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The Proteome Response of *"Hordeum marinum"* to Long-term Salinity Stress

A. Boustani[1], F. Fatehi[2]* and R. Azizinezhad[1]

[1]Department of Plant Breeding Science and Research Branch, Islamic Azad University, Tehran, Iran
[2]Department of Agriculture, Payame Noor University (PNU), Tehran, Iran

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Salinity is a major constraint to crop productivity and mechanisms of plant responses to salinity stress are extremely complex. "*Hordeum marinum*" is a salt tolerant barley species, which could be a good source to evaluate salt-tolerance patterns. Proteomics is a powerful technique to identify proteins involved in plant adaptation to stresses. We applied a proteomic approach to better understanding the mechanism of plant responses to salinity in a salt-tolerant genotype of barley. At the 4-leaf stage, plants were exposed to 0 (control treatment) or 300 mM NaCl (salt treatment). Salt treatment was maintained for 3 weeks. Total proteins of leaf 4 were extracted and separated by two-dimensional gel electrophoresis. More than 290 protein spots were reproducibly detected. Of these, 20 spots showed significant changes to salt treatment compared to the control: 19 spots were upregulated and 1 spot was absent. Using MALDI-TOF/TOF MS, we identified 20 cellular proteins which represented 11 different proteins and were classified into five categories. These proteins were involved in various cellular functions. Upregulation of proteins which involved in protein processing (ribosomal protein, cullin family, cp31AHv protein and RNA recognition motif (RRM) superfamily), photosynthesis (Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) and Ribulose bisphosphate carboxylase/oxygenase activase (rubisco activase)), energy metabolism (cytosolic malate dehydrogenase (cyMDH) and fructokinase), oxygen species scavenging and defense (cystatin and thioredoxin) may increase plant adaptation to salt stress.

Keywords: barley, *Hordeum marinum*, proteomics, salinity, two-dimensional gel electrophoresis

Introduction

Salinity is one of the major environmental stresses affecting crop production worldwide. About 7% of the world's land is affected by salinity (Munns and Tester 2008). The salt effects on plants include osmotic stress, ion toxicity, nutrient imbalance and deficiencies, resulting in membrane damage, decreased cell expansion and division, changes in metabolic processes, oxidative stress and genotoxicity. Thus plant salt tolerance is a highly complex phenomenon that involves alterations in physiological and biochemical processes, which may result in morphological and developmental changes (Neto et al. 2015).

*Corresponding author; E-mail: fatehi.foad@gmail.com

Wild species of crops can be used as important sources of tolerance for breeding against biotic and abiotic stresses. Many wild species of barley were found in habitats with saline soils and might be good sources of salt tolerance. Wild species of barley showed more salt tolerance than cultivated genotypes (Fatehi et al. 2012b). One of these wild type is *Hordeum marinum* (sea barley grass) that inhabits salt marshes (Islam et al. 2007) and is tolerant to salinity (Garthwaite et al. 2005). *Hordeum marinum* (2n = 14) is a diploid form of Hordeum genus with a basic chromosome number of x = 7 and four major groups H, I, X and Y. The long arm of chromosome 7H may be responsible for the improved salt tolerance (Darko et al. 2015).

Barley uses three strategies to tolerate salt stress: accumulation or synthesis of compatible solutes, antioxidant protection by synthesis of antioxidant enzymes (such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and ion homeostasis (Munns and Tester 2008).

Breeding plants for higher yield from saline soils requires a proper understanding of the various mechanisms of salt tolerance, and thus it is necessary to identify the responsible proteins and their roles in the tolerance mechanism. Genomics and transcriptomics have contributed significantly to studying gene expression and cell function. However, it is impossible to directly translate mRNA into post-transcriptional and protein modification because it is extremely difficult to assess a direct correlation between mRNA and protein (Zorb et al. 2011). Therefore, we need proteomics methods in order to directly assess which proteins are influenced by a particular environmental stress in various tissues.

