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A PROTEOMIC APPROACH TO IDENTIFY SALT-RESPONSIVE PROTEINS IN RYE

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Abstract

Salinity stress negatively impacts agricultural yield throughout the world affecting production whether it is for subsistence or economic gain. Despite their great degree of stress tolerance, minor crops have received little attention regarding their response against salinity compared to model plants and major crops. Rye, an economically important minor grain crop, is relatively tolerant to abiotic stresses and grown in marginal lands. Proteome study based on two dimensional gel electrophoresis and subsequent mass spectrometric identification was performed in order to analyze the salt-stress response of rye. Overall changes in the protein complement of leaves after four days exposure to 200 mM NaCl were studied. Seventeen reproducibly resolved, differentially expressed protein spots were identified by MALDI-TOF MS (mass spectrometry). Among them, ten proteins were up-regulated, and seven proteins were down-regulated. Majority of the differentially expressed proteins belonged to the functional category of antioxidant metabolism and photosyntheis. Increased expression of reactive oxygen species-scavenging enzymes eg, catalase, cytosolic, monodehydroascorbate reductase and 2-cys-peroxiredoxin may have important role in salinity-induced ROS metabolism. This is the first proteomic description of salt stress response in rye and the identification of such differentially expressed proteins would provide deeper insight pertaining to the stress physiology of this important crop plant.

Introduction

Most cultivated plants are glycophytes i.e, they are very sensitive towards soil salinity. Soil salinity is a major constraint to food production because it limits crop yield and restricts use of land that were previously uncultivated (Azeem & Ahmad, 2011; Mahmood, 2011; Yousaf, 2011). The United Nations Environment Program estimates that approximately 20% of agricultural land and 50% of cropland in the world is salt-stressed (Flowers & Yeo 1995). As the global population increases, water resource management is deteriorating and environmental pollution is worsening. Consequently, salinization of land is becoming more severe that begun to hinder development of agricultural economics. Soil salinity can be resulted from the capillary transport of a salt laden water table followed by accumulation due to evaporation. It can also be concentrated in soils due to human activity, for example the use of fertilizers and low quality irrigation water.

Exposure to high-salt conditions generally cause alterations in plant metabolism, including reduction in the water potential, ion imbalances and toxicity, reduction in CO_2 assimilation, and susceptibility to injury and oxidative stress. These results in slow growth, wilting or even death of most plant species (Cheeseman 1988, Munns & Tester 2008). Na^+ and Cl^- are the ions that are responsible for the majority of agricultural losses. In most cases, NaCl accumulates in addition to the other soil ions, the osmotic potential of the soil increases, and obstructs the uptake of water. Uncontrolled Na+ uptake can occur by voltage-insensitive monovalent cation channels (White 1999), or members of the H+/K+ transporter family (Rodríguez-Navarro 2000, Rubio *et al*., 1999). The salt tolerance of non-halophytes depends on their ability to restrict Na^+ and Cl^- uptake and to sequester these ions into vacuoles when they do enter the tissues. In addition, cells need to maintain adequate K^+ concentrations and the production of compatible organic solutes, such as glycinebetaine and proline (Islam *et al*., 2007). Salt stress-induced modifications can lead to the accumulation of certain metabolites and they can lead to the appearance or disappearance of some cellular proteins or to decreases or increases in the abundances of others (Kong-Ngern *et al*., 2005). However, only limited information is available on salt-responsive proteins and the molecular mechanisms of salt tolerance in plants.

Stalinization can be managed by changed farm management practices. In rain-fed agriculture, practices such as rotation of annual crops with deep-rooted species may restore the balance between rainfall and water use, thus preventing rising water tables bringing salts to the surface. All such practices will rely on a high degree of salt tolerance, not only of the perennial species used to lower a saline water table, but also of the crops to follow, as some salt will remain in the soil (Munns 2002). Rye (*Secale cereale*) is a grass grown as a grain and as a forage crop. It is closely related to barley and wheat. It has tremendous economic value especially, in developing countries, as it is cultivated in marginal land and grows well in salt-affected soils.

