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Protein mechanisms in response to NaCl-stress of salt-tolerant and salt-sensitive industrial hemp based on iTRAQ technology

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ABSTRACT

Industrial hemp is one of the important natural green fiber crops and, up to now, there has been a lack of research on its salt tolerance and the metabolic molecular mechanisms involved. Study on the adaptive mechanisms to salt stress in hemp through comparative proteome analysis is one important way to solve this problem. In this paper, iTRAQ-based proteomics technology was first used to study the differential proteomics of leaves of seedling under salt stress (600 mM sodium chloride) for 4 days in salt-resistant industrial hemp and salt-sensitive varieties. There were 403 and 252 differential proteins identified through LC-ESI–MS/MS mass spectrometry, respectively, and these were classified into 14 categories: primary metabolism, energy, protein destination and storage, disease/defense, protein synthesis, photosynthesis, transport, transcription, signal transduction, cell structure, secondary metabolism, intracellular traffic, cell growth/division and unknown. Hemp adapted to stress mainly by improved ATP metabolism, regulating photosynthesis according to light intensity, strengthening chlorophyll synthesis, promoting cell relaxation and enlargement, improving synthesis of osmoregulation substances, enhancing flow of inorganic sulfur in the body, regulating aquaporins, strengthening transmission of ion transport signals, improving signal transmission between proteins and between proteins and cytomembranes, improving selective absorption and transportation speed of organic and inorganic molecular, degrading hemicellulose cell walls, controlling transport of cellular material in and out, and promoting metabolism and cell stability. The results provide important reference information for research on molecular mechanisms of salt-stress adaption in hemp.

1. Introduction

Salt damage is one of the most important abiotic stresses in global agricultural regions. Twenty percent of global land area and over 50% of agricultural irrigated land is influenced by salt damage. It is estimated that over 25% of agricultural land in the next 25 years, and even 50% by 2050, will be rendered unusable for farming due to salt (Mahajan and Tuteja, 2005; Yan et al., 2005). Soil salinization not only affects the ecological environment balance, but also seriously restricts sustainable development of the economy and world agriculture (Niu and Wang, 2002).

When the soil salt concentration reaches 40 mM (e.g., of sodium chloride, NaCl), it is considered saline, which seriously affects normal growth of crops (Munns and Tester, 2008). Salt stress simultaneously leads to other secondary stresses of crops; moderate salt-stress results in ion and osmotic stress in plants, and severe stress will destroy the ion balance leading to nutritional deficiency and oxidative stress (Wang et al., 2003). Crops under salt stress often adapt to it through adjusting related metabolic processes and morphological characteristics. This includes inducing expression of cell membrane and cell wall related proteins; changing cell shape; inducing various kinds of osmoprotectants, late-embryogenesis abundant (LEA) proteins, chaperones and detoxification enzymes; protecting cell, cell organelle and proteins; as well as stimulat-

Abbreviation: ACN, acetonitrile; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio] propanesulfonic acid; DTT, dithiothreitol; ESI, electrospray ionization; HSP, heat shock protein; iTRAQ, isobaric tagging for relative and absolute quantitation; LC, liquid chromatography; MS, mass spectrometry; PMSF, phenylmethanesulfonyl fluoride; TEAB, triethylamine borane; TOF, time of flight.

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ing related stress signal transduction pathways (Seki et al., 2003; Shinozaki et al., 2003).

Industrial hemp is a low-toxicity cannabis [THC (Δ-9-tetrahydrocannabinol) < 0.3%] without drug value, and is often used as green fibers. It plays a very important role in the textile and manufacturing industries. The perennially cultivated area of hemp in China is 30 000–50 000 ha, accounting for 1/3–1/2 of the world's cultivated area (Yan, 2014). Hemp has the characteristics of wide distribution, deep root system, fast growth, high biomass, strong resistance to stress and ease of cultivation. It can adapt to the stress environment of saline alkali land, and is a suitable economic crop for the improvement and promotion of saline alkali land; however, there are relatively large differences in salt tolerance among varieties. There is an urgent need to select salt-tolerant varieties through biotechnological methods and improving salt tolerance (Guo et al., 2010). However, there has been no research on the salt-tolerance mechanisms of hemp, and the molecular mechanisms of adaption to salt stress remain unclear. Therefore, it is important to explore the salt-stress related proteins and metabolic pathways through molecular technologies. Study of the life activities of hemp related to adaption to salt stress using proteomics is an important and direct way to address this problem.

In this paper, the currently popular high-throughput proteomic technology (iTRAQ technology) was first used to analyze relative enzyme and metabolic activities adaptive to salt stress in two industrial hemp varieties (salt-tolerant and -sensitive) under salt stress, and to study the important stress-protein mechanisms of photosynthesis, energy metabolism and stress-related metabolism in hemp under salt stress. This provides an important molecular basis and reference information for future study of hemp salt tolerance.

