation)DP TDYFR	5.6e-7	6.6/62 125.0	5.9/64 360	FAD linked oxidase	M	-1.25	1.02	-3.12	0.00051	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
* ₆₆	115468462	6/10.5	AAM(Oxidation)DP TDYFR	1.0e-6	6.6/62 125.0	5.48/6 4845	FAD linked oxidase	M	1.09	-1.27	-1.14	0.018	
* ₇₈	115468462	2/3.5	NVDQG AADVVA R	4.0e-4	6.6/62 125.0	6.27/5 8904	FAD linked oxidase	M	-1.81	-1.19	-3.85	0.0093	
* ₆₇	115468462	6/10.2	LVNANG ELVDR	4.6e-4	6.6/62 125.0	5.98/6 4360	FAD linked oxidase	M	-1.14	1.54	-1.44	0.0056	
* ₆₀	115468462	7/11.9	AAM(Oxidation)DP TDYFR	1.9e-6	6.6/62 125.0	6.06/6 8139	FAD linked oxidase	M	-1.38	1.46	-1.90	0.013	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
86	115436290	1/2.8	DAGVAA GLIR	0.0029	6.2/37 731.0	6.75/5 3587	Class III peroxidase 12	S	-1.10	1.32	-2.03	8.4e-005	
95	115445243	1/4.0	M(Oxidation)GNIG QPSDGE VR	1.9e-5	5.3/34 175.7	4.69/4 8168	Peroxidase 6	S	2.07	1.42	4.81	0.015	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
92	115468442	4/13.2	AGM(Oxidation)AQAVAAEP R	2.4e-5	6.8/33 181.0	6.75/4 8531	Peroxidase 52 precursor	S	1.09	1.59	-2.87	0.0060	
98	115474059	3/11.4	M(Oxidation)GNISP LTGTQG QIR	2.3e-6	5.8/32 890.1	4.51/4 7379	Peroxidase	S	-1.22	1.53	NDT	0.025	
100	115474059	3/8.8	GFSVID NAK	3.7e-4	5.8/32 890.1	4.63/4 7308	Peroxidase	S	-1.36	-1.02	1.92	0.0060	
105	115474059	3/11.4	M(Oxidation)GNISP LTGTQG QIR	3.5e-6	5.8/32 890.1	5.36/4 5497	Peroxidase	S	1.24	6.99	5.35	0.054	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph/ graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
81	115480874	5/16.1	SAPEAT GTTAPIV DR	1.7e-6	4.7/35 700.3	4.81/5 6563	Peroxidase 54 precursor	S	1.53	-1.61	2.06	0.027	
72	115480874	2/5.7	GFPVVD DVK	4.5e-4	4.7/35 700.3	3.73/6 1618	Peroxidase 54 precursor	S	-2.56	1.73	-3.56	0.017	
80	115480874	3/8.6	GFPVVD DVK	3.2e-4	4.7/35 700.3	5.11/5 7765	Peroxidase 54 precursor	S	-1.90	-1.32	-2.09	0.015	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
115	115487710	4/10.4	DAIAM(Oxidation)TGGPSF DVPTGR	5.0e-7	4.7/34 578.4	3.73/4 2333	Peroxidase 43	S	1.26	1.39	-1.72	0.0022	
55	115449517	6/13.7	VVTDKG EEIIADV VLFATG R	1.8e-6	6.2/53 507.5	6.06/7 0534	Glutathione reductase	-	-1.58	-1.03	-1.74	0.0070	
Lipid metabolism													
27	115446991	1/1.6	TLSGNPP AIIAK	3.6e-5	5.7/81 085.1	4.85/8 9957	Glycerophosphoryl diester phosphodies terase, putative	S	-1.19	-1.15	-2.18	0.031	
8	115446991	1/1.1	FANAVS VR	0.0022	5.7/81 085.1	4.72/9 7996	Glycerophosphoryl diester phosphodies terase, putative	S	-1.04	1.32	-1.19	0.020	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
*114	115452473	2/8.8	IPVISNV DASPHS DPDTIK K	5.4e-5	7.5/40 456.8	4.33/4 2716	Hypothetical protein	M	1.13	1.67	1.45	0.036	
99	115480429	3/8.2	SVAVDA AGGGR	0.0011	5.0/39 472.2	4.36/4 7379	Putative Anter-specific proline-rich protein APG	S	1.33	-1.02	-1.35	0.014	
131	115434516	2/11.9	ESGSTM(Oxidation)DVVAA QTK	3.9e-5	5.4/27 063.2	4.87/3 5194	Triosephosphate isomerase	-	-2.60	1.48	1.14	0.0038	
Cell wall biogenesis and degradation													