The basic resources for biotechnology are genetic determinants of salt tolerance and yield stability. Implementation of biotechnology strategies to achieve this goal requires that substantial research effort be focused to on identify salt tolerance effectors and the regulatory components that control these during the stress episode (Hasegawa *et al*., 2000). Proteomics, the large-scale analysis of proteins, have been receiving an increased attention in studying cellular functions. Due to their ability to provide comparative abundance information directly at the level of proteins, that carry out and regulate most functions within a cell, 'differential display' proteomics workflows are gaining increasing momentum in the proteomics field to study various stress response. By focusing on the gene products, proteomics has started to

play an increasingly important role in genome annotation and cloning genes for biotechnological applications (Alam *et al*., 2011). Salt stress-induced proteomic profiling has been studied extensively in crop plants including wheat (Gao *et al*., 2011), sorghum (Swami *et al*., 2011), soybean (Alam *et al*., 2011), sugar beet (Wakeel *et al*., 2011) cabbage (Fernandez-Garcia *et al*., 2011) and tomato (Manaa *et al*., 2011). However, conclusions derived from research conducted on these plants may not be equally applicable to other species. Thus, research on species-specific responses to a particular abiotic stress is needed. Rye grows well in much poorer soils than those necessary for most cereal grains; it is especially valuable crop in molecular studies since they may retain regulatory mechanisms enabling their survival and productivity under adverse condition.

To our knowledge, proteomic analysis has not been reported for this important germplasm. Here, we report a comparative proteomic analysis of rye leaves grown under high salinity stress. The study of global protein expression in response to salinity may help to identify the involved genes and provide a detailed network of the salt-response mechanisms in rye.

Materials and Methods

Plant growth and treatments: Rye (*Secale cereale* cv Paldang) seeds were imbibed in water, planted on commercial potting mix in plastic trays, and allowed to grow in a growth chamber. After 15 days, the seedlings were subjected to treatments. To impose salt stress, the seedlings were irrigated daily with a 200mM NaCl solution. Control group was irrigated with tap water. About 85-90% field capacity was maintained for both the groups. The seedlings were maintained under light condition (500 µmol m^{-2} s^{-1}, 16/8h light/dark period) at 25°C and 55-65% humidity. Following a four-day treatment, leaves were excised from untreated (control) and treated seedlings and were used for proteomic analysis.

Protein extraction and 2-D PAGE: Proteins were extracted from the leaf samples using a phenol extraction method described previously (Alam *et al*., 2010). Briefly, 500 mg of tissue was homogenized with a Mg/NP-40 extraction buffer [0.5 M Tris-HCl, pH 8.3, 2% (v/v) NP-40, 20 mM $MgCl_2$, 1 mM phenyl methyl sulfonyl fluoride, 2% (v/v) β-mercaptoethanol and 1% (w/v) polyvinyl polypyrrolidone] and fractionated with water-saturated phenol, followed by centrifugation at 12,000 ×*g* for 15 min.

The proteins were recovered from the supernatant by precipitation with ammonium acetate in methanol. The protein samples were then quantified using the Bradford assay (Bradford 1976) and subjected to two-dimensional gel electrophoresis (2-DE) using a standard procedure. Immobilized pH gradient (IPG) dry strips were equilibrated for 12-16 hours with 7 M urea, 2M thiourea containing 2% 3-[(3-cholamidopropy) dimethyammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), 1% pharmalyte and respectively loaded with 400 µg of sample.

Isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit and EPS 3500 XL power supply (Amersham Biosciences) following manufacturer's instruction. For IEF, the voltage was linearly increased from 150 to 3,500V during 3 hours for sample entry followed by constant 3,500V, with focusing complete after 96kVh. Prior to the second dimension, strips were incubated for 10 minutes in equilibration buffer (50mM Tris-Cl, pH6.8 containing 6M urea, 2% SDS and 30% glycerol), first with 1% DTT and second with 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS-PAGE gels (20 x 24cm, 10-16%).

