Biochimica et Biophysica Acta 1804 (2010) 929–940

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Abscisic acid pretreatment enhances salt tolerance of rice seedlings: Proteomic evidence

Xiao-Juan Li [a,c,d,1], Ming-Feng Yang [a,1], Hui Chen [a], Le-Qing Qu [a], Fan Chen [b], Shi-Hua Shen [a,*]

[a] Institute of Botany, The Chinese Academy of Sciences, Beijing 100093, PR China
[b] Institute of Genetics and Developmental Biology, The Chinese Academy of Sciences, Beijing 100080, PR China
[c] College of Life Sciences, Agriculture University of Hebei, Baoding 071001, PR China
[d] Graduate University of Chinese Academy of Sciences, Beijing 100039, PR China

ARTICLE INFO

Article history:
Received 17 October 2009
Received in revised form 4 January 2010
Accepted 7 January 2010
Available online 14 January 2010

Keywords:
Abscisic acid
Proteome
Rice
Salt stress
Tolerance

ABSTRACT

Enhanced salt tolerance of rice seedlings by abscisic acid (ABA) pretreatment was observed from phenotypic and physiological analyses. Total proteins from rice roots treated with ABA plus subsequent salt stress were analyzed by using proteomics method. Results showed that, 40 protein spots were uniquely upregulated in the seedlings under the condition of ABA pretreatment plus subsequent salt stress, whereas only 16 under the condition of salt treatment. About 78% (31 spots) of the 40 protein spots were only upregulated in the presence of the subsequent salt stress, indicating that plants might have an economical strategy to prevent energy loss under a false alarm. The results also showed that more enzymes involved in energy metabolism, defense, primary metabolism, etc. were upregulated uniquely in ABA-pretreated rice seedlings, suggesting more abundant energy supply, more active anabolism (nitrogen, nucleotide acid, carbohydrate, etc), and more comprehensive defense systems in ABA-pretreated seedlings than in salt stressed ones.

1. Introduction

Abscisic acid (ABA) is a phytohormone that plays an important role in plant tolerance and adaptation to a variety of stresses [1]. It has been well documented that while endogenous ABA accumulates in plants under abiotic stresses [2], exogenous application of ABA enhances the tolerance of plants or plant cells to cold [3], heat [4], drought [5], anoxia [6] and heavy metal stresses [7]. Several studies also showed that ABA pretreatment enhances plant tolerance to salinity. Larosa et al. first reported that ABA pretreatment enhances the adaptation of cultured tobacco cells to salinity [8]. Later, this was also found to be true for cultured rice cells [9]. Since then, application of exogenous ABA has been shown to improve the salinity tolerance of several plant species, such as finger millet [10], rice [11] and common bean [12]. This phenomenon is generally known as induced resistance, and it suggests that ABA has great agronomic potential for improving the stress tolerance of agriculturally important crops.

Over the years, efforts have been made to understand and characterize the molecular basis for enhanced tolerance of ABA-treated plants to different stresses. The effects of ABA have been characterized into two broad categories; firstly, the morphological and physiological responses, such as induction stomatal closure [13], decrease of electrolyte leakage [14], fluctuation of fatty acid compositions [15], and induction antioxidant enzymes [16]; secondly, gene expression changes in seedlings or cultured cells, such as induction of the osmotin [17], late-embryogenesis abundant protein [18] and histidine-rich protein genes [19]. Many ABA-responsive genes have been identified successfully from some plants [20]. It has been demonstrated that transgenic plants overexpressing some of these ABA-responsive genes show significantly improved tolerance to stresses [21,22]. Recently, transcriptomic analysis of *Arabidopsis* and rice under drought, cold, high salinity and ABA treatments using cDNA microarray [23–25] and Tiling Array method [26], revealed that a panorama of gene expression changes occur during the various treatments. Although the transcriptomic data has deepened our understanding on the possible connection between ABA application and other stresses at transcriptional level, we know little about the changes occurring at the protein level. Several lines of evidence indicate that a group of ABA-responsive proteins are associated with induction of low or high temperature stress tolerances [4,27]. However, apart from proteins such as 26 kDa protein [28], late-embryogenesis abundant protein and histidine-rich protein [29], which are associated with salinity adaptation, only limited data are available regarding synthesis of new proteins after ABA treatment. Due to the limitation of the technology for protein separation and identification, only a few correlated proteins have been obtained and identified. The responses of plants to exogenous ABA treatment at the protein level, especially for induction of salinity tolerance, are still unclear.

Salinity is one of the primary limiting factors in agricultural crop production. Salinization of arable land is increasing, and could possibly have devastating global effects. In order to understand the

* Corresponding author. Tel.: +86 10 62836545; fax: +86 10 62596594.
E-mail address: shshen@ibcas.ac.cn (S.-H. Shen).
[1] These authors contributed equally to this work.

1570-9639/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbapap.2010.01.004

protein regulation underlying the increased salt tolerance by ABA pretreatment, we investigated the proteome pattern of ABA-pretreated rice seedlings under salinity. Our results indicate the important role of ABA in tolerance to abiotic stresses, and also provide new insights into the molecular mechanism of improved salt tolerance by the application of exogenous ABA on crops.

2. Materials and methods

2.1. Plant culture and treatments

Rice (*Oryza sativa* L. ssp. *Indica*) seeds were soaked in water for one day and then sprouted on wet filter paper for one day. The germinated seeds were grown in plastic containers containing complete Kimura B nutrient solution [30] under white light (150 μmol photons/m^2 s; 14-h light/10-h dark photoperiod) at 26 °C in a growth chamber. As outlined in Fig. 1, ten-day-old seedlings were pretreated with 5 μM ABA ((+)-Abscisic acid) in Kimura B nutrition solution for 48 h (PA), while seedlings cultured in Kimura B without ABA were marked as control (PC). PA seedlings were rinsed with distilled water to remove the residual ABA, and subjected to either 150 mM NaCl in Kimura B (AS) or grown in Kimura B (Aa) for two days. PC seedlings were either subjected to salinity (S) or grown in nutrient solution (Ctr) for two days. The entire experiment was repeated three times independently. The samples of each treatment were harvested for analyses of morphology, physiology, endogenous ABA levels and proteomic profile of the seedlings. For the latter two analyses, the samples were frozen in liquid nitrogen immediately, and kept at −80 °C until ABA or protein extraction.

2.2. Growth

The growth rates of rice seedlings were investigated after 48 h of 150 mM NaCl treatment. The fresh weight of five seedlings in each treatment of PC, PA, AS, S, Aa and Ctr were measured. The fresh weight (FW) increase of shoots and roots of seedlings in AS and Aa was calculated by the weight difference as compared to PA, and with PC in case of S and Ctr. The second leaf of rice seedlings was harvested from the shoots. After its FW was measured, the second leaf was dried at 80 °C for 72 h to determine the dry weight (DW). Absolute water content (AWC) was calculated by the following formula: $AWC = (FW - DW) / FW \times 100\%$.

2.3. Relative electrolyte leakage

Membrane damage was assayed by measuring ion leakage from the second leaves of rice seedlings. For each measurement, three leaves were cut into 1 cm long segments and floated on 15 mL of double distilled water and vacuum infiltrated until all the segments sink down. The conductivity of the bathing solution was measured using electrolyte leakage apparatus (value *A*). Afterward, the solution and segments were transferred into sealed tubes, and were boiled for 15 min. After cooling to room temperature the conductivity of the bathing solution was measured again (value *B*). For each measurement, ion leakage was expressed as percentage leakage, *i.e.* (value *A* / value *B*) × 100.

2.4. Determination of proline

One gram of rice root was homogenized in 5 mL of 3% sulphosalicylic acid and the residue was removed by centrifugation. The extract (2 mL) was reacted with 2 mL glacial acetic acid and 3 mL acid ninhydrin (1.25 g ninhydrin was warmed in a mixture of 30 mL glacial acetic acid and 20 mL of 6 M phosphoric acid until dissolved) for 1 h at 100 °C and the reaction was then terminated in an ice bath. The reaction mixture was extracted with 5 mL toluene. The chromophore containing toluene was warmed to room temperature and its optical density was measured at 520 nm. The amount of proline was determined from a standard curve.

2.5. Measurement of endogenous ABA levels

Frozen rice roots of 1.5 g were finely ground in liquid nitrogen and 10 mL of 80% methanol was added together with 0.01 g of ascorbic acid and 0.01 g polyvinylpyrrolidone (PVP) to prevent oxidation reactions during extraction. The homogenate was stirred overnight at 4 °C. After centrifugation (4000 × *g*, 15 min), the supernatant was recovered and adjusted to pH 8.0. The aqueous methanol was evaporated under reduced pressure at 35 °C. The residue was dissolved in 5 mL of water. Then it was frozen and thawed for three cycles. After centrifugation (4000 × *g*, 15 min), the supernatant was recovered and adjusted to pH 2.5 and partitioned against ethyl acetate. Then the solution with the free ABA in ethyl acetate was collected. This process was repeated thrice. After that, the collection was adjusted to pH 8.0 and dried. The resulting dried precipitate was dissolved in 1 mL of 3% methanol containing 0.1 M acetic acid, and was filtered through a 0.45 mm

