

# PROTEOMICS APPROACH FOR IDENTIFYING SALT STRESS RELATED PROTEINS IN BARLEY LEAVES

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In many areas of the world, salinity is one of the major environmental factors limiting plant growth and yield (Parida *et al.*, 2004). Salinity and water deficits are among the most important agricultural problems in semi-arid and arid regions (Glenn and Brown, 1998). These conditions cause significant yield reductions on cultivated lands, inducing a wide range of perturbations at the cellular and whole-plant levels that can lead to plant death and decreases in productivity. However, it has been well established that plants growing naturally in arid and semi-arid areas have evolved many adaptations to counteract salt stress (Khan *et al.*, 2000). The ability to maintain turgor despite the lack of water induced by salt stress may preserve the metabolic processes, and therefore the growth, of a plant (Martinez *et al.*, 2004). To cope with salinity stress, plants respond with physiological and biochemical changes designed to retain water despite of the high external osmotic pressure and to maintain photosynthetic activity even when stomatal opening is reduced to counter water loss. During the onset and development of salt stress within a plant, all the major processes such as photosynthesis, protein synthesis and energy and lipid metabo-

lisms are affected. The earliest response is a reduction in the rate of leaf surface expansion followed by cessation of expansion as the stress intensifies but growth resumes when the stress is relieved (Parida and Das, 2005). A clear understanding of the molecular mechanisms involved in plants response to salt stress is of fundamental importance to plant biology. Knowledge about these mechanisms is also crucial for continued development of rational breeding and transgenic strategies to improve stress tolerance in crops. Dramatic changes in gene expression, biomembrane lipid composition and small molecular accumulation have been found to be closely related to these processes (Claes *et al.*, 1990). Proteomic analysis is a tool that facilitates the visualization/comparison of complex mixtures of proteins and provides a large amount of information about the individual proteins involved in specific biological responses. Recently, a number of plant proteomic studies have been published (Mock and Matros, 2008; Hashiguchi *et al.*, 2010). Improvements in proteomic technology regarding protein separation and detection, as well as mass spectrometry based protein identification, have an increasing impact on the study of plant responses to

salinity stress (Caruso *et al.*, 2008). Differential-expression proteomics is based on comparison of composition of different proteomes. In the field of plant abiotic stress research, the most common case is comparison of proteomes isolated from non-stressed (control) plants and the corresponding proteomes upon stress conditions. Barley (*Hordeum vulgare* L.) is widely grown in the arid and semiarid regions of the Mediterranean for forage purposes and as a grain crop (Al-Karaki, 2001). It is rated as a moderately tolerant forage crop and a highly tolerant grain crop (Francois and Mass, 1999). Salt tolerance of barley has been of interest for a long time and has resulted in a considerable body of data from studies using physiological (Munns and Rawson, 1999), genetic (Mano and Takeda, 1997), and cytogenetic approaches (Forster *et al.*, 1997). Recent molecular characterization of salinity stress in plants has indicated the involvement of multiple genes responsive to salinity. In the current work, we report the identification of salt-stress related proteins in barley using proteomic approach.

## MATERIALS AND METHODS

### *Plant material and salinity stress*

Seeds of barley (*Hordeum vulgare* L. cv. Giza 123) were sown into plastic pots that were filled with clay and sand (50% each). They were grown under controlled environmental condition ( $22 \pm 2^\circ\text{C}$  with 16-h light/8-h dark photoperiod) at conviron growth chamber. Gradual salt stress using sea salt (Sigma, USA) was imposed starting on day 14 after sowing.

Sea salt was brought up to 150 mM by increments of 50 mM per irrigation for two weeks.

### *Protein extraction*

Protein extraction was performed according to Hurkman and Tanaka (1986). Shoots were harvested at 24 h after reaching a final concentration of 150 mM sea salt. Leaf samples (1-2 g fresh weight) were finely ground in a mortar under liquid  $\text{N}_2$  and then suspended with 5 ml of ice-cold acetone containing 10% trichloroacetic acid (TCA) and 0.07% dithiothreitol (DTT). The resulting protein-containing suspension was allowed to precipitate overnight at  $-20^\circ\text{C}$  and then centrifuged at 12,000  $\times g$  for 45 min. The pellet was rinsed three times for 1 h with ice-cold acetone containing 0.07% DTT and 1 mM phenylmethylsulfonyl fluoride (PMSF) at  $-20^\circ\text{C}$ . Finally the protein pellet was air-dried and used for two-dimensional electrophoresis (2-DE) analysis. The white protein precipitates were washed once with 1 ml cold acetone and incubated at  $-80^\circ\text{C}$  for 60 min. Samples were centrifuged and white pellet were air dried.

