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Proteomic changes in kenaf (*Hibiscus cannabinus* L.) leaves under salt stress

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ABSTRACT

High salinity is one of the major abiotic stresses limiting crops productivity and the geographical distribution of many important crops worldwide. To better understand the salinity stress responses in fiber crop, kenaf (*Hibiscus cannabinus* L.), a comparative proteomic analysis was performed using the combination of two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser-desorption ionization-time of mass spectrometry (MALDE-TOF/MS), and identified 42 altered protein spots (36 spots were up-represented and 6 down-represented). The majority of these proteins were associated with energy production and metabolism, protein synthesis and degradation, photosynthesis, defense, and reactive oxygen species (ROS) scavenging. A semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed based on peptide sequences for comparing transcript and protein accumulation patterns for 7 candidate proteins. Of these proteins, 5 patterns of induced transcript accumulation were consistent with those of induced protein accumulation. It is possible that the plants proteomic response to salinity stress is a complex signal pathway and that the identified proteins may contribute to facilitating ion homeostasis when plants exposed to salinity stress.

1. Introduction

Salinity stress, one of the most serious threats to agriculture productivity, adversely affects crops growth and development (Li et al., 2013). It is estimated that nearly 20% of the world's cultivated area and about half of the world's irrigated lands already suffer from salinity problems. Natural phenomena and human practices such as irrigation can accelerate salt accumulation in soil (Dooki et al., 2006; Wiebe et al., 2007). To relieve detrimental effects from excess salt, plants respond with physiological and biochemical changes aim to retain water and maintain photosynthetic activity (Chinnusamy et al., 2005a). When plants are subjected to NaCl, cellular ion homeostasis is altered: excess Na^+ accumulation, K^+ deficiency and cytosol capacity changes; these factors in turn induce ionic and osmotic stress, and can further accumulate reactive oxygen species (ROS) (Ashraf and Harris, 2004; Chinnusamy et al., 2005a). Those free radicals disturb normal metabolism by peroxidizing membrane lipids and denaturing proteins and nucleic acids. To protect cellular and subcellular systems from cytotoxic effects of ROS, some osmolytes such as proline, glycine betaine and sugars, and up-regulation of Na^+/H^+ antiporters, as well as antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) were accumulated in plant cellular (Parida and Das, 2005).

In general, molecular mechanisms underlying in plant cells coping with environmental stresses can be unveiled through transcriptomics and proteomics analysis. Transcriptome profiling, a widely used approach to identify stress-responsive genes, has provided us a better understanding of salinity stress in many species (Jiang and Deyholos, 2006; Kawasaki et al., 2001; Kreps et al., 2002). However, several studies have demonstrated that the levels of transcripts and proteins are not strictly correlated in yeast, or other species (Gygi et al., 1999; Mooney et al., 2006; Swami et al., 2011). Furthermore, many proteins are modified by post-transcriptional modification such as phosphorylation and glycosylation, which can lead to a dramatic increase in proteome complexity without a concomitant increase in mRNA level (Jiang et al., 2007; Rose et al., 2004). Therefore, proteomic profiles have become a necessary and complementary approach, applied for understanding the cellular processes of plants response to environmental stresses.

Kenaf (*Hibiscus cannabinus* L.) is an important fiber crop, short-day, rapid-growing, annual Malvacea native to tropical regions of Asia and Africa. It has a great potential for applications to

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the pulp and paper industry, oil absorption and potting media, board making, filtration media, and animal feed (Ayadi et al., 2011; Bhardwaj et al., 2005). Kenaf has also been identified as an appealing alternative fiber source for the manufacture of a large range of paper products, due to pulping kenaf requires less energy and chemical inputs for processing compared with standard woods sources(Bhardwaj et al., 2005; Villar et al., 2009). However, the fiber yield and quality of kenaf suffers from severe losses under the adverse excess salinity conditions. Although traditional breeding approaches have had some successes to improve abiotic stress tolerance, the quality and biomass productivity of kenaf is still limited and cannot meet commercial demands due to a lack of efficient selection techniques and low levels of genetic variance and fertility (Bartels and Sunkar, 2005; Niu et al., 2015). Fortunately, proteomic approach is a powerful technology that facilitates the visualization/comparison of complex proteins mixtures and provides a large amount of information about the individual proteins involved in specific biological responses (Du et al., 2010; Qiao et al., 2014). In the present study, we analyzed the protein expression profiles for the leaves of kenaf seedling treated with different NaCl concentration (0 mM, 70 mM, 140 mM and 200 mM) by using two-dimensional gel electrophoresis (2-DE) and identified a few differentially expressed proteins using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

2. Materials and methods

2.1. Plant growth and treatment

Kenaf (*H. cannabinus* cv. Fuhong952) seeds were gained from Fujian Agriculture and Forestry University (Fuzhou, China). Seeds were rinsed under running water for 10 min, and sterilized with 75% (v/v) ethanol for 1 min, and 5% (v/v) sodium hypochlorite for 10 min, washed three times with sterile water, and then germinated on moist filter paper in the dark at 28 °C for 3 days. The germinated seedlings were transferred to plastic pots (30 cm diameter and 20 cm depth) containing a mixture of nutrient soil and quartz sand and grown in a growth chamber at 28 °C for 16 h with light and 26 °C for 8 h without light, 60% relative humidity, ~450 μmol m^{-2} s^{-1} light intensity, and seedlings were supplied with water containing 1/4 Hoagland solution once in two days. Seedlings with 8–10 leaves were subjected to different NaCl stress (0 mM, 70 mM, 140 mM and 200 mM) for 6 days, and leaves were harvested in triplicate (at 10 o'clock in the morning). The fresh weights together with stem lengths of sampled plants were measured. All samples were washed with deionized water, blot-dried with filter paper, frozen in liquid nitrogen, and stored at −80 °C until protein extraction. Triplicate leaves samples were used for proteins extraction assay.