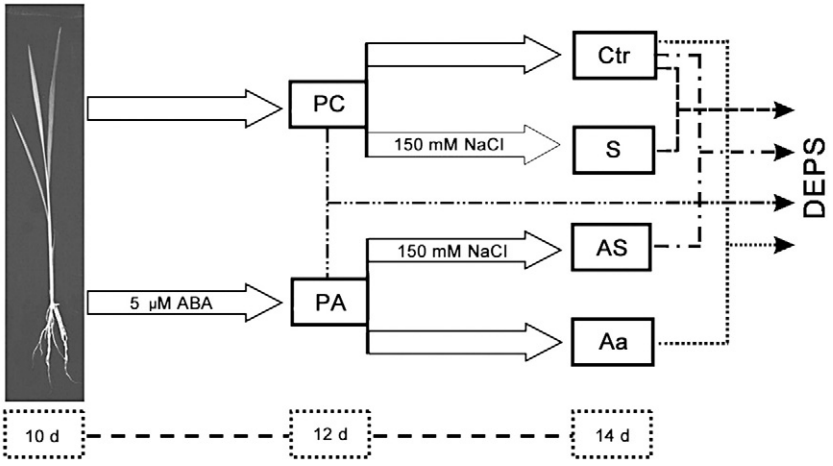


Fig. 1. Experimental flowchart. Ten-day-old rice seedlings were pretreated with 5 μM ABA in Kimura B nutrient solution for 2 days (PA). Seedling cultured in Kimura B without ABA was treated as control (PC). PA seedlings were further split and either shifted to a salt stress of 150 mM NaCl in Kimura B (AS) or grown in Kimura B (Aa) for two days. PC seedlings were either subjected to salinity for two days (S) or grown in nutrient solution for two days (Ctr) and used as controls for AS, Aa and S treatments. Proteins were extracted from roots of these seedlings, and differential proteomics analyses were performed. Differentially expressed protein spots (DEPS) in gels of PA treatment were obtained by comparison with PC; DEPS in gels of AS, Aa and S treatments were obtained by comparison with Ctr (comparing between different treatments is indicated by dotted lines).

membrane filter. The extract (100 μL) was automatically injected and processed by HPLC (Agilent 1100 Series. USA) equipped with a reverse phase column (4.6×250 mm Diamonsic C18, 5 μm). It was eluted with a linear gradient of methanol (3–97%), containing 0.01% acetic acid, at a flow rate of 4 mL/min. The detection was run at 260 nm with a diode array detector. The retention time of ABA was 36.4 min and shifted 0.1 to 0.5 min. Quantification was obtained by comparing the peak areas with those of known amounts of ABA.

2.6. Protein extraction

One gram of frozen rice roots was ground into fine powder in liquid nitrogen in a pre-cooled mortar and pestle, and homogenized in 2 mL homogenization buffer containing 20 mM Tris–HCl (pH 7.5), 250 mM sucrose, 10 mM ethylene glycol-bis (ß-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and 1% Triton X-100. The homogenate was transferred into a microcentrifuge tube and centrifuged at 15 000×*g* for 15 min at 4 °C. The supernatant was transferred to a new tube and protein was precipitated using 1/4 volume 50% cold trichloroacetic acid (TCA) in an ice bath for 30 min. The mixture was centrifuged at 15 000×*g* for 15 min at 4 °C, and the supernatant was discarded. The pellet was washed 3 times with acetone, centrifuged and vacuum-dried. The dried powder was dissolved in sample buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-Cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS), 2% ampholine pH 3.5–10 (GE Healthcare Bio-Science, Little Chalfont, UK) and 1% DTT.

2.7. Two-dimensional gel electrophoresis

The first dimensional isoelectric focusing (IEF) was performed in a 13 cm long glass tube with a diameter of 3 mm. The gel solution consisted of 8 M urea, 3.6% acrylamide, 2% NP-40 and 5% Ampholines (1 part pH 3.5–10, 1 part pH 5–8). IEF was performed at 200 V, 400 V and 800 V for 30 min, 15 h and 1 h, respectively. About 500 μg of protein was loaded. After the first dimensional run, IEF gels were equilibrated in equilibration buffer (62.5 mM Tris–HCl pH 6.8, 2.5% SDS, 10% (v/v) glycerol and 5% 2-mercaptoethanol) for 15 min twice. The second dimension electrophoresis was performed on 15% resolving gels and 5% stacking gels (175×200×1 mm). The gels were stained with 0.1% Coomassie brilliant blue (CBB) R-250. Reproducibility of the 2-D gels was ensured by four technical replicates for each biological replicate of given treatment.

2.8. Image and data analysis

The stained gels were scanned using a UMAX Power Look 2100XL scanner (UMAX Inc., Taipei, China) in transmission mode with a resolution of 300 dots per inch (dpi). The data was analyzed using ImageMaster™ 2D Platinum software 5.0 (GE Healthcare Bio-Science). Three images representing three independent biological replicates for each treatment were grouped to calculate the averaged volume of all the individual protein spots. In order to correct the variability due to CBB-staining and to reflect the quantitative variations in intensity of protein spots, the spot volumes were normalized as a percentage of the total volume in all of the spots present in the gel. The spot volumes in PA were compared with those in PC, and the spot volumes in AS, S and Aa were compared with those in Ctr. Significantly changed protein spots in PA, AS, S and Aa ($p<0.05$), which altered more than 1.5-fold in volume%, were considered as differentially expressed proteins. The experimental p*I* and M_r of each protein were determined by using 2-DE markers (Bio-Rad).

2.9. Protein identification

Protein spots were excised from the gels manually and cut into small pieces. Each small gel piece was destained with 50 mM NH_4HCO_3 in 50% ethanol for 1 h at 40 °C. The protein in the gel piece was reduced with 10 mM DTT in 100 mM NH_4HCO_3 for 1 h at 60 °C and incubated with 40 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min at room temperature in dark. The gel pieces were minced and lyophilized, then rehydrated in 25 mM NH_4HCO_3 with 10 ng sequencing grade modified trypsin (Promega, Madison, WI, USA) at 37 °C overnight. After digestion, the protein peptides were collected, and the gels were washed with 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile thrice to collect the remaining peptides. The peptides were then concentrated and cocrystallized with one volume of saturated α-cyano-4-hydroxycinnamic acid in 50% v/v acetonitrile containing 1% TFA. Tryptic peptide masses were measured with an AXIMA-CFR plus MALDI-TOF mass spectrometry (Shimadzu Biotech, Kyoto, Japan).

The National Center for Biotechnology Information nonredundant protein database (NCBInr) was searched for the acquired peptide mass fingerprinting (PMF) data using the Mascot software available at http://www.matrixscience.com. *O. sativa* was chosen for the taxonomic category. Database queries were carried out for monoisotopic peptide masses using the following parameters: enzyme of trypsin; mass error tolerance of 0.1–0.3 Da; maximum of one missing cleavage site; modifications allowed for carboxyamidomethylation of cysteine. To determine the confidence of the identification results, the following criteria were used: in addition to a minimum of 61 in MOWSE score, sequence coverage of the protein should be no less than 14% by the matching peptides.

2.10. Statistical analysis

The data on fresh weight, absolute water content, relative ion leakage and proline content in seedlings of different treatments were statistically analyzed by one-way Analysis of Variance (ANOVA). The volume changes of protein spots were analyzed by Student's *t*-test.

3. Results

3.1. The tolerance of rice seedlings to salt stress was enhanced by ABA pretreatment

To test if ABA pretreatment could enhance salt tolerance of rice, 10-day-old rice seedlings were pretreated with ABA for two days (termed as PA). These ABA-pretreated seedlings were then subjected to a salt stress of 150 mM NaCl for two days (AS). Two other treatments were established: ABA-pretreated seedlings were cultured in Kimura B nutrient solution (Aa) and seedlings without ABA pretreatment cultured in 150 mM NaCl (S) for two days. Ten-day-old seedlings cultured in Kimura B for two days were used as control for PA (termed as PC), and those cultured for four days were control for AS, Aa and S treatment (Ctr) (Fig. 1).

The relative weight increase of shoots in S, AS, Aa and Ctr was -0.05 ± 0.01, 0.03 ± 0.01, 0.102 ± 0.01 and 0.09 ± 0.01 g, respectively. The weight increase of shoots in AS was significantly higher than that in S ($p<0.05$) (Fig. 2A). This trend was also observed in rice roots (Fig. 2B). These results suggested that the growth of rice seedlings was inhibited significantly by the salt stress, whereas the inhibition was mitigated upon pretreatment of seedlings with ABA.

Salt stress also led to a morphological change in S as observed by the obvious wilting and to a lesser extent, searing of the second leaves, while the AS seedlings did not show any observable change (Fig. 3). The AWC (absolute water content) decrease of the second leaf was determined to be $-0.20\pm1.14\%$, $-0.27\pm1.05\%$ and $0.18\pm0.47\%$ in AS, Aa and Ctr, respectively (Fig. 2C), while in S treatment, it was $7.07\pm0.45\%$, which was significantly higher than those in the former three treatments ($p<0.05$). In contrast, the leaf AWC decrease in AS showed no difference when compared with Aa and Ctr measurements ($p>0.05$). These results indicated that while salt stress leads to substantial water loss from rice cells, this loss is alleviated on pretreating the seedlings with ABA.