SDS-PAGE was performed using Hoefer DALT 2D system (Amersham Biosciences) following manufacturer's instruction. 2D gels were run at 20°C for 1,700Vh. After that, 2D gels were stained by silver staining as described previously (Oakley *et al*., 1980) but fixing and sensitization step with glutaraldehyde was omitted.

Image analysis: Gel images were acquired using a high-resolution scanner (GS-800 Calibrated Imaging Densitometer; Bio-Rad, Hercules, CA, USA. Spots were detected, quantified and then matched using the Bio-Rad PDQuest software (Version 7.2; Bio-Rad).

To compensate for the variability in gel staining, the volume of each spot (spot abundance) was normalized as a relative volume of total spot intensity. A minimum of three gels were generated for each sample. Only spots that showed significant and reproducible (present in three out of three gels) changes of at least 1.5-fold were considered differentially expressed proteins. The standard error (SE) was calculated from three spots in replicated gels.

Enzymatic digestion of proteins: Protein spots were enzymatically digested in-gel in a manner similar to that previously described (Shevchenko *et al*., 1996) and using modified porcine trypsin. Gel pieces were washed with 50% acetonitrile to remove SDS, salt and stain, dried to remove solvent and then rehydrated with trypsin (8-10 ng/µl) and incubated 8-10h at 37°C. The proteolytic reaction was terminated by adding 5 µl 0.5% trifluoroacetic acid. Tryptic peptides were recovered by combining the aqueous phase from several extractions of gel pieces with 50% aqueous acetonitrile. After concentration the peptide mixture was desalted using C_{18}ZipTips (Millipore), and peptides eluted in 1-5 µl of acetonitrile. An aliquot of this solution was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile, and 1 µl of mixture spotted onto a target plate.

MALDI-TOF analysis and database search: Peptide samples were analyzed using a Ettan MALDI-TOF mass spectrometer (Amersham Biosciences). Peptides were evaporated with a N2 laser at 337nm, and using a delayed extraction approach. They were accelerated with 20Kv injection pulse for time of flight analysis. Each spectrum was the cumulative average of 300 laser shots. The online search program MASCOT (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2 &SEARCH =PMF) was used for protein identification by peptide mass fingerprinting. Spectra were calibrated with trypsin auto-digestion ion peak *m/z* (842.510, 2211.1046) as internal standards. The search was performed within all green plants (Viridiplantae)

using the following parameters: the maximum number of missed cleavages was set at one, the complete carbamidomethylation of cysteines and variable oxidation of methionines was assumed, monoisotopic masses were used. Statistically significant heats (confidence 95% or more) were chosen.

Statistical analysis: Results of the spot intensity were statistically analyzed by using analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) to determine significant differences among group means. Significant differences from control values were determined at $p<0.05$ levels. All the results are represented as means ± SE of at least three independent replicates. The statistical program SAS, version 9.1 (SAS Institute, Cary, NC, USA) was used for the statistical analyses.

Results and Discussion

Morphological changes due to stress imposition: After exposure of the plants to 200 mM NaCl, considerable morphological changes were observed. In addition to growth reduction, leaves were wilted because of the loss of water and osmotic stress (Fig. 1). The delay in growth was clearly visible after 2 days of the salt-stress treatment.

