REGULAR ARTICLE

# Proteomic analysis of salt stress-responsive proteins in rice root

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Salt stress is one of the major abiotic stresses in agriculture worldwide. We report here a systematic proteomic approach to investigate the salt stress-responsive proteins in rice (*Oryza sativa* L. cv. Nipponbare). Three-week-old seedlings were treated with 150 mM NaCl for 24, 48 and 72 h. Total proteins of roots were extracted and separated by two-dimensional gel electrophoresis. More than 1100 protein spots were reproducibly detected, including 34 that were up-regulated and 20 down-regulated. Mass spectrometry analysis and database searching helped us to identify 12 spots representing 10 different proteins. Three spots were identified as the same protein, enolase. While four of them were previously confirmed as salt stress-responsive proteins, six are novel ones, *i.e.* UDP-glucose pyrophosphorylase, cytochrome *c* oxidase subunit 6b-1, glutamine synthetase root isozyme, putative nascent polypeptide associated complex alpha chain, putative splicing factor-like protein and putative actin-binding protein. These proteins are involved in regulation of carbohydrate, nitrogen and energy metabolism, reactive oxygen species scavenging, mRNA and protein processing, and cytoskeleton stability. This study gives new insights into salt stress response in rice roots and demonstrates the power of the proteomic approach in plant biology studies.

**Keywords:**
Mass spectrometry / *Oryza sativa* / Salt stress / Two-dimensional gel electrophoresis

## 1 Introduction

Salt stress is a major abiotic stress in agriculture worldwide. It is estimated that about 20% of the earth's land mass and nearly half of all irrigated land are affected by salinity. Increased salinization of arable land is expected to have devastating global effects, with predictions of 30% land loss within the next 25 years, and up to 50% by the year 2050 [1]. This has led to research of salt stress with the aim of improving crop salt tolerance by genetic engineering. Identifying novel genes, determining their expression patterns in response to salt stress, and understanding their functions in stress adaptation will provide us with the basis for effective engineering strategies to improve crop stress tolerance [2].

In general, a high concentration of salt causes ion imbalance, hyperosmotic stress and oxidative damage [3]. Plants can perceive the stress signals and transmit them to the cellular machinery to activate adaptive responses. The adaptation is completed in part by regulating gene expression. One way to study the cellular response on a large scale is to examine gene expression at the mRNA level using techniques such as cDNA microarrays, serial analysis of gene expression, cDNA-amplified fragment-length polymorphism and massively parallel signature sequencing [4]. These techniques allowed identification of many salt stress-responsive genes in plants. For example, using full-length cDNA microarray, 194 transcripts in *Arabidopsis* were found to be increased more than five-fold after high salinity treatment [5]. In another study of gene expression profiles during

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**Abbreviations: ABP**, putative actin-binding protein; **COX**, cytochrome *c* oxidase; **GS**, glutamine synthetase; **NAC**, nascent polypeptide associated complex; **ROS**, reactive oxygen species; **SAMS**, *S*-adenosylmethionine synthetase; **UGPase**, UDP-glucose pyrophosphorylase

 

the initial phase of salt stress in rice, approximately 10% of the transcripts in salt-tolerant rice were found to be significantly up-regulated or down-regulated by salt stress [6]. However, the techniques mentioned above do not offer insights into the quantity and quality of the final gene products, *i.e.* the proteins. The amount of proteins is not always correlated to that of mRNA, especially for proteins of low abundance. Moreover, many proteins undergo post-translational modifications (PTMs) such as removal of signal peptides, phosphorylation and glycosylation, which are extremely important for protein activities and subcellular localization. Therefore, it is necessary to study the salt stress response at the protein level. Proteomics is thus evolving and playing an increasingly important role in addressing these issues. In fact, it has become a necessary and complementary approach in the postgenomic era [7, 8]. The proteomes of various plants in response to different environmental cues have been studied, including drought, salinity, heavy metal, heat, anoxia and elicitor [9–18]. Nevertheless, proteomic plant analysis is still in its infancy compared to that of prokaryotes, yeast and humans [19].

Rice is an important crop worldwide. It is also considered to be a model plant for monocots because of its relatively small genome size. Investigation of salt stress-responsive proteins with the aim of gaining better knowledge about salt stress tolerance in rice has both fundamental and economic importance. The current status of the proteomic study of rice was reviewed by Rakwai and Agrawal [19]. Previously, Ramani and Apte [11] detected 35 induced and 17 repressed polypeptides by salt stress in rice seedlings using *in vivo* radiolabeling followed by two-dimensional gel electrophoresis (2-DE) and autoradiography. However, these polypeptides were not further identified. The development of mass spectrometry (MS) and IPG strips has made proteomic analysis more sensitive, reliable and powerful. Recently, three salt stress-responsive proteins, ASR1-like protein, ascorbate peroxidase and caffeoyl-CoA *O*-methyltransferase were identified in rice by 2-DE and MS analysis [20].

We report here a systematic proteomic analysis of root proteins in rice grown under high salinity conditions. 2-DE analysis revealed 54 differentially accumulated protein spots. Twelve spots representing 10 different proteins, including six novel salt stress-responsive proteins in rice were identified by MS analysis.

