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Research article

Screening for salt-responsive proteins in two contrasting alfalfa cultivars using a comparative proteome approach

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ABSTRACT

A comparative proteomic approach was carried out between two contrasting alfalfa cultivars, nonomu (NM-801; salt tolerant) and vernal (VN; salt intolerant) in terms of salt tolerance. Seedlings were subjected to salt stress (50 and 100 mM NaCl) for three days. Several physiological parameters (leaf water, chlorophyll, root Na^+, K^+, and Ca^{2+}) and root proteome profile were analyzed. Comparison of physiological status revealed that NM-801 is more tolerant to salt than VN. Eighty three differentially expressed proteins were found on 2-DE maps, of which 50 were identified by MALDI-TOF or MALDI-TOF/TOF mass spectrometry. These proteins were involved in ion homeostasis, protein turnover and signaling, protein folding, cell wall components, carbohydrate and energy metabolism, reactive oxygen species regulation and detoxification, and purine and fatty acid metabolism. The comparative proteome analysis showed that 33 salt-responsive proteins were significantly changed in both cultivars, while 17 (14 in VN and 3 in NM-801) were cultivar-specific. Peroxidase, protein disulfide-isomerase, NAD synthetase, and isoflavone reductase were up-regulated significantly only in NM-801 in all salt concentrations. In addition, we identified novel proteins including NAD synthetase and biotin carboxylase-3 that were not reported previously as salt-responsive. Taken together, these results provide new insights of salt stress tolerance in alfalfa.

1. Introduction

Soil salinity is one of the most important constraints on plant growth and productivity worldwide. Approximately 6% of the world's total land and 20% of irrigated land is greatly affected by salinity (Guo et al., 2012). With the rapid expansion of irrigated cropland, the salinity problem is predicted to become more extensive. Therefore, understanding the molecular and physiological mechanisms of salinity tolerance in plants is necessary to sustain productivity. High salinity induces osmotic stress and ion toxicity, which impair the metabolic processes of essential enzymes, limits plant growth, leads to wilting, and ultimately death of the most plant species (Munns and Tester, 2008). Plant roots are the first sensor organ to perceive salinity stress, from where stress signals are transmitted to the entire plant. Plants exhibit a plethora of anatomical, molecular, and biochemical mechanisms to cope with salinity stress (Zhao et al., 2013). Plant cells maintain Na^+ and Cl^- homeostasis to avoid ion toxicity in plant tissues. The maintenance of adequate K^+ concentrations and the production of osmolites including glycinebetaine and proline are also important. A higher level of vacuolar H^+-ATPase plays an important role in stress tolerance in plant roots. Several salinity-responsive genes (*HKT1, NHX1, H^+-ATPase, SOS1*) have pivotal roles in regulating Na^+ influx, K^+ uptake, and long-distance Na^+ transport in plants (Zhao et al., 2013), though little is known about the genes and salinity regulatory networks involved. Large-scale transcriptomic analyses have reported numerous salinity-responsive genes in the roots of

Abbreviations: TBARS, thiobarbituric acid reactive substance; 2-DE, two-dimensional gel electrophoresis; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PMF, peptide mass finger printing; DEPs, Differentially expressed proteins; TCA, tricarboxylic acid; PDHC, pyruvate dehydrogenase complex; V-ATPase, vacuolar-type H^+ -ATPase; Hsp, Heat shock protein.

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plants, including *Arabidopsis*, rice, maize, wheat, tomato, barrel medic, Chinese lyme grass, and alfalfa (Jin et al., 2010; Zhao et al., 2013). These genes are mostly involved in transcription, stress defense, protein turnover, cell wall, energy metabolism, signal transduction, and hormone signaling responses in salinity-stressed roots. In addition, the transcripts may undergo a series of post-transcriptional and post-translational modifications (Zhao et al., 2013), which leads to a lack of correlation between the transcriptome and proteome in plants under stress.

Proteomics is a powerful molecular tool for the systemic analysis of proteins expressed by the genome (Porubleva et al., 2001). It is one of the best strategies to reveal the dynamics response of proteins under different environmental stresses. However, comparative/quantitative proteomics is also the most utilized subarea of proteomics with the aim to establish protein profiles from different individuals or distinct treatments (Valledor et al., 2014). High-throughput quantitative proteomic technologies have facilitated the identification of numerous salinity-responsive proteins in plant roots, allowing the molecular mechanisms in several plants species (Manaa et al., 2011; Guo et al., 2012; Zhao et al., 2013). These reports document major proteins that are involved in signal transduction, protein turnover, carbohydrate and metabolism, cell wall dynamics, and reactive oxygen species (ROS) homeostasis.