Fig. 1. Effect of salt stress on the rye plants. Followed by a two-week growth, the plants were treated with 200 mM NaCl for four days and used for proteomic analysis

Proteomic alteration of rye seedlings in response to salt stress: To investigate the molecular mechanisms involved in the regulation of salinity in rye seedlings, differentially expressed proteins were identified through proteome analysis. To analyze the proteome complement, rye seedlings (2-week-old) were irrigated with 200 mM sodium chloride solution for 4 days. Following the treatments, proteins were extracted from the leaves and were analyzed by 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Fig. 2 shows 2D gel images obtained from leaf proteome treated with 200 mM NaCl- and their corresponding controls. On the silver-stained 2-DE gels, over 750 highly reproducible protein spots were detected. The quantity of differentially expressed proteins on the 2D gels was reproducible. The highest number of spots was detected in the pI range of 4-7 (Fig. 2). The global pattern of proteins remained largely unaltered in terms of numbers and in-gel positions. We analyzed the number of differentially regulated proteins (up-regulated/down-regulated) in controls in comparison to the NaCl-treated plant for leaves). Among the well-resolved spots, densitometric analysis of the replicated gels reveal that 30 protein spots exhibited a greater than 1.5-fold change in abundance following 4 days of salt stress. Among the differentially accumulated protein spots, 17 spots were identified by MALDI-TOF MS (Table 1). Enlarged gel picture showing the differentially expressed spots (Fig. 3). The relative abundance of the protein spots on the gel is shown in Fig. 4. A comparison of the differential expression of the proteins revealed that 10 proteins were up-regulated and 7 were down-regulated (Fig. 4).

As an advantage of using classical two-dimensional electrophoresis, we were able to detect multiple spots differing in p*I* and *M*r as the same protein. For example, two species were detected for Rubisco large subunit (spot 7607, 8612), oxygen-evolving enhancer protein 2 (spot 4203, 4204) and UTP-glucose-1-phosphate uridylyltransferase (UGPase; spot 2601, 2701), which suggests possible post-translational modification(s) of this protein. This type of information cannot be obtained using transcriptomic approaches. The potential to identify these alternative protein forms is an advantage of using classical two-dimensional electrophoresis because that type of information is not accessible by genomic technologies. The identified proteins were broadly classified into several functional categories including (1) photosynthesis and energy metabolism, (2) antioxidant metabolism, (3) stress-responsive proteins and (4) unknown proteins.

Photosynthesis and energy metabolism: We identified several spots representing photosynthesis-related proteins including Rubisco large sub unit, oxygen evolving enhancer (OEE) protein. Rubisco (spot 7607, 8612) and OEE (spot 4203, 4204) were down-regulated by salt stress. Spot 6002 was identified as a unknown protein. The homology search using BLASTP revealed that this protein shares 100% positivity at the amino acid level (data not shown) with Rubisco small subunit suggesting that these are identical. Rubisco plays bifunctional roles as a carboxylase for mediating photosynthetic CO_2 assimilation, and as an oxygenase for catalyzing the first step of the photorespiratory pathway in plants. Except for the up-regulation of spot 8612, all the other protein spots were significantly down-regulated by salt stress. Salt stress suppresses the carboxylase activity of Rubisco while promoting its oxygenase activity (Sivakumar *et al*., 2000). On the other hand, Enhancement of these proteins in maize under salt stress was also reported (Zörb *et al*., 2004), while expression of Rubisco was not altered in salt-treated sorghum (Swami *et al*., 2011). Thus, a decreased expression may reflect on damage of photosynthetic machinery. An increased Rubisco level may reflect an increased Rubisco oxygenase activity. Photorespiration might be enhanced by salt stress as reported by (Di Martino *et al*., 1999). Therefore, we assumed that the accumulation of Rubisco in this study could more likely reflect the increase of photorespiration under salt stress.