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
134	115475445	1/5.9	VVSDDG KTQQVA LTLDL	2.4e-5	5.3/32 096.9	4.69/3 3543	Xyloglucan endotransglycosylase	S	-1.07	-1.66	1.03	0.0050	
132	115475445	1/5.9	VVSDDG KTQQVA LTLDL	2.7e-6	5.3/32 096.9	4.79/3 4774	Xyloglucan endotransglycosylase	S	-1.01	2.56	1.86	0.0012	
128	115475445	4/14.1	VVSDDG KTQQVA LTLDL	8.0e-10	5.3/32 096.9	4.74/3 5513	Xyloglucan endotransglycosylase	S	-1.37	1.29	-1.31	0.013	
142	115483206	1/6.9	ELAAFF GQTSHE TTGGTR	2.9e-9	6.1/27 551.9	4.87/2 8054	Chitinase	S	-1.31	1.90	1.49	0.021	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
135	125551525	2/7.7	VLVGVV ASPEAD R	2.8e-4	5.9/32 535.2	5.45/3 3242	Putative chitinase	S	-1.02	-1.38	-1.82	0.0011	
136	125551525	3/11.1	LGVV(Oxidation)L TATAR	5.3e-5	5.9/32 535.2	5.76/3 2895	Putative chitinase	S	1.60	1.11	2.31	0.045	
103	108709682	3/10.2	YVDV M(Oxidation)TVPK	6.5e-6	6.1/30 227.1	5.13/4 6116	α-1,4-glucan-protein synthase, putative	-	-1.22	1.07	-2.04	0.020	
120	115485779	1/3.1	STGTGF QTR	0.0060	5.7/33 845.0	6.04/4 8835	Putative endoxyloglucan transferase	S	-1.51	-1.40	-1.63	0.038	
Energy metabolism													

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA		Abundance graph/ graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con			
3	115445275	2/2.4	LADLEA APAAVA R	0.0010	5.8/10 9933.6	5.58/1 01591	Phosphoenolpyruvate carboxylase	-	1.04	-2.04	-1.55	0.032		
93	115459078	7/19.9	DAPM(Oxidation)F VVGVNE K	2.4e-5	6.3/36 773.3	6.39/4 9118	Glyceraldehyde-3-phosp hate dehydrogenase		-1.48	-2.04	-2.38	0.041		
75	115469166	2/4.2	FSDPOP DYSAFR	1.9e-5	6.6/55 156.0	6.19/6 0882	Acid phosphatase	S	-1.31	-1.22	-2.25	0.0035		
108	108864048	4/10.4	ATPEQV SDYTLK	4.8e-5	6.1/41 606.8	4.94/4 4751	Fructose-bisphosphate aldolase,	-	-1.29	NDT	1.45	0.034		
Nucleotide metabolism														