A powerful molecular technique to unravel the molecular mechanisms of salt stress and salt tolerance is 2-dimensional polyacrylamide gel electrophoresis (2-DE). This technique separate proteins in two steps: the first-dimension is isoelectric focusing (IEF), which separates proteins according to their isoelectric points (pI); the second-dimension is SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular weights (MW). This technique allows to recognize differentially regulated proteins during salt stress and thus to identify the corresponding genes. Recent progresses in high resolution 2-DE gels, computer-assisted analysis, and protein identification using modern mass spectrometry analysis and bioinformatics tools facilitate such studies (Zorb et al. 2011).

This research aims to analyze protein patterns in *"Hordeum marinum"* leaves grown under high salinity condition and find stress-responsive proteins. Using this information, along with data from other suitable molecular techniques can improve salt tolerance in barley and other similar plants.

Materials and Methods

Plant material and salinity treatment

The seeds used in this study were obtained from a salt-tolerant wild species Barley. Seeds were surface-sterilized by using 1% hypochlorite. Then eight to ten seeds were planted into pots (diameter: 25 cm) containing a mixture of perlite and cocopeat (3:1), the pots

were arranged in a randomized complete block design for a factorial experiment with three replications. During the first week pots were irrigated with tap water, then with a quarter strength Hoagland's solution in second week and after that with half strength Hoagland's solution. Twenty days after sowing, the pots were moved to a growth chamber to satisfy the vernalization requirement by exposing the plants to a temperature of 2–4 °C for 4 weeks. Thereafter the pots were placed back in the glasshouse and thinned to five plants per pot. At the 4-leaf stage, plants were exposed to 0 (control treatment) or 300 mM NaCl (salt treatment). NaCl (50 mM) was added twice a day (at 6:00 am and 6:00 pm) for 3 days to a final concentration of 300 mM, and supplementary $CaCl_2$ was also added to give a final concentration of 12 mM, because of Ca^{2+} deficiency which caused by high concentration of Na^+. Salt treatment was maintained for 3 weeks. After 3 weeks each fourth leaf from barley plants was cut at the base of leaf number one, then quickly wrapped in aluminum foil pouch and immediately frozen in liquid nitrogen and stored at –80 °C.

Na and K measurement

In order to sodium (Na^+) and potassium (K^+) analysis, blades of leaf 4 were washed in distilled water, and then they were dried in an oven at 70 °C for 48 h. Dried leaf blades grounded after being weighed. Na^+ and K^+ concentration was determined from the chloride acid (2 N) extract of the samples that had been burned at 580 °C for 4 h, and were measured by using a flame photometer. Statistical analysis was performed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) and the *t*-test was used to evaluate differences between mean values.

Protein extraction

Harvested leaves were finely ground in liquid nitrogen using mortar and pestle. Then the proteins were extracted following the procedure, with some modifications. Briefly, 1 g of powder was suspended in an ice-cold solution of 10% w/v trichloroacetic acid (TCA) in acetone with 0.07% w/v DTT for at least 1 h at –20 °C, and centrifuged for 20 min at 35,000×*g*. The pellets were rinsed twice with acetone containing 0.07% w/v DTT for 1 h at –20 °C and then lyophilized. The resulting pellet was solubilized in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 35 mM TRIS–HCl, 1% w/v DTT, and 1% v/v Ampholyte, pH 3.5–10) for 1 h at room temperature and then centrifuged at 12,000×*g* for 15 min. The supernatant was carefully collected and aliquoted and stored at –80 °C until electrophoresis. The protein concentrations were quantified according to the Bradford method using BSA as standard (Fatehi et al. 2012a).

Two-dimensional electrophoresis (2-DE)

2-DE was done according to the method described by Görg et al. (1988). At the first dimension proteins were separated according to their isoelectric points by using IPG strips

(pH 4–7, 17 cm length, Bio-Rad). The second dimension was performed on a 12.5% SDS polyacrylamide gel in order to separate proteins according to their molecular weights. Protein spots in analytical gels were visualized with silver nitrate by Blum's protocol. Preparative gels were stained with colloidal CBB G-250.