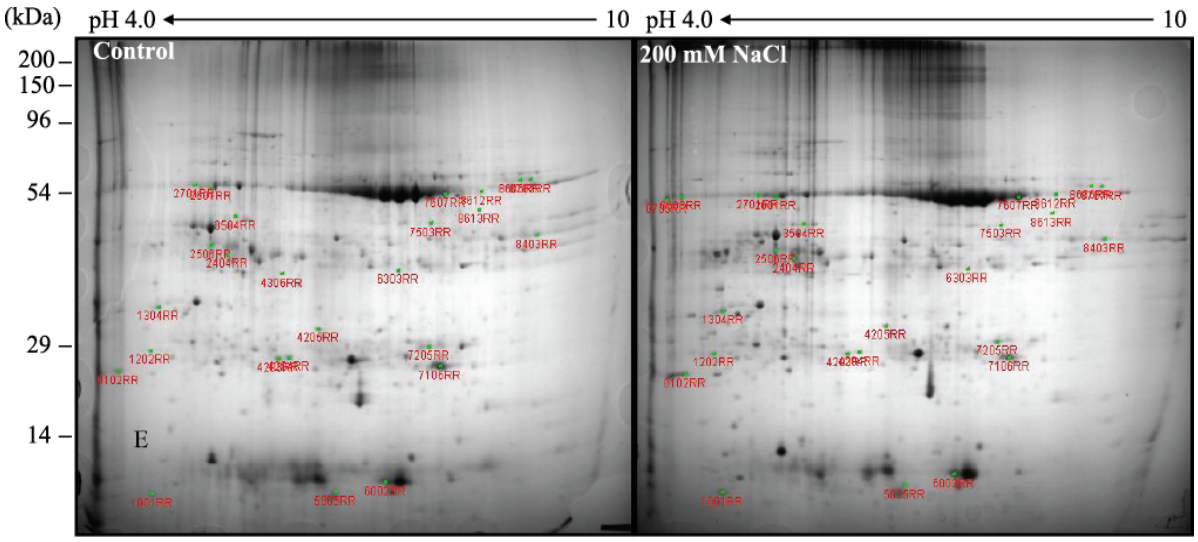

Fig. 2. A 2-DE analysis of rye leaf proteins under salt stress. A total of 150 μg of protein was separated by 2-D as described in the Materials and Methods and visualized with silver stain. A representative 2-DE map of control seedlings or seedlings treated with a 200mM salt for four days. The numbers indicate differentially expressed proteins in response to the salt stress.

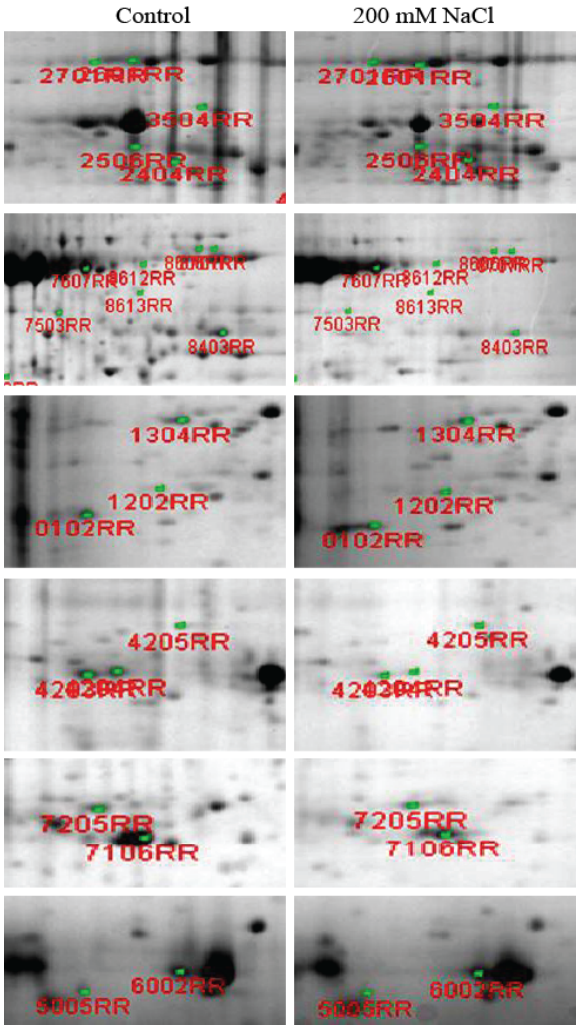

Fig. 3. Enlarged views of gels of the differentially expressed proteins, which are identified (Table 1) and marked in figure 2.