## 2 Materials and methods

### 2.1 Plant materials

Rice seeds (*Oryza sativa* L. cv. Nipponbare) were allowed to germinate in the dark for 24 h at 28°C before being transplanted into nutrient solution [21]. The seedlings were grown at 28°C/25°C (day/night) with a 12 h photoperiod under an irradiance of 350~400 μmol/$m^2$/$s^1$ and a relative humidity of 60~80% in the phytotron (Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences). Three-week-old seedlings were treated with 150 mM NaCl for 24, 48 and 72 h. Roots were harvested, frozen in liquid nitrogen and kept at −75°C.

### 2.2 Protein extraction and 2-DE analysis

Roots were ground in liquid nitrogen and suspended in ice-cold 10% w/v TCA in acetone containing 0.07% w/v DTT, incubated at −20°C for 1 h and centrifuged for 15 min at 35 000 × *g*. The pellets were resuspended in 0.07% w/v DTT in acetone, incubated at −20°C for 1 h and centrifuged for 15 min at 12 000 × *g*. This step was repeated three times and the pellets were lyophilized. The resulting powder was solubilized in lysis buffer (9 M urea, 35 mM Tris, 4% w/v CHAPS, 1% v/v pH 4–7 IPG buffer, 1% w/v DTT) followed by centrifugation for 15 min at 12 000 × *g*. The proteins in the supernatant were precipitated by adding four volumes of ice-cold acetone, incubated at −20°C for at least 2 h and centrifuged for 15 min at 12 000 × *g*. The pellets were dissolved in rehydration buffer (8 M urea, 20 mM DTT, 2% w/v CHAPS, 0.5% v/v pH 4–7 IPG buffer). Protein concentration was determined using the Bradford assay (Sangon, Shanghai, China) using bovine serum albumin as standard.

For 2-DE, 60 μg and 300 μg of proteins were loaded onto analytical and preparative gels, respectively. For IEF, the Ettan IPGphor system (Amersham Biosciences, Uppsala, Sweden) and pH 4–7 IPG strips (13 cm, linear) were used according to the manufacturer's recommendations. The IPG strips were rehydrated for 12 h in 250 μL rehydration buffer containing protein samples. Focusing was performed in three steps: 500 V for 1 h, 1000 V for 1 h and 8000 V for 10 h. The gel strips were equilibrated for 20 min in 10 mL equilibration buffer (50 mM Tris-HCl buffer, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 1% w/v DTT and 0.002% w/v bromophenol blue). SDS-PAGE was performed with 12% gels using the PROTEAN II xi Cell system (Bio-Rad, Hercules, CA, USA). The gels were run at 15 mA *per* gel for the first 30 min and followed by 30 mA *per* gel. The protein spots in analytical gels were visualized by silver staining [22]. Preparative gels were stained with colloidal Coomassie Brilliant blue G-250 [23]. At least three replicates were performed for each sample.

### 2.3 Image and data analysis

The gels were scanned using ScanMaker 4 (Microtek, Carson, CA, USA) at a resolution of 1000 dots *per* inch. Data were analyzed using Melanie 4.0 software (GeneBio, Geneva, Switzerland). The abundance of each protein spot was estimated by the percentage volume (%Vol). Only those with significant and reproducible changes were considered to be differentially accumulated proteins.

 

### 2.4 In-gel digestion and MALDI-TOF MS analysis

Protein spots were excised from the preparative gels, washed three times with ultrapure water, destained twice with 50 mM $NH_4HCO_3$ in 50% acetonitrile, reduced with 10 mM DTT in 50 mM $NH_4HCO_3$, alkylated with 40 mM iodoacetamide in 50 mM $NH_4HCO_3$, dried twice with 100% acetonitrile and digested overnight at 37°C with sequencing grade modified trypsin (Promega, Madison, WI, USA) in 50 mM $NH_4HCO_3$. The peptides were extracted twice with 0.1% TFA in 50% acetonitrile. Extracts were pooled and lyophilized. The resulting lyophilized tryptic peptides were dissolved in 5 mg/mL CHCA containing 0.1% TFA and 50% acetonitrile. MS analysis was conducted with a MALDI-TOF/TOF mass spectrometer 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). Data were analyzed using GPS Explorer software (Applied Biosystem) and MASCOT software (Matrix Science, London, UK). NCBInr and rice was selected as the database and taxonomy, respectively.

## 3 Results and discussion

### 3.1 2-DE analysis of root proteins in salt stressed rice

To counteract salt stress, plants can change their gene expression and protein accumulation. The root is the first organ of plants to sense salt stress. Some salt stress-responsive genes were found to be mainly, or more strongly, induced in roots than in other organs. Rice is not only an important crop but also a model plant. The International Rice Genome Sequencing Project with the Nipponbare cultivar is nearly completed. The resulting substantial sequence information greatly facilitates the proteomic studies especially in protein identification by MS. Therefore, we investigated the salt stress-responsive proteins in the roots of Nipponbare rice.

Three-week-old rice seedlings were treated with 150 mM NaCl for different time periods. The seedlings had obvious stress symptoms but could survive three day treatment. All the seedlings grew well after they were shifted to normal nutrient solution (data not shown). Total proteins in roots were extracted and separated by 2-DE using pH 4–7 IPG strips in IEF. More than 1100 protein spots were reproducibly detected on gels by Melanie 4.0 software. The representative 2-DE maps are shown in Fig. 1, in which two framed regions are enlarged in Fig. 2. The proteins were separated very well in both dimensions. The gel maps were of high quality and can be used as reference 2-DE maps for rice root proteins.