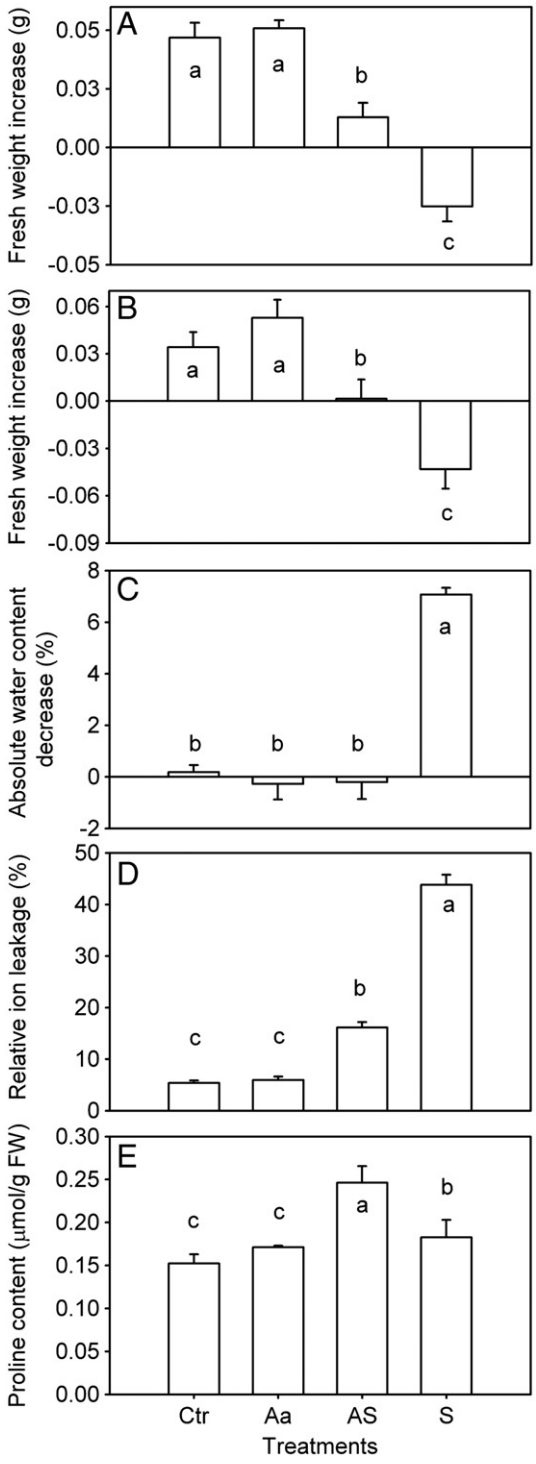


Fig. 2. The effects of different treatments on the growth of rice seedlings. (A) Fresh weight increase of shoots; (B) fresh weight increase of roots; (C) the decrease of absolute water content in the second leaf; (D) the relative ion leakage of the second leaf; (E) the proline content. Values are means ± SD ($n = 3$). Different letters indicate significance at $p < 0.05$. Ctr, Aa, AS and S were different treatments as described in Fig. 1.

At the physiological level, ion leakage is usually considered an instantaneous result of abiotic stress. Ion leakage of rice leaf in S treatment was found to be the most significant (43.8%), and was determined to be 1.87-fold of that in AS conditions ($p < 0.05$) (Fig. 2D). The significant reduction of the ion leakage in AS indicates that ABA pretreatment mitigated membrane damage of leaf cells under salt stress. Proline is beneficial to plant tolerance to stresses. In contrast to ion leakage, significant amounts of proline were accumulated in AS

Fig. 3. The morphological characteristics of rice seedlings under Aa, AS and S treatments. The second leaf of rice seedlings wilted in S treatment (indicated by arrow), not in AS treatment. Ctr, Aa, AS and S were different treatments as described in Fig. 1.

treated rice roots (0.26 μmol/g FW), which was 1.35-fold of that in S conditions (Fig. 2E).

Taken together, the tolerance of rice seedlings to salt stress was enhanced by ABA pretreatment.

3.2. AS treated rice roots accumulated more ABA

ABA content in plants is closely related to the abiotic stress tolerance [31]. Therefore, endogenous ABA content in rice roots was determined. The ABA contents in rice roots of Ctr, S, Aa, and AS were 0.01 ± 0.00, 0.05 ± 0.01, 0.13 ± 0.02, and 0.21 ± 0.01 nmol/g, respectively (Fig. 4), indicating that roots in AS accumulated more ABA than in S.

3.3. Proteins were differentially expressed in roots of AS and S treatments

In order to investigate the protein profile of the ABA-pretreated roots under salt stress, differential proteomics analyses were performed on AS and S, and contrasted with Ctr samples. A total of 800 reproducible protein spots were detected on the gels of total proteins from rice roots in AS and S treatments, and 100 among these spots were differentially expressed (Fig. 5). These 100 protein spots could be subdivided into three groups: i) 47 that were differentially expressed only in AS (e.g. spot 7), ii) 24 that were specific to S (e.g. spot 47), and iii) 29 that were shared between AS and S treatments (e.g. spot 2). This result shows that more protein spots were differentially expressed in AS vs. S; that is, ABA pretreatment prior to salt stress results in the regulation of several proteins which were not otherwise detected under salt stress conditions only.

3.4. Identification of differentially expressed proteins

The differentially expressed protein spots were analyzed by MALDI-TOF/MS. According to the criteria described in the Materials

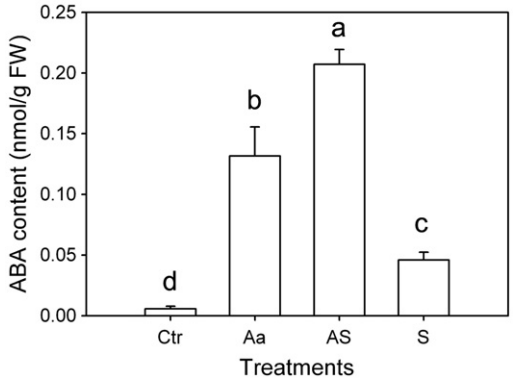


Fig. 4. The level of ABA in rice roots under different treatments. Values are means ± SD ($n = 3$). Different letters indicate significance at $p < 0.05$. Ctr, Aa, AS and S were different treatments as described in Fig. 1.

and methods section, 76 out of the 100 differentially expressed protein spots were identified. These proteins were classified into eight groups according to their functions (Table 1). The differentially expressed protein spots in each functional group were upregulated or downregulated spots, which could be further classified into three groups according to where they were found differentially expressed: differentially expressed only in AS, only in S, and those shared by both (Table 2). Most differentially expressed protein spots identified (about 58%) were in energy metabolism and defense-related groups, and majority of those were upregulated proteins (Table 2). Although defense-related protein spots (19 spots) were less than protein spots grouped into energy metabolism (25 spots), the defense-related proteins (17 proteins) were actually more than those grouped into energy metabolism (10 proteins) (Table 1), because many spots in the energy metabolism group shared the same identity (for example, spots 2, 3, 4, 5 and 6 were aconitate hydratase), which might be due to modifications or the presence of different isoforms as found in previous studies [32]. The eight groups of proteins identified in this report are described in detail in the following paragraph.

3.4.1. Energy metabolism

Proteins in this group are involved in glycolysis (spots 13, 18, 36, 37, 46, 47, 49, 50, 51, 53, 54, 56, 58 and 77), tricarboxylic acid cycle (spots 2, 3, 4, 5 and 6), respiratory chain (spot 7), ATP synthesis (spots 25, 26 and 28), energy releasing (spot 94) and mitochondrial protein processing (spot 15). Most proteins in this group, 21 out of 25 spots, were upregulated proteins. Seven protein spots were upregulated in both AS and S treatments, including aconitate hydratase (spots 2, 3, 4 and 5), cytosolic glyceraldehydes-3-phosphate dehydrogenase (spots 50 and 51) and alcohol dehydrogenase (spot 77). Noticeably, most of these upregulated proteins (14 out of 21 spots) were upregulated only in AS, including aconitate hydratase (spot 6), NADH-ubiquinone oxidoreductase (spot 7), phosphoglycerate mutase (spot 13), mitochondrial processing peptidase (spot 15), isoforms of glyceraldehydes-3-phosphate dehydrogenase (spots 18, 49, 53, 54, 56 and 58), ATP synthase beta subunit (spots 25, 26 and 28), and soluble inorganic pyrophosphatase (spot 94). These results suggest that roots under AS treatment result in a greater involvement of the energy metabolism enzymes as compared to S treated roots, and it is probable that this contributes in countering the effects of salt stress in AS conditions.

3.4.2. Defense related

Proteins in this group are mainly involved in antioxidation (spots 14, 16 and 81), detoxification (spots 31, 40, 42 and 83), and other defense-related functions, as suggested by previous studies [19,33–38]. All proteins (19 spots) in this group were upregulated. Five of these were upregulated both in S and AS, including glutathione reductase (spot 16), annexins (spot 60), endo-1, 3-β-glucanase (spots 69 and 70) and heat shock protein 90 (spot 82). More than half of this group, 10 spots, were upregulated only in AS, including catalase (spots 14 and 81), glyoxalase I (spot 40), formate dehydrogenase 1 (spot 42), putative r40c1 protein (spot 52), universal stress protein (spot 74), aldo-keto reductase (spot 83), aldose reductase (spot 84), group 3 LEA (spot 85) and alpha glucan phosphorylase (spot 86). However, just four spots were found upregulated exclusively in S. This indicates that the defense-related system was possibly more active in the AS treated roots vis-a-vis S treated roots.

3.4.3. Primary metabolism

Proteins in this group are involved in metabolism of nitrogen, nucleotide acid, carbohydrate, *etc*. Among them, glutamine synthetase (spots 34 and 38), transketolases (spot 8), 2-isopropylmalate synthase (spot 12), UMP synthase (spot 87) and aspartate aminotransferase (spot 88), were upregulated only in AS (Fig. 6); while glutamate dehydrogenase (spot 20) and 3-phosphoshikimate 1-carboxyvinyltransferase (spot 100) were upregulated only in S (Fig. 6). In plants, glutamine synthetase, 2-isopropylmalate synthase, aspartate aminotransferase, glutamate dehydrogenase and 3-phosphoshikimate 1-carboxyvinyltransferase participate in biosynthesis of amino acids and nitrogen metabolism. Transketolases catalyze the reversible transfer of an activated two-carbon glycol-aldehyde moiety from a ketose to an aldose, and in these pathways, different sugar phosphate intermediates are synthesized, which can be channeled to carbohydrate metabolism or nucleic acid and amino acid biosynthetic reactions. UMP synthase catalyzes the last two key regulatory steps in the *de novo* synthesis of pyrimidine nucleotides. Pyrimidine nucleotides are abundant molecules with essential functions in a multitude of biochemical processes and particularly important in dividing and elongating tissues as building blocks for nucleic acid biosynthesis. In addition, the pyrimidine nucleotides are directly involved in plant carbohydrate metabolism providing the energy-rich precursor UDP-Glc for many synthetic reactions [39].