We also identified another protein related to Rubisco, namely OEE, which is involved in light-induced oxidation of water for the evolution of oxygen in photosystem II in plants. (Thornton *et al*., 2004) Thus, all of these proteins have important roles in photosynthesis and down-regulation of these proteins shows that one of the target points of salt stress effects on rye plants is photosynthetic system which results in decreased growth. The modulation of photosynthesis in response to salt stress has been demonstrated previously. For example, saline and hyperosmotic stresses have inhibitory effects on the electron transfer activity of photosystem I (Allakhverdiev *et al*., 2000).

Another spot (8403) was identified as glyceraldehyde-3-phosphate dehydrogenase, which is down-regulated in response to salt stress. This protein is involved in glycolysis, indicating a possible reduced glycolytic flux under the salinity stress. In addition, it may participate in nuclear events including transcription, RNA transport, DNA replication and apoptosis. Nuclear functions are probably due to the nitrosylase activity that mediates cysteine S-nitrosylation of nuclear target proteins such as SIRT1, HDAC2 and PRKDC. Another glycolytic protein UDP-glucose pyrophosphorylase (UGPase, EC 2.7.7.9; spot 2601, 2701) was identified as 2 spots. One is up-regulated (spot 2601), while another one is down-regulated (spot 2701). The appearance and different expression pattern might be due to different posttranslational modification. In glycolysis, UGPase catalyzes the reversible production of UDP glucose and pyrophosphate from glucose-1-P and UTP. In sucrose synthesis, the glucose-1-phosphate is converted to UDP-glucose via a specific UGPase that is analogous to the ADP-glucose pyrophosphorylase of chloroplasts. An increased expression in this protein may indicate reduced requirement of photoassimilates due to growth depression.

These could be deposited in the cells as sucrose/starch. During salt stress, these molecules may assist in marinating osmotic stress.