Comparative proteomic analysis was used to investigate the protein profiles under salt stress. In order to distinguish stress responses from developmental changes in protein accumulation, both control and treated roots were harvested at each time point of treatment. Total proteins extracted from control and treated samples were separated at the same time to minimize experimental error. There were not many changes in protein accumulation profiles between control and treated samples until 24 h after initiation of salt stress treatment (data not shown). The changes persisted and maximized at the 72 h time point. Fifty-four protein spots showed significant and reproducible changes in abundance (Fig. 1). Thirty-four of them were up-regulated (U1–U34) (Fig. 1B), 20 were down-regulated (D1–D20) (Fig. 1A). Two protein spots (U5, U29) showed qualitative changes between control and treated samples (Figs. 1, 2). They were visible only in the treated samples, suggesting that they were newly synthesized after salt stress treatment or in very low abundance without stress. The other differentially accumulated proteins showed quantitative changes in a time-dependent manner with the most significant changes at 72 h after initiation of salt stress treatment (Fig. 2). The abundance ratios, *i.e.* the percentage volumes in treated samples/the percentage volumes in control samples, at 72 h time point are shown in Fig. 3. Spots U7, U9, U10, U11, U12, U14, U25, U26, U30, U33 and U34 were increased more than five-fold in abundance (Figs. 3A, 3B), while spots D5, D10 and D12 were decreased less than 20% in abundance (Fig. 3C). Spots D2, D3, D4, D8 and D9 decreased drastically and almost disappeared after 72 h of salt stress treatment (Fig. 3C).

### 3.2 Salt stress-responsive proteins identified by MS

Among 54 differentially accumulated proteins, 28 with relatively high abundance were analyzed by MALDI-TOF/TOF MS. Sixteen of them had no MS/MS data. Although they could be identified by PMF data, their theoretical $M_r$s and *pI*s did not fit well with the experimental ones. Their identities need to be further confirmed. Twelve protein spots representing 10 different proteins were identified with high probability (Table 1). The MS analysis result of spot D13 is shown in Fig. 4 as an example. Spots D3, D4 and D5 were identified as the same protein, enolase (Table 1). They located at different positions on the gels, with similar but different $M_r$ and p*I* (Figs. 1A, 2B), indicating that they might be isoforms of enolase or have different PTMs. Six proteins were enzymes involved in basic metabolism, including triosephosphate isomerase (U32), enolase (D3, D4 and D5), UDP-glucose pyrophosphorylase (UGPase, D6), cytochrome *c* oxidase subunit 6b-1 (COX6b-1, U25), glutamine synthetase (GS) root isozyme (D13) and *S*-adenosylmethionine synthetase 2 (SAMS2, D14). The other four proteins were peroxidase (U18), putative nascent polypeptide associated complex alpha chain (α-NAC, D16), putative splicing factor-like protein (U26) and putative actin-binding protein (ABP, U29).

Triosephosphate isomerase (U32) and enolase (D3, D4 and D5) are enzymes involved in glycolysis. It was shown that triosephosphate isomerase was induced by drought in rice and maize based on 2-DE evidence [9, 24]. Our results show that it was also induced by salt stress in rice (Fig. 1B). Enolase is responsive to many environmental stresses, including salt stress, drought, cold and anaerobic stress in

 