3.4.4. Secondary metabolism

Most of proteins in this group were downregulated. Some were downregulated in both AS and S treatments, including Phe ammonia-lyase (spot 10), S-adenosylmethionine synthetase (spot 23), caffeate *O*-methyltransferase (spot 33) and caffeoyl-CoA *O*-methyltransferase (spot 68), which are enzymes involved in biosynthesis of lignin and/or the phenylpropanoid pathway. UDP-glucuronic acid decarboxylase (spots 44 and 48), which catalyzes the formation of UDP-D-xylose was downregulated only in S treatment. UDP-D-xylose is an important nucleotide sugar required for the synthesis of numerous glycoconjugates [40]. This indicates that the synthesis of macromolecules for cell growth was inhibited by salinity, and that ABA pretreatment could partially overcome this inhibition.

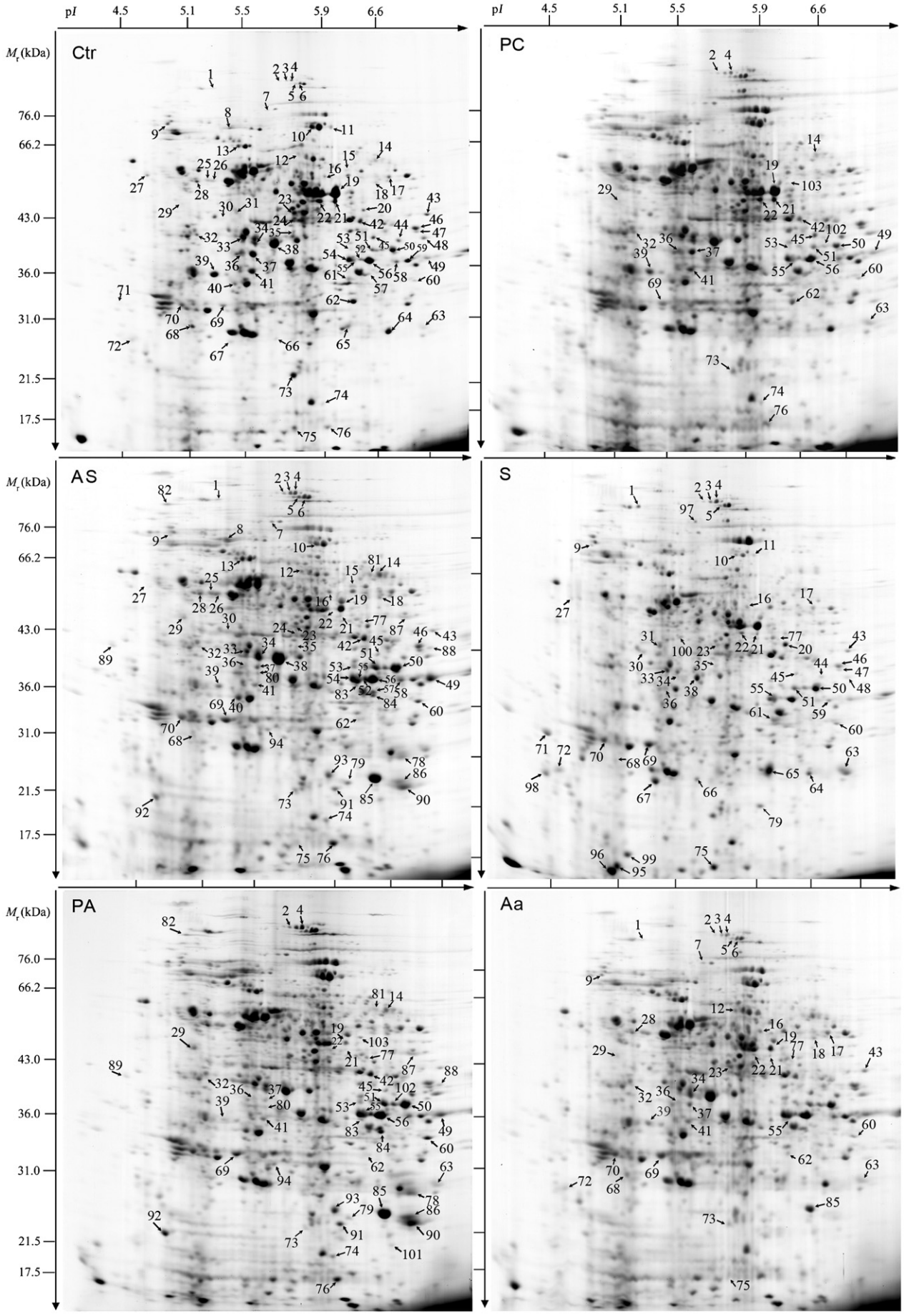
3.4.5. Protein turnover

Proteins in this group are parts of enzyme complexes involved in protein degradation or protein synthesis. Beta 7 subunit of 20S proteasome (spot 78) is a part of 26S proteasome. The eukaryotic 60S acidic ribosomal protein P0 (spot 80) is a part of the lateral stalk of the 60S ribosomal subunit. Both were induced only in AS treatment.

Other functional categories are cell growth/division, signal transduction and proteins with unknown function (Table 1).

3.5. Proteins differentially expressed in PA and Aa

A total of 40 protein spots were upregulated uniquely in AS relative to S treatment (Table 2), and these proteins might contribute to higher salt tolerance of ABA-pretreated seedlings. To understand if the unique upregulation was due to the ABA pretreatment, their expression patterns were compared with corresponding protein spots in PA, Aa treatments (Fig. 5). Among these 40 protein spots, 78% (31 spots) showed no differential expression in Aa treatment (Table S1). This suggests that these 40 protein spots were upregulated under the


Ctr
PC
AS
S
PA
Aa
pI
4.5
5.1
5.5
5.9
6.6
M_r(kDa)
76.0
66.2
43.0
36.0
31.0
21.5
17.5

condition of ABA pretreatment plus subsequent salt treatment; however, the upregulation of most of these proteins could not be maintained without the subsequent salt treatment. Among these 31 protein spots mentioned above, 21 were upregulated in PA treatment (spots 14, 42, 49, 53, 56, 74, 76, 78, 80, 81, 83, 84, 86, 87, 88, 89, 90, 91, 92, 93 and 94) while 9 were not (spots 8, 13, 15, 25, 26, 40, 52, 54 and 58). This suggested that some proteins (9 spots) could not be upregulated solely by ABA pretreatment or by salt stress; they could be upregulated, instead, under the condition of ABA pretreatment followed by subsequent salt treatment. Among those protein spots which were upregulated specifically in AS (not in S or Aa), 25 were indentified, and 24 have known functions (Fig. 6), most of which were energy metabolism proteins (spots 13, 15, 25, 26, 49, 53, 54, 56, 58 and 94) and defense-related proteins (spots 14, 40, 42, 52, 74, 81, 83, 84 and 86) (Table 1). Among the 40 protein spots upregulated uniquely in AS (not in S), 58% (23) were upregulated in both AS and PA. Seventeen out of these 23 proteins were identified, most of which were defense-related proteins (spots 14, 42, 74, 81, 83, 84, 85 and 86), followed by energy metabolism (spots 49, 53, 56 and 94), primary metabolism (spots 87 and 88), protein turnover (spots 78 and 80), and signal transduction proteins (spot 29) (Table 1).

4. Discussion

4.1. The differentially expressed proteins are related to the enhanced salt tolerance of ABA-pretreated rice seedlings

The salinity tolerance of rice seedlings was significantly enhanced by ABA pretreatment (Fig. 2), which is consistent with previous observation [11]. Our results corroborate the function of ABA as a trigger to initiate the process for salt adaptation and enhance salt tolerance in higher plants. A previous study that monitored expression profiles of rice genes under salt stress and abscisic acid application using cDNA microarray indicated that 57 and 43 genes were induced by high salinity and ABA, respectively [24]. In this report, however, among the 32 and 37 protein spots upregulated by high salinity and ABA respectively, only the identities of a few proteins overlapped with the gene identities in previous study, such as catalase, LEA protein and actin. Among these overlapped proteins, the sequence of actin protein identified in our report has a high identity (about 97%) with the actin reported previously, suggesting they are probably homologous genes in different rice varieties (*O. sativa* var. Nipponbare in the study of Rabbani et al. and var. Indica in our report). The sequence of LEA identified in our report is much different from the LEAs in previous studies [24,41], and it is probably a different member of the LEA family, given the fact that LEAs are a large family. The little overlapping of protein identities between these studies may be attributed to absence of the corresponding probes in the microarray, posttranscriptional regulation, and the differences in plant tissues used and treatment conditions.

It is shown that protein synthesis is necessary for improvement of stress tolerance in ABA-pretreated plant cells [42]. Under salt stress, only 16 protein spots were upregulated uniquely in rice seedlings without ABA pretreatment, while 40 were upregulated uniquely in ABA-pretreated seedlings (Table 2). This indicates that the upregulation of a bunch of proteins involved in many biological processes post-ABA treatment is associated with the enhanced salt tolerance. The most significant changes at protein level were found in those enzymes involved in energy metabolism and defense-related systems followed by primary metabolism, secondary metabolism, signal transduction, protein turnover, etc.