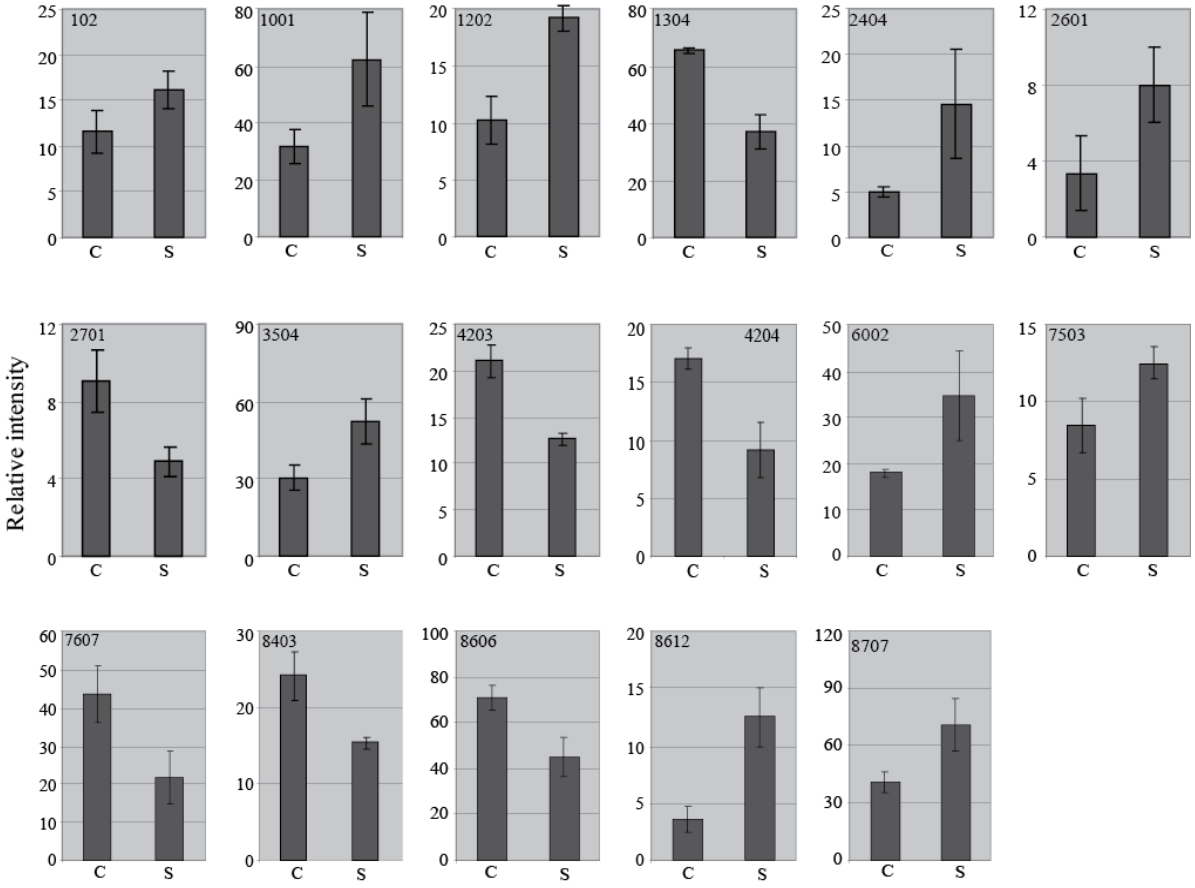

Fig. 4. The expression levels of the identified proteins compared to those of controls. Bars indicates the relative expression level of control (C) and salt stress (S) of individual spots mentioned. Spot intensities were measured using a densitometer and compared to those of the controls. The data represent the mean values and SE of three independent experiments. Different letters above the bars indicate statistically significant differences ($p<0.05$).

Proteins associated with antioxidant metabolism: Salt stress is complex and imposes a water deficit because of osmotic effects on a wide variety of metabolic activities (Cheeseman 1988). This water deficit leads to the formation of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$). These cytotoxic activated oxygen species can seriously disrupt normal metabolism through oxidative damage to lipids and to protein and nucleic acids. Since internal O_2 concentrations are high during photosynthesis chloroplasts are especially prone to generate activated oxygen species. To prevent damage to cellular components by ROS, plants have developed a complex antioxidant system. The primary components of this system include carotenoids, ascorbate, glutathione and tocopherols, in addition to enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), peroxidases, and the enzymes involved in the ascorbate-glutathione cycle (Foyer & Halliwell 1976), such as ascorbate peroxidase (APX) and glutathione reductase (GR). Many components of this antioxidant defense system can be found in various sub-cellular compartments (Hernández *et al*., 2000). In our experiment a number of proteins were increased in expression, which are associated with the different antioxidation metabolism pathway were altered under high salinity. These proteins include 2-cys peroxiredoxin-like protein Catalase-1 (spot 8606, 8707) and cytosolic monodehydroascorbate reductase (spot 3504). The scavenging of ROS by increased activation of antioxidant enzymes can improve salt tolerance (Alscher *et al*., 1997). A relationship between salt tolerance and increased activation of antioxidant enzymes has been demonstrated in *Plantago* (Hediye Sekmen *et al*., 2007), pea (Hernández *et al*., 2000), *Arabidopsis*, rice (Dionisio-Sese & Tobita 1998), soybean (Phang *et al*., 2008), tomato and maize (de Azevedo Neto *et al*., 2006).