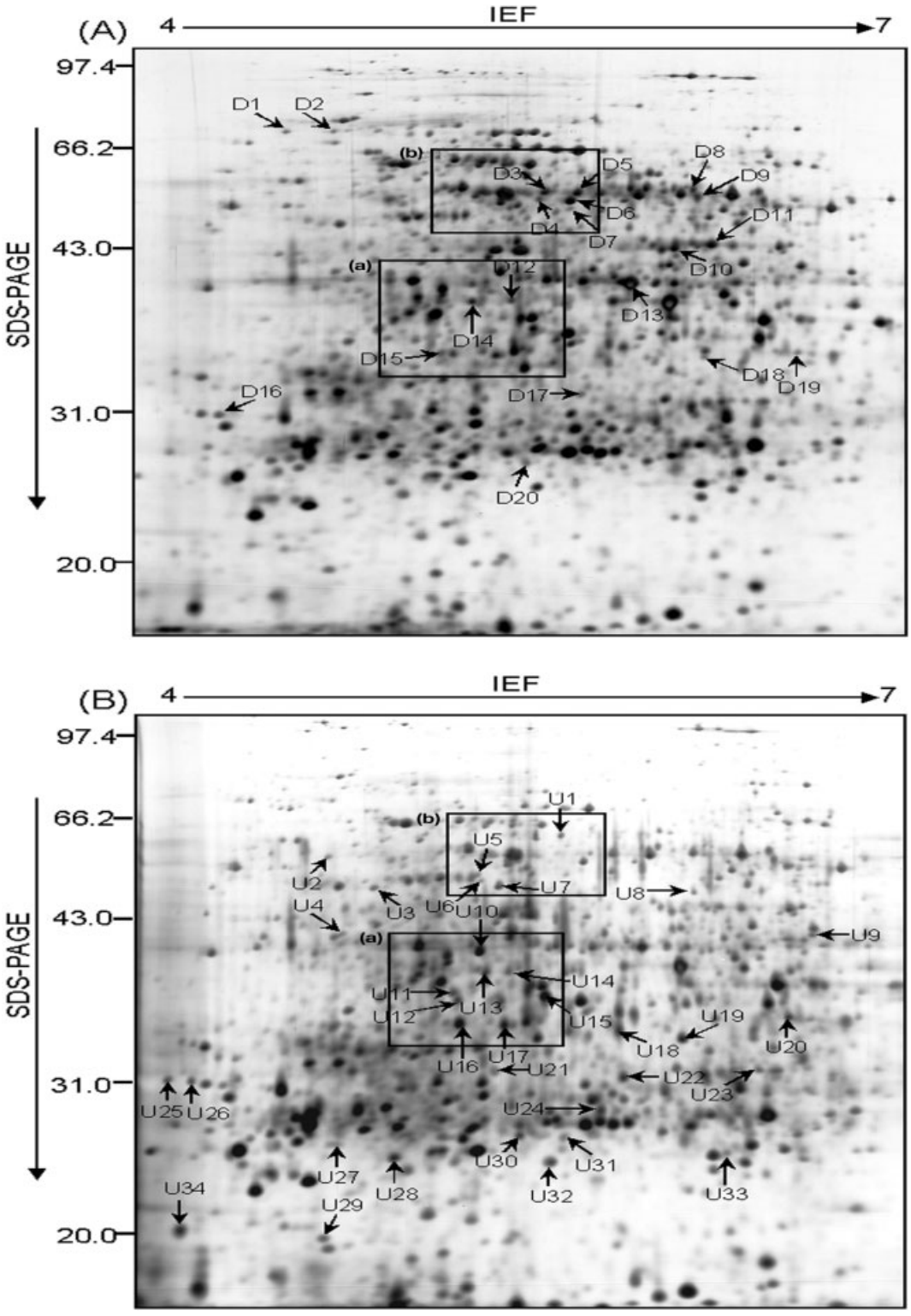


**Figure 1.** Representative 2-DE maps of rice root proteins. Three-week-old rice seedlings were treated with 150 mM NaCl for 72 h. Total root proteins were extracted and separated by 2-DE. In IEF, 60 μg of proteins were loaded onto pH 4–7 IPG strips (13 cm, linear). SDS-PAGE was performed with 12% gels. The spots were visualized by silver staining. Differentially accumulated protein spots are indicated by arrows. Twenty down-regulated spots (D1–D20) are indicated in the map of control sample (A) and 34 up-regulated spots (U1–U34) are indicated on the map of NaCl-treated sample (B). The framed regions are enlarged in Fig. 2.

 