Despite these advancements, there have been only a few reports on the forage root proteome. Most forage plants are poorly represented in the sequence databases. The successful application of several molecular tools such as the cDNA microarrays, cDNA fragment fingerprinting and serial analysis of gene expression (SAGE) are practically limited to model organisms. Orthologous gene sequences are rarely identical from one species to another, as they are usually riddled with nucleotide substitutions. In this case, proteomic approach is more powerful (Carpentier et al., 2008; Sharmin et al., 2013). Alfalfa is a perennial forage legume crop cultivated for its higher feed value and yield potential (Lacefield et al., 1997). Additionally, alfalfa improves the soil structure through fixing atmospheric N_2. However, the above mentioned benefits are hampered by its susceptibility to several environmental stresses, including salinity (Zahran, 1999). Therefore, it is imperative to understand the effects of salinity at the physiological, biochemical, and molecular levels in alfalfa roots. Molecular events have been studies in alfalfa using transcriptomic approach in response to salinity (Postnikova et al., 2013), and drought (Kang et al., 2011). The global protein expression profiles of alfalfa cultivars differing in salinity response would be helpful for identifying novel proteins/genes with detailed molecular networks of salinity tolerance. The objective of this study was to identify differentially expressed protein species in two contrasting alfalfa cultivars under high salinity as well as to explain their biological role of each system. Despite the availability of the proteomic study on alfalfa, comparisons of proteome dynamics between salt-tolerant and salt-sensitive varieties are yet to be studied, especially under a range of salt concentrations in order to provide new insight into the salinity stress responses of forage legume.

2. Materials and methods

2.1. Plant growth and salinity treatment

Two contrasting alfalfa (*Medicago sativa* L.) cultivars, vernal (VN; salt intolerant) and nonomu-801 (NM-801; salt-tolerant, obtained from the Institute of Botany, Chinese Academy of Science, Beijing, China), were used as the research materials. Seeds were placed to wet filter paper and kept in a growth chamber maintained at 25 °C under white inflorescent light (480 μmol $m^{-2}.s^{-1}$) with a 16-h photoperiod and 60%–65% humidity for 2 days. Then the seedlings were transferred to an aerated hydroponic system with Hoagland nutrient solution (Phytotechnology Laboratories, USA). The nutrient solution was replaced at a 3-day interval. After 2 weeks of growth, the seedlings were subjected to 50 mM and 100 mM NaCl for 3 days. The roots were then excised, washed with deionized water, and quickly frozen in liquid nitrogen. The samples were stored at −80 °C, further it used in proteomic and biochemical analyses.

2.2. Measurement of relative leaf water and chlorophyll contents

The physiological parameters of plants were examined under salinity stress conditions. The relative water content (RWC) and chlorophyll content of leaf were measured after 3 days in salt-treated plants. RWC (%) was calculated as [(FW-DW)/(TW-DW)] × 100 (FW = leaf fresh weight, TW = turgid weight). The chlorophyll content was measured as leaf greenness using a portable diagnostic chlorophyll meter (SPAD-502, 2900P, Spectrum Technologies, Ireland). Based on the quantification of the light intensity (peak wavelength: 650 nm, red LED) an instantaneous and non-destructive reading on plants leaf was taken by the meter. A second peak (peak wavelength: approximately 940 nm, infrared LED) is emitted simultaneous with red LED for to compensate the thickness of leaf. Third trifoliate leaves were chosen for measuring SPAD values, where the mean of four readings were obtained by the chlorophyll meter for individual leaf.

2.3. Determination of ion accumulation in root tissue

Root samples were dried in a dry oven at 80 °C for 72 h and pulverized into a fine powder. Approximately 1 g of fine powder for each treatment group was digested using a ternary solution (HNO_3/H_2SO_4/$HClO_4$, 10/1/4, v/v) as described previously (Sharmin et al., 2012); the total soluble ions (Na^+, K^+ and Ca^{2+}) were measured by inductively coupled plasma atomic emission spectrometry (ICP; Optima 5300DU, Perkin Elmer, USA). Three different biological replicates of root samples were used for the analyses.

2.4. Measurement of lipid peroxidation and H_2O_2 levels

Lipid peroxidation was estimated by measuring the content of 2-thiobarbituric acid-reactive substances (TBARS) as the malondialdehyde (MDA; $\varepsilon = 155\ mM^{-1}\ cm^{-1}$) content as described previously (Hodges et al., 1999). MDA was determined at A_{532} and corrected for nonspecific turbidity at A_{600}. A measurement at A_{440} was included to subtract the absorbance of non-MDA compounds at 532 nm due to presence of interfering substance such as sugars and anthocyanins. The hydrogen peroxide (H_2O_2) content was measured spectrophotometrically according to the method of Jana and Choudhuri (1982). H_2O_2 was extracted by homogenizing 200 mg root tissue with 3 ml phosphate buffer (50 mmol l^{-1}, pH 6.8) containing 1 mmol l^{-1} hydroxylamine (catalase inhibitor). The homogenate was centrifuged at 6000× g for 25 min. To determine the H_2O_2 accumulation, 3 ml of the extracted solution was mixed with 1 ml of 0.1% titanium sulfate in 20% (v/v), and the mixture was centrifuged at 6000× g for 15 min. The absorbance of the supernatant was determined at 410 nm, and the H_2O_2 content was calculated by the extinction co-efficient (0.28 $\mu mol^{-1}\ cm^{-1}$).

2.5. Measurement of free proline and soluble sugar

The free proline accumulation was estimated using the acid-ninhydrin reaction (Bates et al., 1973). For this, 400 mg of pulverized sample was homogenized with 3% (w/v) sulfosalicylic acid at