2-Cys Prx is a member of peroxiredoxins (Prx) family, which are ubiquitous enzymes that use their highly reactive cysteine residues to decompose peroxides (Wood *et al*., 2003). Based on the number of conserved Cys residues that participate in the catalytic cycle, Prxs are largely divided into 2 groups, 1-Cys Prxs and 2-Cys Prxs. Prxs exert their protective antioxidant role in cells through their peroxidase activity ($ROOH+2e^{-}\rightarrow ROH+H_2O$), whereby hydrogen peroxide, peroxynitrite and a wide range of organic hydroperoxides (ROOH) are reduced and detoxified. The redox biochemistry of Prx recycling between disulfide/sulfenic acid to the sulfhydryl form (Rhee *et al*., 2005) may be related to the fact that Prxs protect against several stresses (Demasi *et al*., 2006; Huang *et al*., 2003). 2-Cys-prx is involved in multiple cellular processes such as antioxidant defense (Neumann *et al*., 2003), H_2O_2-mediated

cellular signaling (Choi *et al*., 2005) and molecular chaperones (Jang *et al*., 2004). Although, 2-Cys-Prx serves as an important antioxidant component, the peroxidase activity is completely inactivated by oxidative stress and oligomerized into the thylakoid membrane of plant chloroplasts (König *et al*., 2002). The stress dependent structural and functional switching of C2C-Prxs could play pivotal roles in plant cells to adjust antioxidative redox signaling cascades and to protect denaturation of intracellular macromolecules from a broad range of external stresses. Overexpression of an *Arabidopsis* 2-Cys-Prx gene protected photosynthetic machinery during heat stress in tall fescue.

Catalase is involved in the degradation of hydrogen peroxide into water and oxygen, is the most effective antioxidant enzymes in preventing oxidative damage (Mittler 2002). In our present experiment, two spots were identified as catalase; one is upregulated, another one is downregulated. Similar to our findings, increased catalase activity differing in salt tolerance were found in maize (de Azevedo Neto *et al*., 2006). The changes in catalase activity may depend on the species, the development and metabolic state of the plant, as well as on the duration and intensity of the stress (Chaparzadeh *et al*., 2004). This study lends further support to those findings.

Monodehydroascorbate reductase (MDAR; EC 1.6.5.4) is an FAD enzyme that uses NAD(P)H as electron donor to reduce MDA to ascorbate (AsA) (Miyake & Asada, 1994). It is crucial for AsA regeneration and essential for maintaining a reduced pool of AsA. AsA is a major antioxidant and free-radical scavenger in plants. It is considered to be of paramount importance as an electron donor for H_2O_2 detoxifications via APX in plant cells (Noctor & Foyer 1998). Overexpression of MDAR confers enhanced tolerance to ozone, salt and polyethylene glycol stresses in transgenic tobacco (Eltayeb *et al*., 2007). Thus and increased expression of MDAR might be associated with highly enhanced antioxidant metabolism under salinity stress.

Defense and miscellaneous proteins: DNAJ heat shock family protein (spot 1202) was up-regulated due to salt stress. HSPs are involved in a wide range of cellular functions, including protein folding, the correct assembly of oligomeric proteins and import of proteins across membranes. Generally, HSPs are able to maintain partner proteins in a folding-competent, folded or unfolded state, thereby minimizing the aggregation of non-native proteins. Alternatively, HSPs can target non-native or aggregated proteins for degradation and removal from the cell (Hoekstra *et al*., 2001). During salt stress, this survival strategy also prevents protein unfolding. In this study, the up-regulation would thus likely have detrimental effects on proteins at multiple levels. A putative aspartate transaminase (AspAT; spot 16), which catalyzes the reversible transfer of an α-amino group between aspartate and glutamate and, as such, is an important enzyme in amino acid metabolism, was significantly up-regulated specifically by the salt stress. Aspartate aminotransferase are important for transfer of nitrogen-containing groups in plant cells. Differential changes in levels of this enzyme have been reported in salt-stressed *Aeluropus lagopoides*-a halophyte plant (Sobhanian *et al*., 2010). In addition to the proteins described here, several proteins (spot 1001, 2404) was identified as a protein with unknown function. We were unable to correlate the role of this protein in relation to salt stress. Further studies are needed to address their possible role in relation to salinity stress.

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