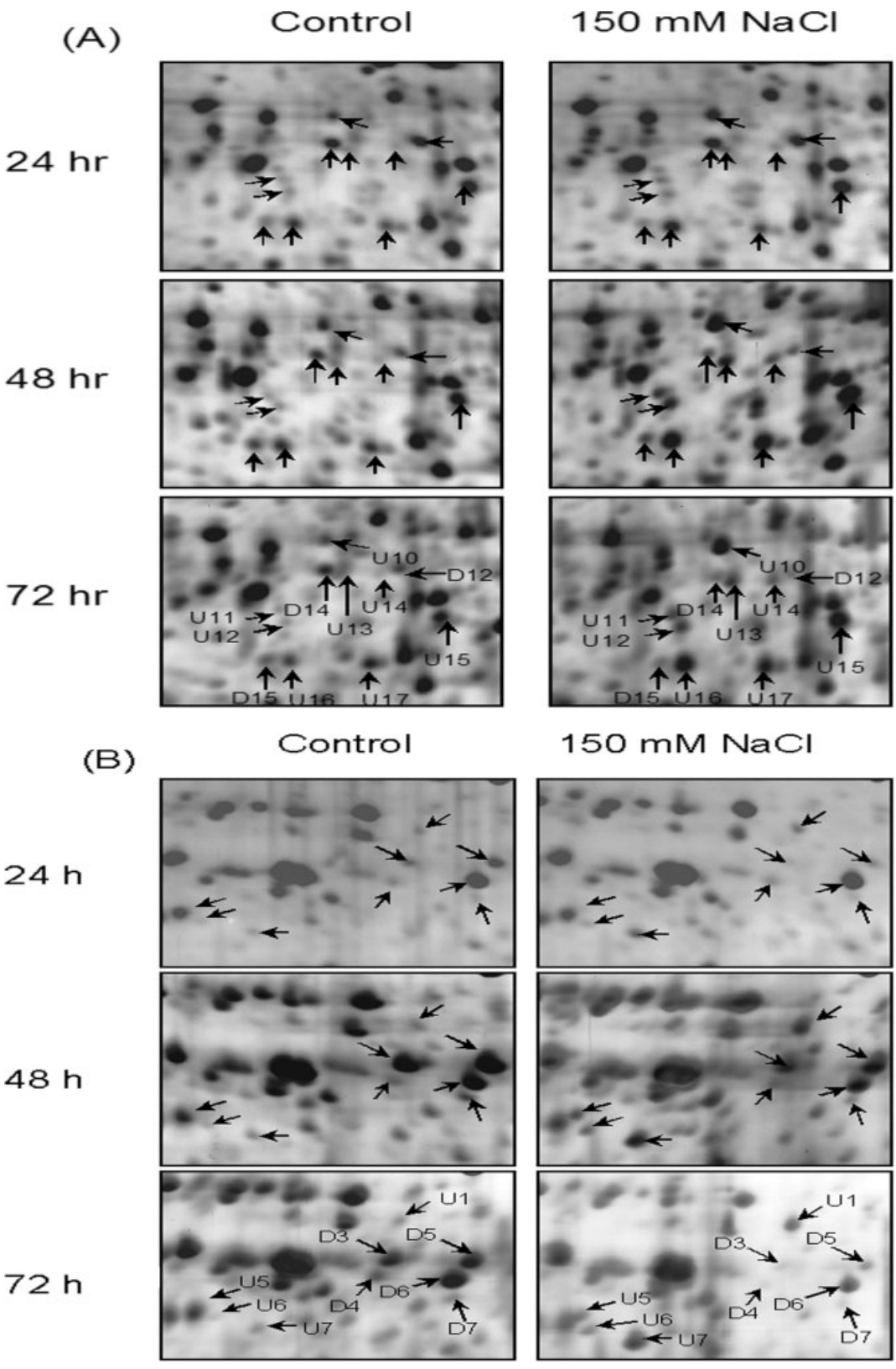


**Figure 2.** Time-dependent changes of the differentially accumulated proteins. Proteins in roots were extracted from both control and NaCl-treated samples after 24, 48 and 72 h treatment and separated by 2-DE. A and B correspond to the framed regions (a) and (b) in Fig. 1.

 

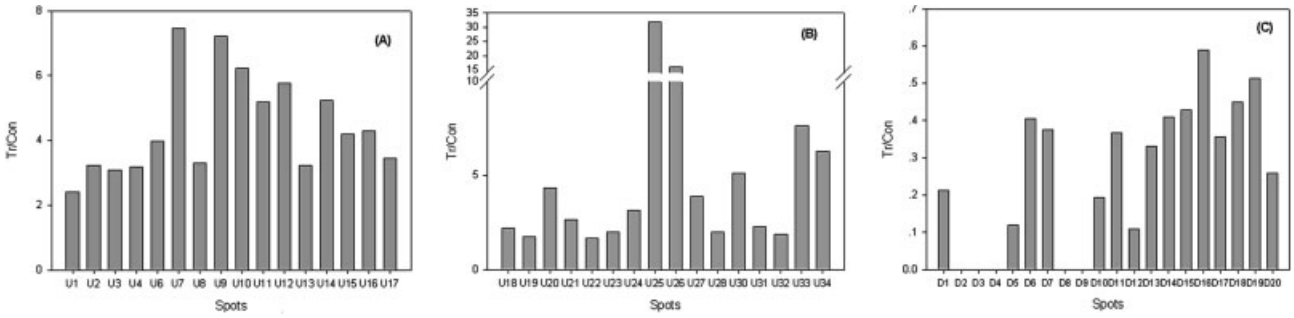


**Figure 3.** Abundance ratio of the differentially accumulated proteins after 72 h of salt stress treatment. The %Vol of each spot was considered as the abundance of each spot. The abundance ratio of each spot was calculated by %Vol in treated samples/%Vol in control samples. A and B show the up-regulated proteins, C shows the down-regulated proteins. Spots U5 and U29 were absent from control samples and their abundance ratios are not shown.

**Table 1.** Differentially accumulated proteins identified by MS

| Spot no. | Protein name | Sequence [a] | MP/C [b] | Accession no. |
|---|---|---|---|---|
| D3 | Enolase | AAVPSGASTGVYEALELR | 20/45 | Q42971 |
| D4 | Enolase | AAVPSGASTGVYEALELR | 13/32 | Q42971 |
| D5 | Enolase | AAVPSGASTGVYEALELR | 25/62 | Q42971 |
| D6 | UDP-glucose pyrophosphorylase | VQLLEIAQVPDEHVNEFK | 27/66 | BAB69069 |
| D13 | Glutamine synthetase root isozyme | IIAEYIWVGGSGIDLR | 15/41 | P14654 |
| D14 | S-adenosylmethionine synthetase 2 | FVIGGPHGDAGLTGR | 11/30 | P93438 |
| D16 | Putative nascent polypeptide associated complex alpha chain | IEDLSSQLQTQAAEQFK | 8/35 | BAB89723 |
| U18 | Peroxidase | DSVVALGGPSWTVLLGR | 7/27 | AAC49818 |
| U25 | Cytochrome c oxidase subunit 6b-1 | APSLAEEYSLPPQEAPVEK | 9/58 | BAA76393 |
| U26 | Putative splicing factor-like protein | GPGGSREYMGR | 15/27 | BAB64646 |
| U29 | Putative actin-binding protein | YAVYDHDFTVSDATATAAA GEGGEAPR | 8/76 | AAO65861 |
| U32 | Triosephosphate isomerase | VATPDQAQEVHDGLR | 10/36 | AAB63603 |

a) The amino acid sequence of the peptide with the highest score identified by MS/MS.
b) The number of matched peptides/the percentage of sequence coverage

different plant species [24–27]. While the enzymatic activity and mRNA abundance of enolase were much increased by stresses, its protein abundance was increased only 1.4-fold in maize [24] or not at all in rice plant [26]. In our experiment, enolase proteins were markedly down-regulated and even disappeared after 72 h of salt stress treatment (Fig. 2B). These results suggest that enolase is regulated at the transcriptional, post-transcriptional, translational and post-translational levels. UGPase (D6) is responsible for the synthesis and pyrophosphorolysis of UDP-glucose, the key precursor of sucrose and cell wall components (*e.g.* cellulose, β-glucans). In *Arabidopsis,* it was strongly induced by sucrose, light, cold stress and phosphate deficiency but reduced by drought and flooding [28, 29]. We show here for the first time that the UGPase protein was markedly reduced by salt stress (Fig. 2B).