Among the energy related proteins, many proteins were upregulated only under AS treatment (Tables 1 and 2). Introduction of glyceraldehydes-3-phosphate dehydrogenase in potato has previously been shown to improve salt tolerance [43]. We also detected the upregulation of several isoforms of glyceraldehydes-3-phosphate dehydrogenase upon AS treatment, and it is thus likely that these proteins play similar roles in enhancing salt tolerance in rice seedlings. Mitochondrial processing peptidase is involved in the processing of mitochondrial proteins, most of which are respiration related and correlated with energy generation [44]. Its upregulation possibly results in the activation of non-functional fusion mitochondrial proteins [44], which consequently may contribute towards increasing energy generation in the mitochondria. The energy thus available to the AS treated roots could be utilized to overcome the adverse effects of salt stress. Thus the processes of energy generation were more activated in AS treatment than in S treatment in order to cope with the salt stress.

An efficient and effective defense mechanism comprises one of the most important strategies that plants utilize to survive and thrive under various stress conditions. When ABA-pretreated seedlings were under salt stress, 10 defense-related protein spots were upregulated uniquely when compared with salt stressed seedlings. The upregulation of catalase (spots 14 and 81) indicates that the antioxidant system is involved in the salt tolerance of ABA-pretreated seedlings. Prasad et al. found that after ABA pretreatment, the activity of catalase in mitochondrion of maize seedlings is several folds of control seedlings, and this change was considered to be the underlying cause for the improved cold tolerance [16]. The detoxification system is also involved in the protection of ABA-pretreated rice from salt stress. Glyoxalase I (spot 40) is a member of glyoxalase system that carries out the detoxification of methylglyoxal and other reactive aldehydes produced in metabolism of plants. Formate dehydrogenases catalyze the oxidation of formate to bicarbonate and play a detoxification role in plants [45]. The upregulation of formate dehydrogenase (spot 42) in our report is in agreement with a previous study, in which the formate dehydrogenase mRNA was shown to increase under stresses [46], indicating this enzyme might have a bona fide role in stress tolerance. Aldo-keto reductases (spot 83) are a large superfamily of related proteins that carry out NADPH-dependent reduction of various aldehydes and ketones, and they may aid in the detoxification of toxic lipid peroxidation products [47]. In addition to above-mentioned enzymes involved in antioxidant- and detoxification systems, many other defense-related proteins, which have been shown to be helpful in plant salt resistance in previous studies, were also observed as upregulated proteins uniquely in AS treatment. For example, r40c1 protein (spot 52) is involved in water loss prevention during salt stress of rice [19]. Universal stress proteins (spot 74) can mediate survival of *Escherichia coli* cells exposed to osmotic stress [33] and play a role in the adaptation of rice to submergence stress [34]. Aldose reductase (spot 84) catalyzes D-glucose to sorbitol, which serves as an osmoregulator in plants under salt stress [35]. An elevated level of aldose reductase gene expression is associated with the induction of freezing tolerance in cultured bromegrass cells pretreated with ABA [36]. LEA protein (spot 85), which can be induced by exogenous application of ABA [29] and play important roles during stresses [37], was also induced in AS treatment. α-glucan phosphorylase (spot 86) is a key enzyme in glucan catabolism. α-glucan phosphorylase-deficient plants are more sensitive to transient water and salt stress and phosphorylase may play an important role in stress tolerance [38]. The experimental molecular weight of α-glucan phosphorylase identified in this report is much smaller than its theoretical molecular weight possibly due to degradation (Supplementary Table S1). Previous studies have also found degradation of this enzyme and it has been reported that the partially degraded/cleaved proteins have a higher level of activity than the native proteins [48,49].

The five defense-related protein spots upregulated both in S and AS, as members of the defense systems, probably also contribute to the enhanced salt tolerance of ABA-pretreated seedlings. Glutathione

Fig. 5. Comparison of the proteome patterns of rice seedlings under different treatments. Differentially expressed protein spots in each treatment are indicated by arrows and numbered as in Table 1. Ctr, PC, AS, S, PA and Aa were different treatments as described in Fig. 1.

Table 1
Differential protein spots identified from rice roots by MALDI-TOF MS.

Spot no.	M_r/p*I*	Protein identity	Accession no.	Treatments			
				AS	S	Aa	PA
Energy metabolism							
2	98601/5.67	Putative aconitate hydratase, cytoplasmic	Q6YZX6	U	U	U	U
3	98601/5.67	Putative aconitate hydratase	Q6YZX6	U	U	U	
4	98601/5.67	Putative aconitate hydratase	Q6YZX6	U	U	U	U
5	99402/5.8	Putative aconitate hydratase	EEE58288	U	U	U	
6	99402/5.8	Putative aconitate hydratase	EEE58288	U		U	
7	82167/5.86	NADH-ubiquinone oxidoreductase	NP_001051072	U		U	
13	60980/5.42	Putative phosphoglycerate mutase	EEC71703	U			
15	54141/6.25	Putative mitochondrial processing peptidase	BAD72225	U			
18	54868/6.8	Glyceraldehyde-3-phosphate dehydrogenase	AAM00227	U		U	
25	45265/5.26	Putative ATP synthase beta subunit	BAD82522	U			
26	45265/5.26	Putative ATP synthase beta subunit	BAD82522	U			
28	45265/5.26	Putative ATP synthase beta subunit	BAD82522	U		U	
36	41812/6.07	Fructose-bisphosphate aldolase, chloroplast precursor	ABA91631	D	D	D	D
37	41812/6.07	Fructose-bisphosphate aldolase, chloroplast precursor	ABA91631	D		D	D
46	39245/6.96	Fructose-bisphosphate aldolase cytoplasmic isozyme	P17784	D	D		
47	39245/6.96	Fructose-bisphosphate aldolase cytoplasmic isozyme	P17784		D		
49	36719/7.68	Glyceraldehyde-3-phosphate dehydrogenase	NP_001047348	U			U
50	36644/6.61	Glyceraldehyde-3-phosphate dehydrogenase	AAA82047	U	U		U
51	36644/6.61	Glyceraldehyde-3-phosphate dehydrogenase	AAA82047	U	U		U
53	36644/6.61	Glyceraldehyde-3-phosphate dehydrogenase	AAA82047	U			U
54	36924/6.34	Glyceraldehyde 3-phosphate dehydrogenase	NP_001053139	U			
56	36924/6.34	Glyceraldehyde 3-phosphate dehydrogenase	NP_001053139	U			U
58	36719/7.68	Glyceraldehyde-3-phosphate dehydrogenase	NP_001047348	U			
77	41580/6.32	Alcohol dehydrogenase 1	CAA34363	I	I	I	I
94	24294/5.59	Soluble inorganic pyrophosphatase	NP_001054331	I			I
Defense-related							
14	57074/6.75	Catalase	CSRZ	U			U
16	53936/6.24	Glutathione reductase	BAA11214	U	U	U	
31	41334/5.25	12-oxophytodienoic acid reductase, OPR-1	ABV45434		U		
40	32867/5.51	Glyoxalase I	BAB71741	U			
42	41445/6.87	Formate dehydrogenase 1	Q9SXP2	U			U
52	42247/6.25	Putative r40c1 protein	AAN64997	U			
60	35693/7.13	Annexin-like protein	EAY87564	U	U	U	U
61	35701/7.01	Beta-1,3-glucanase	BAA77785		U		
67	27933/6.09	Chitinase	NP_922578		U		
69	34837/5.18	Endo-1,3-beta-glucanase	BAD88028	U	U	U	U
70	34837/5.18	Endo-1,3-beta-glucanase	BAD88028	U	U	U	
74	18321/6.49	Universal stress protein family	NP_001066983	U			U
81	57074/6.75	Catalase	CSRZ	I			I
82	93045/4.89	Heat shock protein 90	AAL79732	I	I		I
83	38234/6.28	Aldo-keto reductase	NP_001043588	I			I
84	35857/6.32	Putative aldose reductase	NP_001055826	I			I
85	20539/6.45	Group 3 LEA type I protein	CAA92106	I		I	I
86	105091/5.38	Alpha glucan phosphorylase	AAK15695	I			I
96	17004/4.88	Root specific pathogenesis-related protein 10	BAD03969		I		
Primary metabolism							
8	69416/5.43	Putative transketolase 1	BAD67886	U			
12	65838/6.28	2-isopropylmalate synthase	EEC67654	U		U	
17	51806/7.16	Serine–glycine hydroxymethyltransferase	NP_001067846		D	D	
20	44871/6.21	Glutamate dehydrogenase 2	NP_001053457		U		
34	38784/5.73	Gln1-orysa glutamine synthetase root isozyme	AAN05339	U	D	U	
38	38784/5.73	Gln1-orysa glutamine synthetase root isozyme	AAN05339	U	D		
41	33743/5.38	Predicted amidohydrolase	EAY86155	D		D	D
87	51000/6.72	UMP synthase 1	XP_463746	I			I
88	44650/7.75	Aspartate aminotransferase	XP_463436	I			I
100	42088/5.82	3-phosphoshikimate 1-carboxyvinyltransferase	BAA32276		I		
Secondary metabolism							
10	76031/6.07	Phenylalanine ammonia-lyase	NP_001047481	D	D		
11	76031/6.07	Phenylalanine ammonia-lyase	NP_001047481		D		
23	43649/5.74	S-adenosylmethionine synthetase	AAT94053	D	D	D	
24	43338/5.68	S-adenosylmethionine synthetase 2	P93438	D			
30	42173/5.29	Actin	CAA33874	U	U		
33	40072/5.41	Putative Caffeic acid 3-*O*-methyltransferase	NP_001061031	D	D		
44	39455/7.16	UDP-glucuronic acid decarboxylase	BAB84334		D		
48	39455/7.16	UDP-glucuronic acid decarboxylase	BAB84334		D		
68	27928/5.11	Putative caffeoyl-CoA *O*-methyltransferase	NP_001062142	D	D	D	