Image analysis

The silver-stained 2-DE gels were scanned using a GS800 Calibrated densitometer (Bio-Rad) in transmissive mode, and the scanned gels saved as TIF images for subsequent analysis. Spot detection, quantification, and matching were carried out using Melanie 6.02 software (Genebio, Geneva, Switzerland). The volume of each spot was normalized based on total spot volume, quantified, and subjected to a *t*-test ($p = 0.01$). Only the spots with significant differences which were consistently present in all three replications were considered for further analysis.

Protein identification and data analysis

Protein spots of interest were manually excised from preparative CBB stained gels and destained for 1 h at room temperature using a freshly prepared wash solution consisting of 50% acetonitrile/50 mM ammonium bicarbonate (NH_4CHO_3). Then reduction and alkylation of protein spots were done. Wash solution was removed and spots were left to dry. Proteins were digested using a trypsin solution, this reaction was left to proceed during the night at 37 °C. Peptides were eluted by 50% acetonitrile and 0.1% TFA in water and deposited onto the MALDI target plate and left to dry in air. Peptide mixtures were then analyzed using MALDI-TOF/TOFMS at university of York (England). Data from mass spectrometry were analyzed by Mascot program and NCBI non-redundant protein database.

Results

Ion concentration in leaf 4

In the leaf 4 Na^+ and K^+ concentrations were significantly increased under salinity conditions. But the K^+/Na^+ ratio was decreased under salinity (Table S1*).

Two-dimensional gel electrophoresis (2-DE) analysis

More than 290 protein spots were reproducibly detected on silver stained gels. Of these, 20 spots showed significant differences between the salt treatment and the control treatment: 19 spots were upregulated and 1 spot was absent by the salt stress (Fig. S1).

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

Identification of salt responsive proteins

The 20 salt-responsive proteins were identified by MALDI-TOF/TOF MS. Mass data were analyzed using Mascot (Table S2). The 20 responsive proteins were classified into five categories.

Spots 7, 10 and 19 were identified as hypothetical protein (a protein whose existence has been predicted, but for which there is a paucity of experimental evidence that it is expressed *in vivo*), so we searched them against NCBI and they revealed a 21% match with cullin-4 [*Vitis vinifera*], 9% match with Ty3/Gypsy family of RNase HI in long-term repeat retroelements and 20% match with RNA recognition motif (RRM) superfamily, respectively. The identified proteins included ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) (spots 1, 2, 3, 5, 8, 9, 11 and 18), rubisco activase (spots 15 and 17), ribosomal proteins (spots 4 and 13), chloroplast RNA binding protein (spot 12), cystatin (spot 6), malate dehydrogenase (spot 14), thioredoxin h (Trx) (spot 20), fructokinase (spot 16).

Discussion

The mentioned responsive proteins were involved in different fundamental process which classified into four categories.

Photosynthesis-related proteins

Two different proteins were identified in this group including ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and Rubisco Activase, both of them showed increased expression under salinity stress. Rubisco is a critical enzyme involved in photosynthetic carbon assimilation and by increasing photosynthetic carbon assimilation in C3 plants could improve crop yield (Raines 2011). So the plants with higher amount of rubisco could give high yield along with better tolerance under environmental stress. Increased abundance of Rubisco was reported in different plants exposed to abiotic stresses (Cui et al. 2015; Cheng et al. 2015). In addition we observed upregulation of Rubisco in our last investigation on cultivated barley genotypes (Fatehi et al. 2012a), what indicates that *H. marinum* and barley cultivars could enhance the products of photosynthesis and yield with same method. No distinct differences in amount or downregulation of this vital enzyme was also observed in salt-sensitive cultivars of plants under salt stress, leading to decline in plant growth and crop yield (De Abreu et al. 2014; Cheng et al. 2015).