### *Two-dimensional electrophoresis*

Samples were solubilized in Urea/CHAPS buffer (7 M urea, 2 M thiourea, 4% CHAPS (w/v), 40 mM DTT, 0.5% (v/v) immobilized pH gradient (IPG) buffer 3-11) and protein concentration were determined using Bradford assay kit (Bio-RAD, USA) with the same buffer

without the addition of DDT. One milligram of protein in a final volume of 450  $\mu$ l was loaded onto 24-cm dry polyacrylamide gel strips forming an immobilized non-linear 3-11 pH gradient (Bio-RAD, USA) for the first dimension. Isoelectric focusing (IEF) was performed in the IPGphor™ Isoelectric Focusing System (Bio-RAD, USA) for 10 h at 50 V following by 1 h 30 min-each-step gradient of 500; 1000; 2000; 4000 and 8000 V, reaching a total of 66,300 V-h. Prior to second dimension, IPG strips were equilibrated with 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, traces of bromophenol blue and 10 mg ml<sup>-1</sup> DTT during 15 min, followed by a second equilibration step in the same buffer but containing 25 mg ml<sup>-1</sup> iodoacetamide instead of DTT, for a further 15 min with gentle shaking. The focused strips were loaded and run onto SDS-PAGE 13% polyacrylamide gels at 2.5 W/gel overnight. Proteins were visualized with Coomassie Brilliant Blue G-250 (CBB) staining (Sigma, USA).

### ***Protein identification***

Protein spots that showed statistically significant changes in response to salt stress were excised from the CBB-stained gels and analyzed using a Bruker Daltonics- Autoflex II MALDI TOF/TOF mass spectrometer (Chemistry and Biochemistry Department, Montana State University, USA) as described previously (Sarhadi *et al.*, 2010). The database search was run against the NCBI nonredundant protein database.

## **RESULTS AND DISCUSSION**

### ***Analysis of differentially expressed proteins using 2D-gel***

To identify salt stress-regulated proteins, we carried out a comparative proteomic analysis. In this study, we focused on leaf tissues. 2D-gel was used to investigate the changes of protein profiles under salt stress. Proteins from leaves of control and salt-treated barley plants were separated on 2D-gel (Fig. 1). A total of 15 protein spots showed considerable changes in salt-stressed samples compared to control samples.

### ***Identification of differentially expressed proteins by MALDI TOF/TOF mass spectrometer***

The differentially expressed protein spots were excised, in-gel trypsin digested and analyzed using tandem MS. Proteins in all the protein spots were successfully identified after searching the MS/MS spectra against BLAST P. In barley leaves under salt stress, we detected differential expression of proteins involved in carbohydrate metabolism such as the glycolysis and tricarboxylic acid cycle. The accumulation of four proteins, 6-phosphogluconate dehydrogenase, enolase, Malate dehydrogenase and glyceraldehyde-3 phosphate dehydrogenase (spots 4, 5, 12 and 14, respectively) indicative of some metabolic activity was enhanced under salt stress (Table 1 and Fig. 1). Increased expression of enolase may be expected under hypoxic conditions that limit oxidative phosphorylation of

ADP and favor ATP regeneration via glycolysis and the ethanol fermentative pathway (Zhao *et al.*, 2004). Enhanced glycolysis and increased expression of related enzymes under abiotic stresses have been reported (Suzuki *et al.*, 2005). Malate dehydrogenase is an enzyme in the citric acid cycle that catalyzes the conversion of malate into oxaloacetate (using  $\text{NAD}^+$ ) and vice versa, it is also involved in gluconeogenesis, the synthesis of glucose from smaller molecules (Minarik *et al.*, 2002). Malate dehydrogenase is essential for ATP production. ATP is required for many biosynthetic pathways in plant cells, and during external stress maintenance of energy requirements may increase considerably (Dooki *et al.*, 2006). 6-phosphogluconate dehydrogenase (6PGDH) is involved in the oxidative pentose phosphate pathway, in which  $\text{NADPH}$  is generated and used to prevent oxidative stress. 6PGDH was up-regulated in response to both chilling stress in rice roots (Lee *et al.*, 2009) and salt stress in rice shoots (Huang *et al.*, 2003). Also, chloroplastic ATP synthase subunit alpha in leaves (spot 3) was up-regulated by salt stress to ensure adequate energy for other important processes during salt stress. A trend that has also been reported in the proteomic analysis of potato shoots (Aghaei *et al.*, 2008), Arabidopsis cell suspension cultures (Ndimba *et al.*, 2005) and rice leaf lamina (Parker *et al.*, 2006) under salt stress. The membrane-bound protein (spot 7) is one of the up-regulated proteins. The role of membrane-bound proteins such as  $\text{Na}^+/\text{H}^+$  antiporters (NHX) in ion homeostasis is well documented