2.2. Protein extraction and 2-DE

For analysis of total protein, leaves samples were harvested at 6 days after the treatments. Protein extraction was carried out using a modified version of the phenol extraction method described by Carpentier et al. (2005). Leaves samples (1.0 g fresh weight) were ground into fine powder in liquid nitrogen and suspended with 5 ml ice-cold extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM L-ascorbic acid, 100 mM KCl, 50 mM disodium tetraborate decahydrate, 1% (v/v) Triton X-100, 2% (v/v) 2-ME) on ice for 10 min. The resulting suspension was vortexed with 5 ml ice-cold Tris-buffered phenol (pH 8.0), and then centrifuged at 15,000*g* for 15 min at 4 °C. The phenolic phase was transferred into another tube and mixed with 30 ml 0.1 M ammonium acetate/methanol at −20 °C, vortexed, and kept at −20 °C before centrifuging at 15,000*g* for 15 min at 4 °C. The pellet was washed twice with acetone containing 0.07% (v/v) 2-ME. After further centrifugation, the pellet was vacuum-dried and proteins were extracted using lysis buffer. Total protein content was estimated using a Bradford (1976) assay and then stored at −80 °C for further analysis.

Two-dimensional electrophoresis was carried out according to the method described by Sheoran et al. (2005). Isoelectric focusing (IEF) was carried out previously (Siefritz et al., 2002). IPG strips of nonlinear pH 4–7 (13 cm) were loaded with 250 μl of protein sample in a rehydration tray for 12 h. Following rehydration, the IPG strips were run on an Ettan IPGphor III electrophoresis system (GE Healthcare, UK). IEF was conducted in a stepwise manner: 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, and gradient 8000 V for 6 h. Focused IPG strips were equilibrated for 15 min with an equilibration buffer [50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 1% (w/v) DDT, trace amounts of bromophenol blue], followed by another 15 min in equilibration buffer containing 2.5% (w/v) iodoacetamide. For the second dimension, the proteins were separated on a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein spots were visualized using Coomassie Brilliant Blue (CBB) G-250 described by Kang et al. (2002). At least three replicates were performed for each sample.

2.3. Image analysis and protein identification

The 2-DE gels were scanned at 300 dpi using an image scanner (GE Healthcare, UK). The images were analyzed using ImageMaster™ 2-D Platinum Version 7.0 analysis software (Amersham Pharmacia). The optimized parameters were as follows: smooth 5.0, min area 50 and saliency 400–600. Dividing each spot volume value by the sum of the total spot volume values yielded individual relative spot volumes. The differences in expression between control and treatment were analyzed using a Duncan test with $P \leq 0.05$ considered significant.

Protein spots with a 2.0-fold difference ($P \leq 0.05$) in abundance between control and treatment were selected and excised from the gel, washed, and digested with trypsin as previously described by Veeranagamallaiah et al. (2008). Each sample was suspended in 0.7 μl of matrix solution (*a*-cyano-4-hydroxycinnamic acid in acetonitrile/water (1:1, v/v) acidified with 0.1% (v/v) TFA), and the resulting mixture was deposited onto the MALDI target and allowed to dry and crystallize. MS analysis was performed with a MALDI-TOF/TOF mass spectrometer 4700 Proteomics Analyzer (Applied Biosystems, USA). Peptide mass fingerprint (PMF) data were submitted to the GPS Explorer software (Applied Biosystem) and MASCOT v.1.9 software (Matrix Science, London, UK) for protein identification. The analysis was carried out with NCBInr and other green plants as database. Search parameters were: maximum of one missed cleavage by trypsin, fixed modification of oxidation, charged state of +1, peptide mass tolerance of 50 ppm, and fragment mass tolerance of ±1.0 Da. Only significant hits, as defined by the MASCOT probability analysis ($P \leq 0.05$), were accepted. Currently, a complete kenaf protein database is not yet established. Thus, the availability of only ESTs and partial genome sequence data was used for further identification of all affected proteins.

2.4. Bioinformatic and statistical analysis

In all cases, homology searches were performed using the BLAST protein algorithm against the GenBank non-redundant protein database at http://www.ncbi.nlm.nih.gov. The theoretical molecular weights and isoelectric points were determined by the pH/MW algorithm at expasy server (http://ca.expasy.org). Cluster of orthologous group (COG) for the identified proteins was determined by COGNITOR program at http://www.ncbi.nlm.nih.gov/COG/. The identified homologues in kenaf were identified by Pfam database