Rubisco Activase can activate the RuBisCO enzyme by carbamylation, so higher amount of rubisco activase could lead to more carbon assimilation and crop yield. Upregulation of rubisco activase was reported in stress-tolerant genotypes under various stresses (Caruso et al. 2009; Sobhanian et al. 2010; Budak et al. 2013; De Abreu et al. 2014). Moreover, an increase in Rubisco Activase was seen in our last experiment on cultivated barleys during salinity (Fatehi et al. 2012a). Downregulation of rubisco activase was reported in sensitive plants under stress conditions (Sobhanian et al. 2010;

Beritognolo et al. 2011). The increase in Rubisco Activase may result from their chaperon function that needs more study (Fatehi et al. 2012a). So a significant increase in abundance of Rubisco and Rubisco Activase in our study on *H. marinum* and other barley cultivars can attenuate the salt stress injury by altering the expression of some critical enzymes and therefore crop photosynthesis and yield would be increased.

Protein translation and processing

Four different proteins were identified in this group including Ribosomal protein P1, cp31AHv protein, (RRM) superfamily and cullin family. They all showed increased expression under salinity stress. Ribosomal protein P1 is a component of large ribosomal subunit (60S), which is an important member of the eukaryotic ribosomal translation complex (Rich and Steitz 1987). Several ribosomal proteins are down-regulated in response to abiotic stress, resulting in retardation of growth and productivity in various plants (Sobhanian et al. 2010), these downregulations may lead to salt sensitivity in the mentioned plants. Upregulation of ribosomal proteins was observed in high yield and stress-tolerant plants (Ma et al. 2007). Also, enhanced expression in all ribosomal proteins was observed during salinity in genotypes of cultivated barley (Fatehi et al. 2012a). An increase in the expression of ribosomal protein P1 was reported in *H. vulgare* cultivar "Pallas" under severe salt treatment as well (Alikhani et al. 2013).

Cp31AHv is a kind of RNA-binding proteins (RBPs). RBPs are involved in the regulatory processes of gene expression. An increase and decrease in chloroplast RNA binding protein was observed in salt-tolerant and salt-sensitive barley genotypes, respectively (Fatehi et al. 2012a). Two chloroplast RNA-binding proteins, Cp31AHv and Cp31BHv, increased in abundance, and one of these (Cp31BHv) also increased under long-term nitrogen deficiency in salt-tolerant *H. vulgare* L. cultivar (Møller et al. 2011).

RRM, also known as RBD (RNA binding domain) or RNP (ribonucleoprotein domain), is a highly abundant domain in eukaryotes found in proteins involved in gene expression processes (Marchler-Bauer et al. 2015). Cp31AHv protein is a species of RNP and the related explanations are expressed in the previous paragraph.

Cullin-RING (CRL) complexes are involved in all developmental processes and physiological responses of plants, including acclimation to abiotic environmental factors (Guo et al. 2013). It was reported that cullin complex expression is involved in response to stress condition in barley (Reiner et al. 2016). In addition it was expressed that a combination of Clathrin Adaptor Complex (CAC) and cullin (CUL) as reference should be better for gene expression normalization in sugarcane under salinity and drought stresses (Guo et al. 2014). As a result upregulation of these proteins (Ribosomal protein P1, cp31AHv, RRM and cullin family) illustrates some of the reasons about tolerance of barley to the inhibitory effect of NaCl on protein biosynthesis.

Energy metabolism

Two different proteins were identified in this group including Cytosolic malate dehydrogenase (cyMDH) and fructokinase (FRK). These two proteins were up-regulated under salinity stress. cyMDH play a critical role in citric acid cycle (Krebs) which lead to produce energy carrier molecules like ATP. In addition, cyMDH play an important role to protect membrane by improving proton pumps, it can elevate the tolerance to osmotic-associated stresses by osmotic modulation (Yao et al. 2011). It was reported that upregulation of cyMDH can promote growth and tolerance to various abiotic stress in different plants (Ding and Ma. 2004; Yao et al. 2011). Upregulation of MDH was also seen in our investigation on *H. spontaneum* (a salt-tolerant barley) under salinity stress and furthermore MDH play a role as an antioxidant protein in order to tolerate salinity (Fatehi et al. 2012b).