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
1	56784348	12/7.2	TGNASE AGVIQL YR	2.2e-7	5.24/1 54389.7	4.65/1 09841	Putative formylglycineamide ribotide amidotransferase	C	NDC	NDC	NDC	0.012	
146	115448841	4/23.8	VLVVDG GGSLR	5.3e-4	5.6/18 049.9	5.12/2 2736	S-adenosyl methionine: 2-demethyl menaquinone methyltransferase-like	-	-1.89	1.46	NDT	0.0014	
104	115439033	2/6.8	LTGM(Oxidation)PT ETFEQIS R	2.1e-6	5.1/37 165.0	4.15/4 6185	Ribokinase- like	C	1.19	1.07	-1.52	0.0072	
Amino acid metabolism													
41	115489652	7/13.8	YGAGIG PGVYDI HSPR	3.9e-8	5.9/84 585.4	5.72/8 3326	5-methyltetrahydropteroylirriglutamate-homocysteine methyltransferase-like		1.07	1.07	-2.96	0.027	
46	115489652	7/13.8	YNEVKP ALTNM(Oxidation)VSAAK	8.5e-6	5.9/84 585.4	6.11/8 1838	5-methyltetrahydropteroylirriglutamate-homocysteine methyltransferase-like		1.07	1.07	-2.48	0.037	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
35	115489654	18/29.5	YGAGIG PGVYDI HSPR	8.9e-9	5.9/84	5.96/8	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, putative		1	6	12	0.0032	
					666.5	6643							
32	108862992	5/7.2	YGAGIG PGVYDI HSPR	2.8e-7	5.9/84	5.42/8	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, putative		1	6	12	0.0078	
					636.4	7954							
83	100801530	2/6.4	FVIGGP HGDAAGL TGR	1.0e-7	5.7/42	5.92/5	S-adenosyl methionine synthetase	-	-1.47	1.09	-1.61	0.029	
					535.6	6309							

Cytoskeleton

62	1076737	2/4.3	FPGQLN SDLR	3.2e-5	4.8/50	4.09/6	β-tubulin R1623	-	-1.19	-1.16	-1.84	0.039	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
63	1076738	7/15.5	AVLM(Oxidation) DLEPGT M(Oxidation)DSVR	1.8e-7	4.7/49 864.5	4.25/6 6622	β-tubulin R2242	-	1.36	1.12	-2.48	0.0067	
Other mechanisms													
125	115452831	1/3.5	M(Oxidation)AQSV PGIK	3.9e-5	5.4/28 562.7	5.24/3 8628	Hydroxyacylglutathione hydrolase, putative	-	1.16	1.34	-2.0	0.046	
*130	115472485	1/3.7	ALGRPN AIDGTIK	0.0027	5.4/37 225.2	5.4/35 619	Thiamine biosynthetic enzyme	C	1.93	2.94	3.35	0.020	
9	115450773	10/15.7	LVVDEA TNDDNS VIGM(Oxidation)H PDTM(Oxidation)E K	7.2e-7	5.1/28 9744.9	4.6/97 409	Cell division cycle protein 48, putative	-	-1.17	1.08	-1.46	0.034	
17	115450773	6/7.5	GSSVGD AGGAA DR	2.4e-4	5.1/89 744.9	4.39/9 5239	Cell division cycle protein 48, putative	-	-1.09	-1.37	-1.65	0.037	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph/ graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
6	110289141	5/6.6	GILLYGP PGSGK	1.0e-4	5.1/89 811.9	4.5/97 849	Cell division cycle protein 48, putative	-	1.01	2.26	-1.08	0.0065	
20	110289141	7/10	ELQETV QYPVEH PEKFEK	2.7e-5	5.1/89 811.9	4.86/9 4527	Cell division cycle protein 48, putative	-	1.19	-1.09	-1.52	0.05	
16	110289141	7/9.2	GSSVGD AGGAA DR	1.3e-5	5.1/89 811.9	4.79/9 4811	Cell division cycle protein 48, putative	-	1.24	-1.10	-1.52	0.023	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph/ graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
71	115438572	3/11.4	M(Met-Ioss+Acetyl)AAAAAA VAAVAA AAAAAE PTVSK	9.7e-8	5.0/46 498.4	4.25/6 3783	10-deacetyl baccatin III-10-O-acetyl transferase-like	S	-1.11	-2.18	1.29	0.0029	
76	115451343	1/3.0	ALLPDK STVLDR	8.9e-4	5.0/45 327.2	4.37/6 0974	COP9	-	-1.51	1.06	-2.21	0.0054	
144	115461070	1/5.8	LLAATA EYAAAGD IAR	1.5e-6	5.0/27 306.3	4.59/2 4252	Os04g0659 300 (dof 26 domain)	S	-1.90	3.43	-2.79	0.019	
91	115461070	1/5.8	LLAATA EYAAAGD IAR	1.4e-5	5.0/27 306.3	3.75/4 9118	Os04g0659 300 (dof 26 domain)	S	-1.05	1.57	-1.65	0.013	

Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA		Abundance graph ^f
					theoretical	experimental			1 h/con	6 h/con	12 h/con			
122	108862565	1/4.3	LVFVTN NSTK	0.0027	9.4/26 313.3	4.62/3 9925	P-nitrophenylphosphatase, putative	C	-2.03	2.30	1.70	0.020		
Unknown function														
14	125602446	19/34.6	INPLVPV DLVIDH SVQVDV AR	5.0e-10	5.6/94 602.6	5.6/94 602	Hypothetical protein OsJ_025254	-	1.23	1.27	1.08	0.029		
38	115466224	10/23.6	AM(Oxidation)PNI LM(Oxidation)LRP ADGNET AGAYK	1.5e-8	5.4/73 449.8	5.07/8 4841	Hypothetical protein	-	-1.16	-1.28	-2.38	0.018		
150	115484453	1/5.7	EM(Oxidation)SIVG GSGK	8.8e-4	6.7/18 105.7	6.32/1 9566	Dirigent-like protein	S	NDC	NDC	NDC	0.028		
77	115485339	3/6.3	IGDDM(Oxidation)ELFAK	1.1e-6	5.8/50 322.3	5.26/6 0427	S-type apyrase	S	-1.46	-1.24	-3.44	0.016		

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Spot number ^a	Accession number	UP/C ^b	Peptide sequence (MS/MS)	Best expect value	pI/MW (Da) ^c		Protein homologue	Location ^d	Average ratio ^e			1-ANOVA	Abundance graph/
					theoretical	experimental			1 h/con	6 h/con	12 h/con		
56	115462933	2/2.1	M(Oxidation)VPESPNFK	0.0023	5.4/56 810.0	4.85/7 0639	Hypothetical protein	S	-1.24	1.57	2.16	0.020	
101	115487692	2/6.4	SPLTTVY AAAR	4.1e-5	6.1/34 716.7	5.74/4 6463	Hypothetical protein	-	-1.25	1.10	-3.76	0.00014	
106	115487692	4/12.8	SPLTTVY AAAR	5.6e-6	6.1/34 716.7	5.54/4 5840	Hypothetical protein	-	-1.89	-1.27	-5.85	0.0092	

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^aThe spot number corresponds to the position number in the BVA module of the DeCyder software; apoplastic proteins are shaded in grey,

^{*} non-classical secretory proteins,

^bThe number of unique peptides/the percentage of sequence coverage.

^cTheoretical pI/MW.

^dLocalization was predicted by TargetP (<http://www.cbs.dtu.dk/services/TargetP/>). S: contained a signal peptide in the secretory pathway, M: contained a mitochondrial targeting peptide, C: contained a chloroplast transit peptide, -: any other location.

^eAverage ratio is expressed as a ratio of the standardized protein abundance ratio between the control and salt-treated spots. Positive and negative values indicates increases and decreases in abundance, respectively. For some spots, the average ratio cannot be calculated due to the absence of the spot in either the treated or control samples; NDC: not detected in control, NDT: not detected in treatment.

^fThe abundance graph was generated from the BVA module of the DeCyder software; it represents the relative protein abundance levels at 0, 1, 6, and 12 h of salt treatment.