COX6b-1 (U25) was strongly up-regulated by salt stress (Figs. 1, 3B). Cytochrome *c* oxidase (COX), the terminal enzyme of the respiratory chain, oxidizes cytochrome *c* and transfers electrons to molecular oxygen to form molecular water. The COX of higher plants is composed of at least ten subunits [30]. In rice, two genes *OsCOX6b1* and *OsCOX6b2* encoding 6b subunit have been cloned [31, 32]. COX6b1 was induced by salt stress in our experiment (Fig. 1B), indicating its involvement in salt stress tolerance. It can probably facilitate energy generation through the respiratory chain under stress conditions.

Under salt stress the enzyme activities are affected and the basic metabolisms are disturbed. In order to maintain homeostasis under stress conditions, plants need to fortify the resistance mechanisms, such as ion transport, reactive oxygen species (ROS) scavenging and osmolyte synthesis. These processes require an extra energy supply. Regulation of triosephosphate isomerase, enolase, UGPase and COX6b-1 may be essential for activation of the entire energy-producing pathway. It can be postulated that plants can reduce glucose consumption in sucrose synthesis by down-regulating UGPase and thus provide more glucose for the glycolysis

 

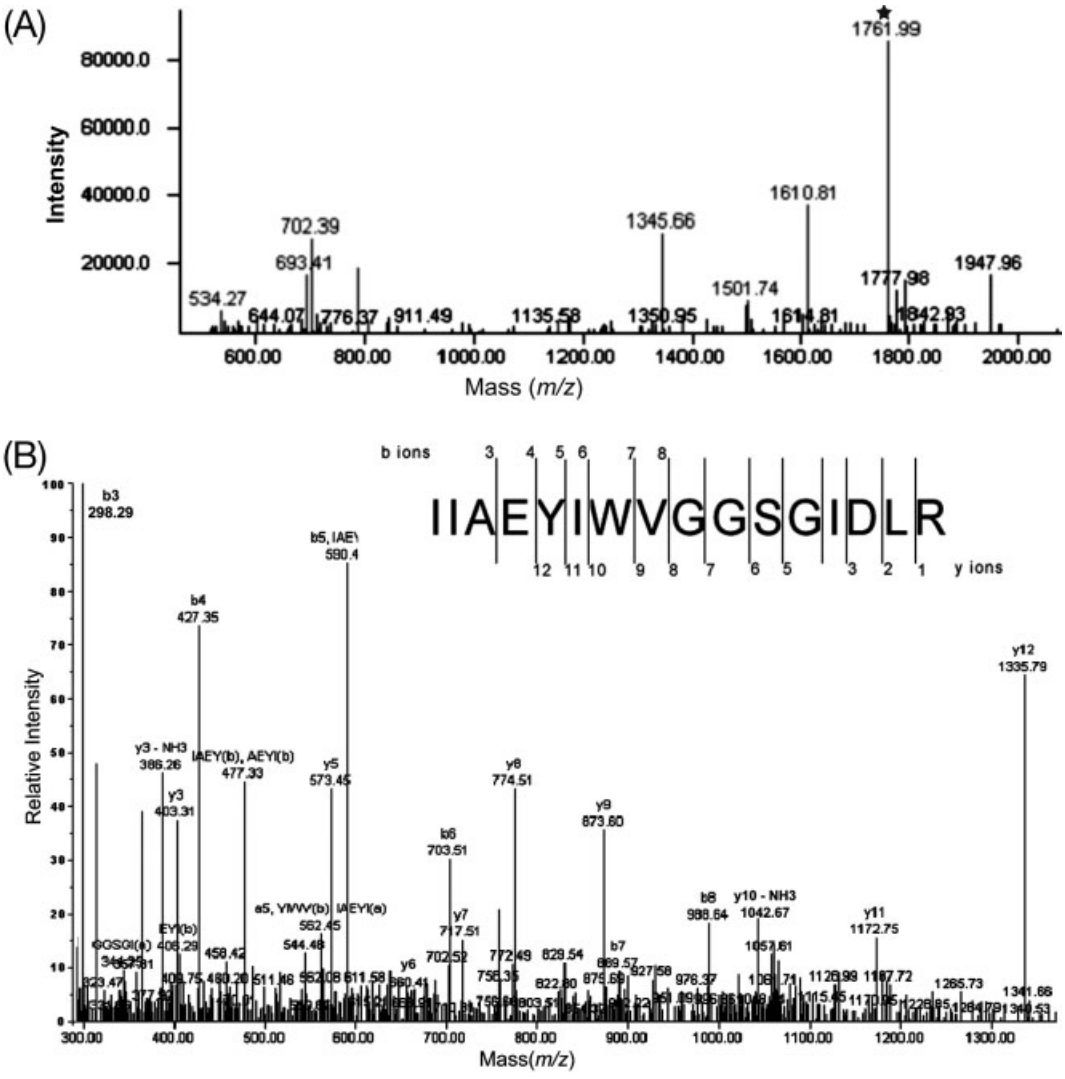


**Figure 4.** MS analysis of spot D13. The protein excised from gels was digested with trypsin and the resulting peptides were analyzed using a 4700 Proteomics Analyzer. A MALDI-TOF MS analysis. The spectral peaks show the intensities of different peptides. The 1761.99 *m/z* ion (marked with an asterisk) was further analyzed by MS/MS. B, MALDI-TOF MS/MS analysis of the 1761.99 *m/z* ion. The b ions, y ions and the resulting peptide sequence were shown. The y1 (175.14 *m/z*) and y2 (288.25 *m/z*) ions were not included in this region. Database searching using MASCOT software against NCBInr database identified the protein as glutamine synthetase root isozyme.

pathway, in which a key enzyme such as triosephosphate isomerase is up-regulated. The components of the respiratory chain such as COX6b-1 are also up-regulated to generate more ATP by oxidizing more NADH from glycolysis. However, the reason for down-regulation of enolase remains unknown.

A peroxidase (U18), a putative splicing factor-like protein (U26) and a putative ABP (U29) were up-regulated by salt stress (Fig. 1B). Salt stress is often accompanied by accumulation of ROS such as singlet oxygen ($O_2^1$), superoxide radical ($O_2^-$), hydroxyl radical ($OH^-$) and hydrogen peroxide ($H_2O_2$) in plant cells [33]. These ROS cause membrane damage and attack macromolecules. Plants have developed enzymatic and nonenzymatic systems to scavenge these toxic compounds. One of the antioxidant enzymes is peroxidase, which can detoxify $H_2O_2$ by oxidizing specific substrates such as ascorbate. Peroxidases have been found to be up-regulated by salt stress in many plants. For example, the transcripts of phospholipid hydroperoxide glutathione peroxidase and ascorbate peroxidase were strongly induced by salt stress in the pea [34]. Moreover, overexpression of a cDNA encoding an enzyme with the dual activities of glutathione *S*-transferase (GST) and glutathione peroxidase (GPX) in tobacco increased the GST and GPX activities and resulted in higher salt-stress tolerance [35]. Plants possess a large number of peroxidase isoenzymes that are encoded by

 