Fructokinase (FRK) is a crucial enzyme for physiological and developmental processes at the whole-plant level, coordinating carbohydrate availability with growth (Granot et al. 2013), FRK has a specific role in developing vascular tissues as well (Roach et al. 2012). Upregulation of FRK was reported in different plants in response to various stress conditions (Klotz et al. 2006; Zorb et al. 2011; Fulda et al. 2011). So it might be involved in responses of plants to abiotic stress. Sobhanian et al. (2010) reported that FRK was down-regulated in the hypocotyls/root of soybean (salt-susceptible) under NaCl treatment. FRK was down-regulated in response to salt stress in barley cultivars (Witzel et al. 2009) whereas upregulation of FRK was observed in our present study on *H. marinum* (as a salt-tolerant wild barley). Therefore, we can suggest that upregulation of fructokinase and cyMDH could elevate plant tolerance to abiotic stress by improving the energy metabolism pathways.

Defense and ROS scavenging

Two different proteins were identified in this group including Cystatin Hv-CPI2 and Thioredoxin (TRx). Both are up-regulated under salinity stress in the present study. Cystatin is a cysteine protease inhibitor that is involved in defense. Increased amount of Cystatin was observed in several plants such as cultivated barley and hop in response to biotic and abiotic stresses (Abraham et al. 2006; Wang et al. 2008).

Thioredoxin (TRx) serve general functions of reserve mobilization, metabolism resumption, and ROS scavenging. Different types of TRxs were induced and regulated by abiotic stress in several plants (Zhang et al. 2011; Chattopadhyay et al. 2011; Ji et al. 2016). An increase in TRx was seen in barley genotypes during salinity as well (Fatehi et al. 2012a). These results indicate that upregulation of Cystatin and TRx could enhances plant tolerance to high salinity.

Evolution

Ty3/Gypsy LTR retroelements constitute a family of retrotransposons. This mobile genetic elements are important components of plant genomes, and majority of them remain quiescent during normal growth and development. So gene silencing might be the key mechanism controlling the activation of these elements. The retrotransposon protein spot was absent under salinity in our study on *H. marinum*. Many studies have indicated that few retrotransposons are responsive to stresses (Ma et al. 2010). It was reported that stress conditions caused the movement of retrotransposon in *H. vulgare* calli as well (Bayram et al. 2012). Therefore, they might be important in response to stresses.

The results indicated that effective regulatory protein expression related to protein processing, photosynthesis, energy metabolism, oxygen species scavenging and defense all played important roles in the salt response of *H. marinum*. In detail, Rubisco, Rubisco Activase, Ribosomal protein, MDH, Cystatin and TRx proteins were up-regulated under stress conditions in both wild and cultivated barleys. Moreover, cullin and retrotransposons were involved in response to stress in wild and barley cultivars. Cp31AHv was increased in wild and salt-tolerant cultivated barleys but decreased in salt-sensitive cultivated barley, and fructokinase was down-regulated in response to salt stress in cultivated barleys while upregulation of FRK was observed in our present study on *H. marinum* (as a salt-tolerant wild barley).

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Table S1.* Mean of Na^+ and K^+ concentrations (μmol g^{-1} DW) in leaf 4 of *H. marinum*

Table S2. Salt responsive proteins of *H. marinum* leaves during long-term salt stress identified with MALDI-TOF/TOF-MS analysis

Figure S1. 2-DE protein profile of leaf 4. Protein (120 mg) was loaded on 18 cm IPG strip with a linear gradient (pH 4–7) and SDS-PAGE was performed with a 12% gel. Proteins were visualized by silver staining. The identified protein spots are marked. A: control treatment, B: salt treatment