Table 1 (*continued*)

Spot no.	M_r/pI	Protein identity	Accession no.	Treatments			
				AS	S	Aa	PA
Signal transduction							
9	60924/4.72	Calreticulin	NP_001052692	D	D	D	
27	48973/4.49	Putative calreticulin precursor	BAC82933	D	D		
29	42618/4.94	Putative calcium-binding protein	ABB46938	U		U	U
Protein turnover							
78	28515/7.07	Beta 7 subunit of 20S proteasome	BAD34432	I			I
80	34470/5.38	60S acidic ribosomal protein P0	XP_479931	I			I
Cell division							
1	90509/5.09	Cell division cycle protein 48, putative	AAP53974	U	U	U	
Unknown							
19	53430/6.13	Unnamed protein product	CAA28475	D		D	D
21	53421/6.14	Unnamed protein product	CAA28475	D	D	D	D
32	45185/5.68	Phosphoribulokinase precursor	NP_001047825	D		D	V
43	43837/8.53	OSJNBa0053K19.11	CAE03503	D	D	D	
57	30953/6.35	Hypothetical protein OsI_00040	EAY72188	U			
59	39725/7.29	Hypothetical protein OsI_27370	EAZ05175		D		
62	29115/6.35	Os01g0791033	BAH91327	D		D	D

M_r/pI indicates theoretical values for molecular weight and isoelectric point. U and D represent upregulated and downregulated spots, respectively. U or D in AS, S and Aa were resulted from comparison with Ctr, whereas in PA, with PC. Significantly changed protein spots in PA, AS, S and Aa ($p<0.05$), which altered in volume% more than 1.5-fold, were shown. The protein spots marked by I (induction) or V (vanishment) indicate that these spots were only undetectable in their controls or treatments, respectively, by the methods used in this report; thus they were still mentioned as upregulated or downregulated protein spots in text. PA, 10-day-old seedlings pretreated with ABA; PC, control of PA (in Kimura B nutrient solution without ABA); Ctr, seedlings in PC remained in Kimura B; S, seedlings in PC shifted to 150 mM NaCl; AS, seedlings in PA shifted to 150 mM NaCl; Aa, seedlings in PA shifted to Kimura B. All seedlings were treated for two days. For clarity, see Fig. 1.

reductase (spot 16) has been proved to elevate concentrations of glutathione, conferring tolerance to oxidative stress in plants [50]. Annexins (spot 60) are a family of Ca^{2+}-dependent membrane binding proteins and they may play important roles in plant defense against stresses [51], and may function as ion channels in the osmotic adjustment of plant cells [52]. Endo-1, 3-β-glucanase (spots 69 and 70) accumulates in rice in response to drought and ABA treatment [53]. Heat shock protein 90 (spot 82) also plays important role during stresses [54].

In proteins grouped into primary metabolism, the upregulation of glutamine synthetase and aspartate aminotransferase in seedlings of AS might result in enhanced glutamate production, to reassimilate the excess ammonia released during stress and ensure the production of stress related nitrogenous compound [55]. Glutamine synthetase is considered as a determinant component for salt stress tolerance [56,57]; thus the upregulation of glutamine synthetase can confer high salinity tolerance to the ABA-pretreated rice seedlings. Also, the upregulation of glutamate dehydrogenase in S treatment is reasonable, as previous studies have reported that when glutamine synthetase is inhibited, glutamate dehydrogenase often plays a complementary role in the reassimilation of excess ammonia released during stress conditions [58]. The upregulation of transketolases (spot 8) and UMP synthase 1 (spot 87) indicate that more sugar phosphate intermediates and pyrimidine nucleotides were produced to sustain growth under AS treatment. These are consistent with the morphological changes that rice seedlings in AS had higher growth rate than in S. On the other hand, the improved biosynthesis of amino acids and other organic solutes might be an efficient way for osmotic adjustment, leading to the better water status in seedlings of AS treatment (Fig. 2).

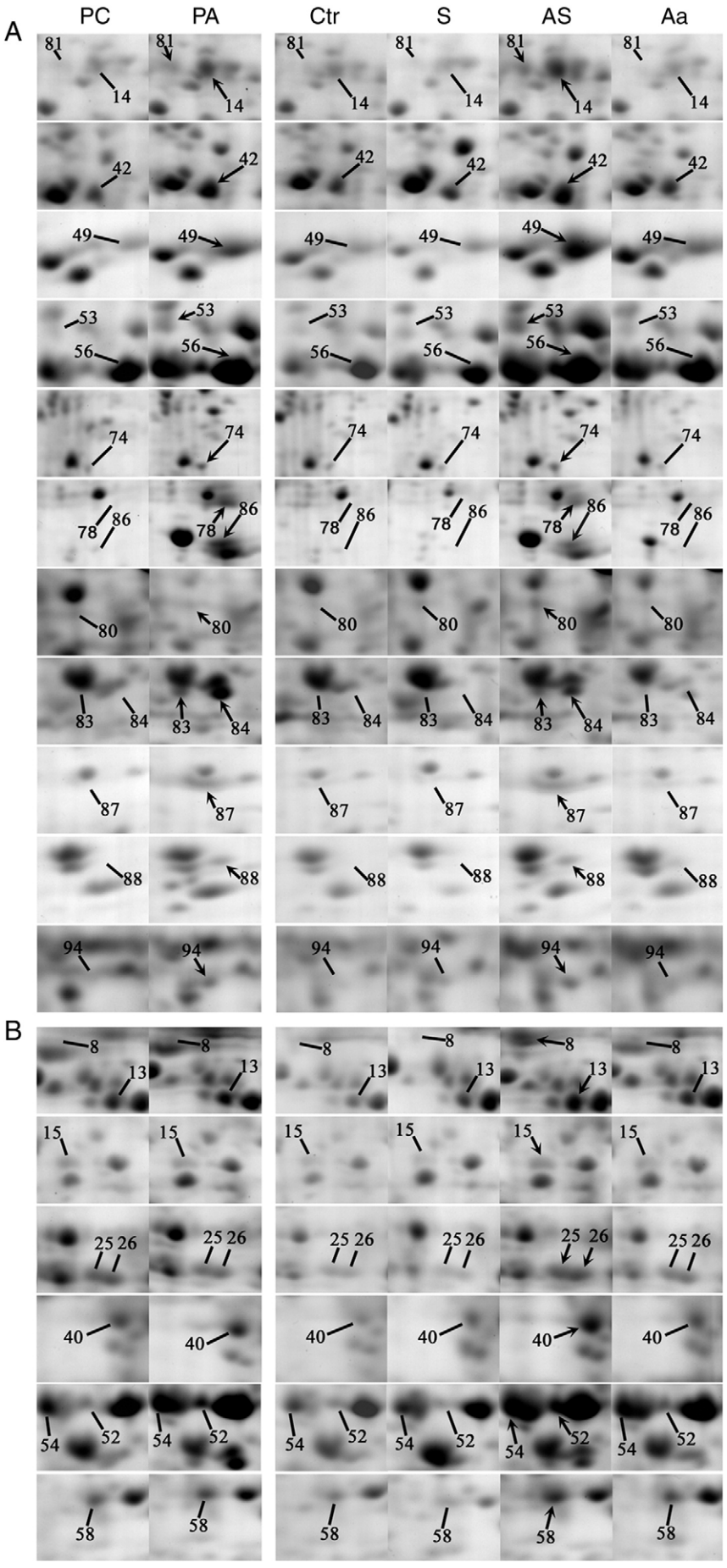
A previous study indicates that stress conditions impose increased demands for ubiquitin/proteasome-mediated proteolysis in plant cells [59]. The main function of the proteasome is to degrade unneeded or damaged proteins by proteolysis. Proteasomes are part of a major mechanism by which cells regulate the concentration of particular proteins and degrade misfolded proteins to synthesize new proteins. The induction of 20S proteasome (spot 78) in AS indicates that the activation of proteolysis to re-establish cellular homeostasis after stresses [60] and may be an important strategy to cope with salt stress. Studies on maize roots under flooding stress and frogs under anoxia or freezing stress indicate that regulation of 60S ribosomal subunit can help to promote selective translation of certain transcripts, which are potentially important to for them to survive stresses [61,62]. Similarly, 60S acidic ribosomal protein P0 (spot 80) induced in AS may play an adaptive role, *i.e.* translating transcripts selectively for survive salt stress. The induction of these proteins suggests a faster protein turnover and therefore a timely re-establishment of cellular homeostasis in seedlings under AS treatment when compared with those under S treatment.

Taken together, many more enzymes involved in energy metabolism, defense, primary metabolism, etc. were upregulated uniquely in AS, suggesting more abundant energy supply, more active anabolism

Table 2
The number of the differentially expressed protein spots in seedlings of AS and S treatments.

Function groups	Upregulated spots			Downregulated spots			Total
	AS	Share	S	AS	Share	S	
Energy metabolism	14	7		1	2	1	25
Defense-related	10	5	4				19
Primary metabolism	6		2	1		1	10
Secondary metabolism		1		3	4	1	9
Signal transduction	1				2		3
Protein turnover	2						2
Cell division		1					1
Unknown	1			1	2	3	7
Unidentified	6	2	10	1	3	2	24
Total	40	16	16	7	13	8	100

The differentially expressed proteins were classified into eight function groups and those proteins unidentified. These protein spots in each group were upregulated or downregulated spots, which were further classified into three subgroups according to where they were found differentially expressed: differentially expressed only in AS, only in S, and those shared by both. To avoid confusion, two protein spots classified in primary metabolism group (spots 34 and 38, see Table 1), which were upregulated in AS and downregulated in S, were only counted in those upregulated in AS.