multigene families. It was suggested that different members of the peroxidase gene family are differently regulated in response to various environmental cues. The up-regulation of the peroxidase identified in this study indicates that it might play an important role in ROS scavenging under salt stress.

Spot U26 was identified as a putative splicing factor-like protein. It was highly induced by salt stress (Fig. 1B). Pre-mRNA splicing is a fundamental step in both constitutive and regulated gene expression. Many proteins are involved in this process [36]. Accumulating evidence suggests that pre-mRNA processing is a new target of salt toxicity in eukaryotic cells. Genetic and biochemical data showed that toxic ion $Li^+$ could inhibit the activities of various RNA processing enzymes in yeast, probably by replacing $Mg^{2+}$ from its binding sites [37]. It is also possible that toxic ions such as $Li^+$ and $Na^+$ can directly interfere with some of the protein-protein, protein-RNA and/or RNA-RNA interactions, which are crucial for RNA processing. Considering the evolutionary conservation, we have grounds to believe that splicing factors in plants are also salt toxicity targets. It was reported [38] that overexpression of an *Arabidopsis* SR-like splicing factor conferred salt tolerance to yeast and transgenic plants. The increased salt stress tolerance was not due to altered ion homeostasis or toxic ion sequestration but nonspecific stimulation and protection of the RNA processing in the presence of salt. We report here that a putative splicing factor-like protein was up-regulated at the protein level in rice, which provides new evidence for the importance of maintaining efficient RNA processing under salt stress conditions. It will be very interesting to carry out more work to study its function in salt tolerance.

Spot U29, a putative ABP was one of the most markedly up-regulated proteins and was only detectable after salt stress (Fig. 1). ABPs play key roles in remodeling of actin cytoskeleton. They are classified into several functional categories, including profilins, formins, actin-depolymerizing factors (ADFs)/cofilins and cyclase-associated proteins [39, 40]. The putative ABP identified in our experiment shares high homology with ADFs from many plant species, *e.g.* with 76% similarity to a wheat ADF (accession no. AAC49404) at the amino acid level. It probably represents a novel ADF in rice. ADFs modulate the dynamic organization of actin cytoskeleton by promoting filamentous actin disassembly [41]. ADFs were induced by salt stress, drought and cold in cereal plants [9, 42], suggesting that ADFs might be required for osmoregulation under osmotic stress. Indeed, osmotic stress regulation of actin organization correlates well with $K^+$ channel activity in guard cells [43]. Depolymerization of actin filaments either by actin drug cytochalasin D or osmotic stress potentiates the inward $K^+$ current in guard cells [43, 44]. Therefore, actin filaments may serve as an osmosensor and target inward $K^+$ channels in guard cells for turgor regulation. It is tempting to postulate that in root cells increased ADF levels under salt stress may result in depolymerization of actin filaments and enhanced $K^+$ influx through inward rectifying potassium channels, and help to restore the ion homeostasis. Further investigation is needed to confirm this hypothesis.