(Pardo *et al.*, 2006). The overexpression of NHX antiporters contribute to improving plant salt-resistance (Apse and Blumwald, 2007), while decreased NHX activity results in increased salt sensitivity (Sottosanto *et al.*, 2007). Moreover, Heat shock protein 70 (spot 2) was up-regulated under salt stress. Previous studies have shown that the levels of HSP70 proteins were increased in *Salicornia europaea* under salt stress (Wang *et al.*, 2009). These results indicate that members in the HSP family might have diverse functions in plant salt tolerance. The expression of Oxygen-evolving enhancer protein 2 (spot 1) was increased by salinity. Oxygen-evolving enhancer protein 2 (OEE2) is involved in light-induced oxidation of water in photosystem II of plants (Tanaka *et al.*, 2005). Increased levels of expression of OEE2 under salt stress have been reported to occur in mangrove and in rice (Abbasi and Komatsu, 2004; Sugihara *et al.*, 2000). The increased expression levels of OEE2 might be needed to repair protein damage caused by dissociation and to maintain the evolution of oxygen. 20S proteasome alpha subunit A (spot 8) was up-regulated in barley under salt stress and also up-regulated in rice under osmotic stress (Zang and Komatsu, 2007). Proteasomes and its structural core particle, 20S proteasome alpha subunit A, are located in the nucleus and the cytoplasm. The main function of the proteasome is to degrade unneeded or damaged proteins by proteolysis, a chemical reaction that breaks peptide bonds. Proteasomes are part of a major mechanism by which cells regulate the concentration of particular

proteins and degrade misfolded proteins. Higher plants have active oxygen-scavenging systems consisting of multiple defense enzymes that can modulate the steady state level of reactive oxygen species (ROS) (Parida and Das, 2005). We found that several antioxidant enzymes were accumulated (spots 6, 9, 10, 11, 13 and 15, viz. glutathione reductase, ascorbate peroxidase, peroxiredoxin Q, chloroplast precursor, mitochondrial peroxiredoxin, glutathione S-transferase and superoxide dismutase) under salinity. Several reports indicated the up-regulation of these proteins during salt stress (Vaidyanathan *et al.*, 2003; Tsai *et al.*, 2005). Moreover, it was suggested that, in *M. crystallinum* and in halophytes as a group, key metabolic pathways including osmolytes and ROS scavenging enzymes might act as an integrated strategy to curtail oxidative burden caused by salt stress and other abiotic factors (Borland *et al.*, 2006). Finally, the functions described above appear to be closely connected with defense mechanism and regulation of cellular state in order to defend cells against harmful salt stress condition.

### SUMMARY

To understand the molecular basis of the salt stress response in barley, a proteomic approach was used to identify the salt stress-responsive proteins. Here we report proteomic analysis of barley leaves under 150 mM sea salt treatment. Proteins from control and salt treated samples were extracted and separated using two-dimensional gel electrophoresis. A total of

15 protein spots were exhibited considerable changes. The protein spots were identified by mass spectrometry and database searching. Identified proteins included those involved in metabolism, antioxidant enzymes, energy, photosynthesis, and protein degradation.

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Table (1): List of differentially synthesized proteins (protein spots) identified by MALDI TOF/TOF MS.