A
PC
PA
Ctr
S
AS
Aa
81
14
42
49
53
56
74
78
86
80
83
84
87
88
94
B
8
13
15
25 26
40
54
52
58

(nitrogen, nucleotide acid, carbohydrate, etc), and more comprehensive defense systems in ABA-pretreated seedlings than in salt stressed ones.

4.2. The possible mechanisms underlying the different regulation of proteins in AS and S

Plants are known to display "cross talking" between abiotic stresses and ABA. In fact, ABA plays a major role in plant responses to salt stress [1], and many enzymes should be regulated by the same or overlapping stress signaling pathways. Therefore, ABA pretreatment resulted in somewhat similar regulation of proteins as in salt treatment. However, for rice seedlings in AS treatment, they had undergone ABA treatment prior to salt stress. More than half (58%) of the protein spots upregulated uniquely in AS treatment (compared to S) were also upregulated in ABA-pretreated seedlings (PA). These proteins shared by AS and PA treatments were analyzed by MALDI-TOF/MS, and bulk of the identified ones were classified as defense-related proteins. These proteins appear much earlier in AS than in S, and they are ready for fighting with the subsequent salt stress. In all, if the plants fail to respond in time and the appropriate defenses are activated too late, as happened in S of this report, the plants are adversely affected by the stress; whereas the ABA pretreatment activates appropriate defenses beforehand resulting in a more effective tolerance of the stress by the plants.

A previous report showed that the presence of NaCl is necessary for osmotin, a protein associated with salt adaptation, to accumulate after the induction by exogenous ABA [17]. In this study, the upregulation of many proteins differentially expressed in AS (not in S) was dependent on the presence of salt stress after the stimulation of ABA (Fig. 6). The molecular mechanism of this phenomenon may be complex. First, ABA accumulated to a higher level in AS treatment than in S (Fig. 4), which might lead to a stronger response and thereby more differentially expressed proteins. Second, promoters of some ABA and salt responsive genes harbor similar regulatory elements that are recognized by the same set of transcription factors [31,63]. After ABA treatment, some of the transcription factors had already accumulated, and a second stress can possibly result in increased concentration of the regulatory factors and thus result in subsequent re-activation of the signal pathway. But only the second stress (in this case salt) cannot activate the signal process because of the absence of the pre-accumulated factors. Perhaps this is why some proteins were not upregulated in S treatment. Third, after ABA pretreatment, the transcripts of these proteins might increase, resulting in the upregulation of some proteins or, possibly, insignificant upregulation of some proteins. However, the subsequent salinity stress might be required for preferential translation of these transcripts or for reduction of the turnover of some proteins, resulting in significant upregulation of most proteins. A 60S acidic ribosomal protein P0 (spot 80), which might have the function of translating transcripts selectively for survive salt stress, was induced in AS. Further work is needed to understand the complex mechanism leading to unique protein regulations in AS. Nevertheless, this might be an economical strategy that plants have evolved over time with the ABA signal serving as an adaptive mechanism that readies the plants in anticipation of environmental stresses, thus ensuring greater chance of tolerating stress and surviving; while in the absence of such stresses, the biological processes can be further adjusted by controlling enzyme turnover to save metabolites and energy for growth and development instead.

Acknowledgements

This work was supported by the National High-Tech Research and Development Program of China (2007AA10Z109), the National Basic Research Program of China (2007CB108905-3), and the Ministry of Science and Technology of China (602007DFA3077001).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbapap.2010.01.004.

References

[1] J.H. Zhang, W.S. Jia, J.C. Yang, A.M. Ismail, Role of ABA in integrating plant responses to drought and salt stresses, Field Crops Res. 97 (2006) 111–119.
[2] L. Xiong, K.S. Schumaker, J.K. Zhu, Cell signaling during cold, drought, and salt stress, Plant Cell (14 Suppl) (2002) S165–S183.
[3] H.H. Chen, P.H. Li, M.L. Brenner, Involvement of abscisic acid in paotato cold acclimation, Plant Physiol. 71 (1983) 362–365.
[4] A.J. Robertson, M. Ishikawa, L.V. Gusta, S.L. MacKenzie, Abscisic acid-induced heat tolerance in *Bromus inermis* Leyss cell-suspension cultures. Heat-stable, abscisic acid-responsive polypeptides in combination with sucrose confer enhanced thermostability, Plant Physiol. 105 (1994) 181–190.
[5] S. Lu, W. Su, H. Li, Z. Guo, Abscisic acid improves drought tolerance of triploid bermudagrass and involves H_2O_2- and NO-induced antioxidant enzyme activities, Plant Physiol. Biochem. 47 (2009) 132–138.
[6] H. Kato-Noguchi, Abscisic acid and hypoxic induction of anoxia tolerance in roots of lettuce seedlings, J. Exp. Bot. 51 (2000) 1939–1944.
[7] Y.T. Hsu, C.H. Kao, Role of abscisic acid in cadmium tolerance of rice (*Oryza sativa* L.) seedlings, Plant Cell Environ. 26 (2003) 867–874.
[8] P.C. Larosa, A.K. Handa, P.M. Hasegawa, R.A. Bressan, Abscisic acid accelerates adaptation of cultured tobacco cells to salt, Plant Physiol. 79 (1985) 138–142.
[9] P.B.K. Kishor, Salt stress in cultured rice cells: effects of proline and abscisic acid, Plant Cell Environ. 12 (1989) 629–633.
[10] P. Aarati, B.T. Krishnaprasad, B.T. Krishnaprasad, M. Ganeshkumar, R. Savitha, Gopalakrishna, G. Ramamohan, M. Udayakumar, Expression of an ABA responsive 21 kDa protein in finger millet (*Eleusine coracana* Gaertn.) under stress and its relevance in stress tolerance, Plant Sci. 164 (2003) 25–34.
[11] J.S. Bohra, H. Dörffling, K. Dörffling, Salinity tolerance of rice (*Oryza sativa* L.) with reference to endogenous and exogenous abscisic acid, J. Agron. Crop Sci. 174 (1995) 79–86.
[12] M. Khadri, N.A. Tejera, C. Lluch, Sodium chloride-ABA interaction in two common bean (*Phaseolus vulgaris*) cultivars differing in salinity tolerance, Environ. Exp. Bot. 60 (2007) 211–218.
[13] J. Pérez de Juan, J.J. Irigoyen, M. Sánchez-Díaz, Chilling of drought-hardened and non-hardened plants of different chilling sensitive maize lines: changes in water relations and ABA contents, Plant Sci. 122 (1997) 71–79.
[14] Y. Jiang, B. Huang, Protein alterations in tall fescue in response to drought stress and abscisic acid, Crop Sci. 42 (2002) 202–207.
[15] J. Zou, G.D. Abrams, D.L. Barton, D.C. Taylor, M.K. Pomeroy, S.R. Abrams, Induction of lipid and oleosin biosynthesis by (+)-abscisic acid and its metabolites in microspore-derived embryos of *Brassica napus* L.cv Reston (biological responses in the presence of 8[prime]-hydroxyabscisic acid), Plant Physiol. 108 (1995) 563–571.
[16] T.K. Prasad, M.D. Anderson, C.R. Stewart, Acclimation, hydrogen peroxide, and abscisic acid protect mitochondria against irreversible chilling injury in maize seedlings, Plant Physiol. 105 (1994) 619–627.
[17] N.K. Singh, D.E. Nelson, D. Kuhn, P.M. Hasegawa, R.A. Bressan, Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential, Plant Physiol. 90 (1989) 1096–1101.
[18] A. Moons, A. DeKeyser, M. VanMontagu, A group 3 LEA cDNA of rice, responsive to abscisic acid, but not to jasmonic acid, shows variety-specific differences in salt stress response, Gene 191 (1997) 197–204.
[19] A. Moons, J. Gielen, J. Vandekerckhove, D. Van der Straeten, G. Gheysen, M. Van Montagu, An abscisic-acid- and salt-stress-responsive rice cDNA from a novel plant gene family, Planta 202 (1997) 443–454.
[20] K. Skriver, J. Mundy, Gene expression in response to abscisic acid and osmotic stress, Plant Cell 2 (1990) 503–512.
[21] Y. Xiang, N. Tang, H. Du, H.Y. Ye, L.Z. Xiong, Characterization of OsbZIP23 as a key player of the basic leucine zipper transcription factor family for conferring abscisic acid sensitivity and salinity and drought tolerance in rice, Plant Physiol. 148 (2008) 1938–1952.
[22] J.A. Godoy, J.M. Pardo, J.A. Pintor-Toro, A tomato cDNA inducible by salt stress and abscisic acid: nucleotide sequence and expression pattern, Plant Mol. Biol. 15 (1990) 695–705.
[23] M. Seki, J. Ishida, M. Narusaka, M. Fujita, T. Nanjo, T. Umezawa, A. Kamiya, M. Nakajima, A. Enju, T. Sakurai, M. Satou, K. Akiyama, K. Yamaguchi-Shinozaki, P. Carninci, J. Kawai, Y. Hayashizaki, K. Shinozaki, Monitoring the expression pattern of around 7,000 *Arabidopsis* genes under ABA treatments using a full-length cDNA microarray, Funct. Integr. Genomics 2 (2002) 282–291.

Fig. 6. Identified protein spots upregulated in AS and PA but not in S or Aa treatments (A) and protein spots upregulated in AS but not in S, Aa or PA treatments (B). Upregulated protein spots in AS or PA are indicated by arrows, while lines indicate the protein spots or invisible ones at the corresponding positions in other treatments. PC, PA, Ctr, S, AS and Aa were different treatments as described in Fig. 1.