GS root isozyme (D13), SAMS2 (D14) and putative α-NAC (D16) were down-regulated by salt stress (Fig. 1A). GS is the key enzyme involved in assimilation of inorganic nitrogen into organic forms. It catalyzes the ATP-dependent condensation of ammonium with glutamate to yield glutamine, which then provides nitrogen groups for the biosynthesis of all nitrogenous compounds in the plant. Two different classes of GS have been identified in angiosperms: GS1 in the cytosol and GS2 in the chloroplasts [45]. GS1 is the predominant isoform in roots and other non-photosynthetic tissues and is encoded by a small multigene family. GS is essential to synthesize the precursors of proline, which can serve as an osmolyte. It was shown that transgenic tobaccos expressing an antisense cytosolic *GS1* gene produced less proline than wild-type plants and were more sensitive to salt stress [46]. Another report also showed that proline accumulation was due, at least in part, to the increased GS activity under salt stress in the cashew [47]. GS activities were induced or reduced by salt stress in the salt-tolerant and salt-sensitive rice leaves, respectively [48]. These data suggest that GS might be a determinant component for salt stress tolerance. The down-regulation of GS protein and consequently reduced GS activity in rice of the Nipponbare cultivar may result in less proline production, which might be part of the reason for its salt sensitivity. Therefore, it is possible to improve salt tolerance in rice by overexpressing the *GS1* gene.

SAMS catalyzes the biosynthesis of *S*-adenosyl-L-methionine (SAM) from L-methionine and ATP. SAM is a universal methyl group donor in several transmethylation reactions [49]. SAMSs are encoded by small gene families in all plants. The transcriptional regulation of SAMS genes in response to salt stress was studied in tomato, *Catharanthus roseus* and rice suspension cultured cells [50–52]. Different isogenes were differently regulated and several SAMS genes were induced by salt stress [50, 52]. It was thought that induction of SAMS genes by salt stress might be necessary to cope with a higher demand of SAM for the increased lignification because lignin monomers were methylated before polymerization. This was supported by the observation that increased lignification was detected in water- or salt-stressed plants [53, 54]. Despite the potential importance of SAMS in salt tolerance, the mRNA of SAMS2 was down-regulated in both salt-tolerant and salt-sensitive rice [6]. The protein level of SAMS2 was also down-regulated by salt stress (Figs. 1A, 2A). How SAMS activities in rice change under salt stress remains unknown. Considering the different regulation mechanisms and the existence of other SAMS genes in rice, we presume that some SAMS might be up-regulated by salt stress. A study of *C. roseus* SAMS showed that there is no simple correlation between the changes of transcript amounts, protein amounts and enzyme activities [51]. Therefore, it is necessary to investigate gene expression, protein accumulation and enzyme activities of SAMS family for a better understanding of their functions in salt stress-response.

 

Spot D16 was identified as putative α-NAC. Nascent polypeptide associated complex (NAC) is a heterodimeric complex of α chain and β chain. It can reversibly bind to eukaryotic ribosomes and is probably the first cytosolic protein to contact nascent polypeptide chains emerging from ribosome. The function of NAC is still obscure. It has been suggested that NAC is involved in protein sorting and translocation. It can prevent mistargeting of nascent polypeptide chains to endoplasmic reticulum [55]. Several lines of evidence suggested that the α-NAC could function as a transcriptional coactivator [56, 57]. Down-regulation of α-NAC mRNA and protein in human was related to Alzheimer's disease and Down's syndrome [58]. Although plant NAC has not been reported yet and its function remains largely unknown, the high sequence homology shared by plant and mammalian NAC suggests that plant NAC may have a similar function to that in mammalian cells. Our results showed that the protein of α-NAC was down-regulated by salt stress (Fig. 1A). The decreased α-NAC protein level might affect the overall NAC function and ultimately affect the process of gene transcription, protein translation and targeting, and inevitably lead to disordered metabolism. Considering the basic function of α-NAC, we suggest that α-NAC is sensitive to salt stress and represents a novel target of salt toxicity. It will be interesting to examine whether overexpression of α-NAC confers salt tolerance in plants.

## 4 Concluding remarks

In recent years, proteomic approaches are providing unprecedented insights into plant biology, microbiology, human disease, *etc*. We report here a systematic proteomic analysis of the root proteins in rice under high salinity conditions. Ten different salt stress-responsive proteins were identified, which are involved in a wide range of cellular processes, *e.g.* carbohydrate, nitrogen and energy metabolisms, ROS scavenging, mRNA and protein processing and cytoskeleton stability. Four of them (triosephosphate isomerase, enolase, SAMS2 and peroxidase) were previously shown to respond to salt stress either at the RNA level or at the protein level. Six of them are novel salt stress-responsive proteins, *i.e.* UGPase, COX6b-1, GS root isozyme, putative α-NAC, putative splicing factor-like protein and putative ABP. These results provide a starting point for further investigation into their functions using genetic and other approaches.

The proteome of any organism is highly dynamic with an endless number of possible variations. It is estimated that the rice genome contains 32 000 to 50 000 genes and each gene may give rise to multiple proteins by means of alternative splicing or PTM. The proteins analyzed in this work represent only a small part of the rice proteome. Many other salt stress-responsive proteins still need to be identified. For example, the noncytosolic proteins such as membrane proteins and nuclear proteins are believed to play key roles in osmosensing, ion transport and signal transduction. Deeper proteomic analysis may help us to better understand the salt stress response in rice.

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