Spot No.	Accession No.	Protein name	Source	Theoretical pI
1	Q96334	Oxygen-evolving enhancer protein 2, chloroplastic	<i>Brassica juncea</i>	4.91
2	ACM42161	Heat shock protein 70	<i>Ageratina adenophora</i>	5.18
3	P06450	ATP synthase subunit alpha, chloroplastic	<i>Spinacia oleracea</i>	5.16
4	NP_001056586	6-phosphogluconate dehydrogenase	<i>Oryza sativa</i>	5.90
5	AAQ18140	Enolase	<i>Gossypium barbadense</i>	6.16
6	NP_001048485	Glutathione reductase	<i>Oryza sativa</i>	6.20
7	Q68KI4	Na <sup>+</sup> /H <sup>+</sup> antiporters (NHX)	<i>Arabidopsis thaliana</i>	6.73
8	O48551	20S proteasome alpha subunit A	<i>Glycine max</i>	6.80
9	AAL35365	Ascorbate peroxidase	<i>Capsicum annum</i>	7.74
10	Q6UBI3	Peroxiredoxin Q, chloroplast precursor	<i>Suaeda salsa</i>	9.45
11	CAG30523	Mitochondrial peroxiredoxin	<i>Pisum sativum</i>	8.43
12	AEE78292	Malate dehydrogenase	<i>Arabidopsis thaliana</i>	8.66
13	CAC94001	Glutathione S-transferase	<i>Triticum aestivum</i>	6.35
14	ABE82032	Glyceraldehyde-3-phosphate dehydrogenase-like	<i>Medicago truncatula</i>	6.55
15	CAA42737	superoxide dismutase	<i>Pisum sativum</i>	7.10

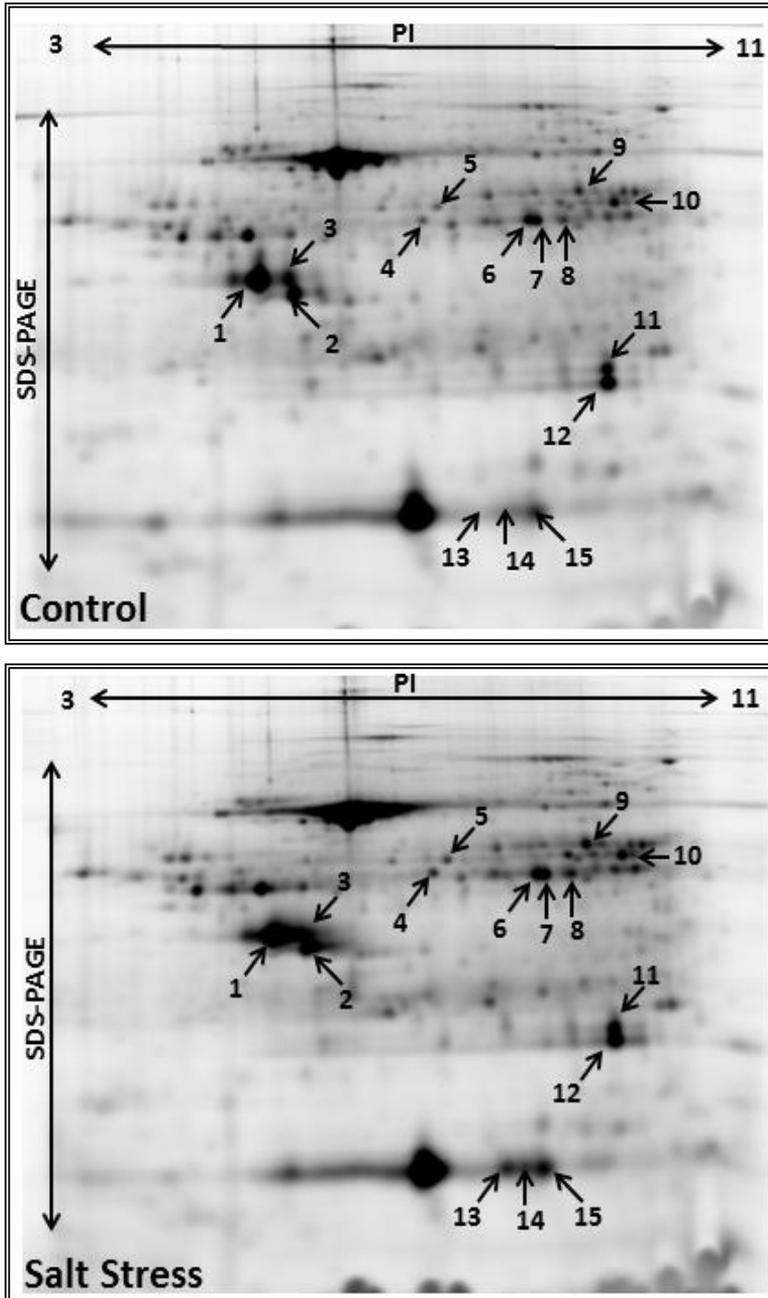


Fig. (1): Effects of salt stress on 2-DE protein profiles in barley leaves. Numbered arrows show changes in proteins under salt treatment. The positions of the same proteins are also marked in almost the same direction and position for clarity in the control. The first dimension (left to right) is designated as IEF (pI 3–11) and the second (top to bottom) is designated as SDS-PAGE (13%).