[24] M.A. Rabbani, K. Maruyama, H. Abe, M.A. Khan, K. Katsura, Y. Ito, K. Yoshiwara, M. Seki, K. Shinozaki, K. Yamaguchi-Shinozaki, Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA get-blot analyses, Plant Physiol. 133 (2003) 1755–1767.
[25] D.Y. Chao, Y.H. Luo, M. Shi, D. Luo, H.X. Lin, Salt-responsive genes in rice revealed by cDNA microarray analysis, Cell Res. 15 (2005) 796–810.
[26] A. Matsui, J. Ishida, T. Morosawa, Y. Mochizuki, E. Kaminuma, T.A. Endo, M. Okamoto, E. Nambara, M. Nakajima, M. Kawashima, M. Satou, J.M. Kim, N. Kobayashi, T. Toyoda, K. Shinozaki, M. Seki, *Arabidopsis* transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array, Plant Cell Physiol. 49 (2008) 1135–1149.
[27] Z. Xin, P.H. Li, Alteration of gene expression associated with abscisic acid-induced chilling tolerance in maize suspension-cultured cells, Plant Physiol. 101 (1993) 277–284.
[28] N.K. Singh, P.C. Larosa, A.K. Handa, P.M. Hasegawa, R.A. Bressan, Hormonal regulation of protein synthesis associated with salt tolerance in plant cells, Proc. Natl. Acad. Sci. U. S. A. 84 (1987) 739–743.
[29] A. Moons, G. Bauw, E. Prinsen, M. Van Montagu, D. Van der Straeten, Molecular and physiological responses to abscisic acid and salts in roots of salt-sensitive and salt-tolerant Indica rice varieties, Plant Physiol. 107 (1995) 177–186.
[30] N. Shimada, in: Y. Noguchi, S. Kawada (Eds.), Dictionary of Agriculture, Yokendo, Tokyo, 1987, p. 817.
[31] K.N. Kim, Y.H. Cheong, J.J. Grant, G.K. Pandey, S. Luan, *CIPK3*, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in *Arabidopsis*, Plant Cell 15 (2003) 411–423.
[32] L. Debellis, R. Tsugeki, A. Alpi, M. Nishimura, Purification and characterization of aconitase isoforms from etiolated pumpkin cotyledons, Physiol. Plant 88 (1993) 485–492.
[33] T. Nyström, F.C. Neidhardt, Expression and role of the universal stress protein, UspA, of *Escherichia coli* during growth arrest, Mol. Microbiol. 11 (1994) 537–544.
[34] M. Sauter, G. Rzewuski, T. Marwedel, R. Lorbiecke, The novel ethylene-regulated gene *OsUsp1* from rice encodes a member of a plant protein family related to prokaryotic universal stress proteins, J. Exp. Bot. 53 (2002) 2325–2331.
[35] S.M. Bagnasco, S. Uchida, R.S. Balaban, P.F. Kador, M.B. Burg, Induction of aldose reductase and sorbitol in renal inner medullary cells by elevated extracellular NaCl, Proc. Natl. Acad. Sci. U. S. A. 84 (1987) 1718–1720.
[36] S.P. Lee, T.H. Chen, Molecular cloning of abscisic acid-responsive mRNAs expressed during the induction of freezing tolerance in bromegrass (*Bromus inermis* Leyss) suspension culture, Plant Physiol. 101 (1993) 1089–1096.
[37] D. Xu, X. Duan, B. Wang, B. Hong, T. Ho, R. Wu, Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice, Plant Physiol. 110 (1996) 249–257.
[38] S.C. Zeeman, D. Thorneycroft, N. Schupp, A. Chapple, M. Weck, H. Dunstan, P. Haldimann, N. Bechtold, A.M. Smith, S.M. Smith, Plastidial alpha-glucan phosphorylase is not required for starch degradation in *Arabidopsis* leaves but has a role in the tolerance of abiotic stress, Plant Physiol. 135 (2004) 849–858.
[39] M. Schröder, N. Giermann, R. Zrenner, Functional analysis of the pyrimidine *de novo* synthesis pathway in solanaceous species, Plant Physiol. 138 (2005) 1926–1938.
[40] M. Bar-Peled, C.L. Griffith, T.L. Doering, Functional cloning and characterization of a UDP-glucuronic acid decarboxylase: the pathogenic fungus *Cryptococcus neoformans* elucidates UDP-xylose synthesis, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 12003–12008.
[41] D.W. Kim, J. Shibato, G.K. Agrawal, S. Fujihara, H. Iwahashi, D.H. Kim, I.S. Shim, R. Rakwal, Gene transcription in the leaves of rice undergoing salt-induced morphological changes (*Oryza sativa* L.), Mol. Cells 24 (2007) 45–59.
[42] Z. Xin, P.H. Li, Abscisic acid-induced chilling tolerance in maize suspension-cultured cells, Plant Physiol. 99 (1992) 707–711.
[43] M.J. Jeong, S.C. Park, M.O. Byun, Improvement of salt tolerance in transgenic potato plants by glyceraldehyde-3 phosphate dehydrogenase gene transfer, Mol. Cells 12 (2001) 185–189.
[44] F.U. Hartl, N. Pfanner, D.W. Nicholson, W. Neupert, Mitochondrial protein import, Biochim. Biophys. Acta 988 (1989) 1–45.
[45] R. Li, M. Moore, P.C. Bonham-Smith, J. King, Overexpression of formate dehydrogenase in *Arabidopsis thaliana* resulted in plants tolerant to high concentrations of formate, J. Plant Physiol. 159 (2002) 1069–1076.
[46] C. Hourton-Cabassa, F. Ambard-Bretteville, F. Moreau, J. Davy de Virville, R. Remy, C.C. Francs-Small, Stress induction of mitochondrial formate dehydrogenase in potato leaves, Plant Physiol. 116 (1998) 627–635.
[47] D. Xu, X. Liu, C. Guo, J. Zhao, Methylglyoxal detoxification by an aldo-keto reductase in the cyanobacterium *Synechococcus* sp. PCC 7002, Microbiology 152 (2006) 2013–2021.
[48] H.M. Chen, S.C. Chang, C.C. Wu, T.S. Cuo, J.S. Wu, R.H. Juang, Regulation of the catalytic behaviour of L-form starch phosphorylase from sweet potato roots by proteolysis, Physiol. Plant 114 (2002) 506–515.
[49] N. Imin, T. Kerim, B.G. Rolfe, J.J. Weinman, Effect of early cold stress on the maturation of rice anthers, Proteomics 4 (2004) 1873–1882.
[50] R.T. el-Khatib, E.P. Hamerlynck, F. Gallardo, E.G. Kirby, Transgenic poplar characterized by ectopic expression of a pine cytosolic glutamine synthetase gene exhibits enhanced tolerance to water stress, Tree Physiol. 24 (2004) 729–736.
[51] X. Gidrol, P.A. Sabelli, Y.S. Fern, A.K. Kush, Annexin-like protein from *Arabidopsis thaliana* rescues delta oxyR mutant of *Escherichia coli* from H_2O_2 stress, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 11268–11273.
[52] S. Lee, E.J. Lee, E.J. Yang, J.E. Lee, A.R. Park, W.H. Song, O.K. Park, Proteomic identification of annexins, calcium-dependent membrane binding proteins that mediate osmotic stress and abscisic acid signal transduction in *Arabidopsis*, Plant Cell 16 (2004) 1378–1391.
[53] T. Akiyama, M.A. Pillai, Molecular cloning, characterization and in vitro expression of a novel endo-1, 3-β-glucanase up-regulated by ABA and drought stress in rice (*Oryza sativa* L.), Plant Sci. 161 (2001) 1089–1098.
[54] W. Wang, B. Vinocur, O. Shoseyov, A. Altman, Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response, Trends Plant Sci. 9 (2004) 244–252.
[55] A. Santa-Cruz, M. Acosta, A. Rus, M.C. Bolarin, Short-term salt tolerance mechanisms in differentially salt tolerant tomato species, Plant Physiol. Biochem. 37 (1999) 65–71.
[56] S. Yan, Z. Tang, W. Su, W. Sun, Proteomic analysis of salt stress-responsive proteins in rice root, Proteomics 5 (2005) 235–244.
[57] A.C. Sahu, S.K. Sahoo, N. Sahoo, NaCl-stress induced alteration in glutamine synthetase activity in excised senescing leaves of a salt-sensitive and a salt-tolerant rice cultivar in light and darkness, Plant Growth Regul. 34 (2001) 287–292.
[58] H.S. Srivastava, R.P. Singh, Role and regulation of L-glutamate dehydrogenase activity in higher plants, Phytochem 26 (1987) 597–610.
[59] W.R. Belknap, J.E. Garbarino, The role of ubiquitin in plant senescence and stress responses, Trends Plant Sci. 1 (1996) 331–335.
[60] T. Grune, Oxidative stress, aging and the proteasomal system, Biogerontology 1 (2000) 31–40.
[61] J. Bailey-Serres, S. Vangala, K. Szick, C.H. Lee, Acidic phosphoprotein complex of the 60S ribosomal subunit of maize seedling roots. Components and changes in response to flooding, Plant Physiol. 114 (1997) 1293–1305.
[62] S. Wu, K.B. Storey, Up-regulation of acidic ribosomal phosphoprotein P0 in response to freezing or anoxia in the freeze tolerant wood frog, *Rana sylvatica*, Cryobiology 50 (2005) 71–82.
[63] K. Shinozaki, K. Yamaguchi-Shinozaki, Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways, Curr. Opin. Plant Biol. 3 (2000) 217–223.