2. Materials and methods

2.1. Plant growth

The experimental materials included salt-tolerant variety Yun Ma No. 5 (YM) and salt-sensitive variety Ba Ma (BM), provided by the Industrial Crops Institute of Yunnan Academy of Agricultural Sciences and the Guangxi Academy of Agricultural Sciences, respectively.

Pot culture was applied using pots of 19 cm in height and 16 cm in diameter with holes in the bottom. An equivalent weight of matrix was put in each pot (red soil and humus mixed in 1:1 ratio), and five hemp seeds were planted in it. Each variety was planted in 30 pots, making a total of 60 pots. All pots were placed in a sheltered and transparent greenhouse.

Every variety had two treatments with 15 pots each: one was salt-stress treatment of 600 mM NaCl; and the other was control, treated with water. The stress treatment was applied when seedlings had four pairs of true leaves (about 20 cm high). The pots for salt stress treatment were watered with 500 mL of 600 mM NaCl once, while controls were watered with 500 mL of fresh water. After that all pots were watered every 2 days to supplement the evaporated water, and three pot per treatment was randomly selected respectively every 2 days after stress treatment for determination of physiological and biochemical. For each treatment, 1–2 g (per pot) of young leaves sampled(at four-leaf stage), repeated twice, which were quick frozen in liquid nitrogen and stored at −80 °C in a refrigerator for protein extraction.

2.2. Determination of physiological and biochemical indexes

The physiological and biochemical indexes including soluble protein, soluble sugar, chlorophyll and superoxide dismutase (SOD) activity (Wang, 2015) in the antepenultimate pair of leaves in hemp seedlings were determined every 2 days from the beginning of the stress treatment.

2.3. Protein extraction

Proteins were extracted from two biological replicates per treatment. Dry proteins from hemp leaves were prepared according to the extraction procedure of Deng et al. (2014). Dry protein powder was treated with 0.5 mL of lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris–HCL, 5 mM EDTA, 1 mM PMSF and 10 mM DTT, pH 8.0). The samples were sonicated three times for 5 min on ice using a high intensity ultrasonic processor. The remaining debris and unbroken cells were removed by centrifugation at 30,000 × *g* at 4 °C for 15 min. To the supernatant was added 1 mmol/L PMSF and 2 mmol/L EDTA, and then 5 min later 10 mmol/L DTT was also added. Four volumes of prechilled 10% (w/v) TCA in acetone solution were added to the supernatant and kept at −20 °C overnight. Then the mixture was centrifuged at 30,000 × *g* for 15 min at 4 °C and the supernatant was discarded. The precipitate was washed with cold acetone before being placed in a freezer at −20 °C for 1 h. The centrifugation step was repeated and the supernatant was collected. After vacuum drying, 500 μL of lysis buffer was added to the precipitated dry protein powder. Then the mixture was sonicated for 15 min on ice and centrifuged at 30,000 × *g* at 4 °C for 15 min. The supernatant was transferred to a new tube, reduced with 10 mM DTT for 1 h at 56 °C and alkylated with 55 mM iodoacetamide for 45 min at room temperature in darkness. The centrifugation step was repeated and the supernantant was collected. Protein content was determined with Bradford (Biorad) or 2-D Quant kit (GE Healthcare) according to the manufacturer's instructions.

2.4. iTRAQ labeling

Approximately 100 μg of protein for each sample was digested with trypsin (Promega, Madison, WI) overnight at 37 °C in a 1:50 trypsin-to-protein mass ratio. After trypsin digestion, peptides were reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for 8-plex iTRAQ kit (Applied Biosystems). Two control samples of YM were labeled with iTRAQ tags 113 and 114, two salt-treatment samples of YM were labeled with iTRAQ tags 115 and 116, two control samples of BM were labeled with iTRAQ tags 117 and 118 and two salt-treatment samples of YM were labeled with iTRAQ tags 119 and 121. Briefly, one unit of iTRAQ reagent was thawed and reconstituted in 70 μL of isopropanol. Peptides from treatment and control subgroups were labeled with different respective iTRAQ tags, by incubation at room temperature for 2 h. The peptide mixtures were then pooled and dried by vacuum centrifugation. The pooled mixtures of iTRAQ-labeled peptides were fractionated by strong cationic exchange (SCX) chromatography.

2.5. Fractionation by SCX chromatography

For SCX chromatography using the Shimadzu LC-20AB HPLC Pump system, the iTRAQ-labeled peptide mixture was reconstituted with 4 mL of buffer A (25 mM NaH_2PO_4 in 25% ACN, pH 3.0) and loaded onto a 4.6 × 250 mm Ultremex SCX column containing 5-μm particles (Phenomenex). The peptides were eluted at a flow rate of 1 mL/min with a gradient of buffer A for 10 min, 5–35% buffer B (25 mM NaH_2PO_4 and 1 M KCl in 25% ACN, pH 3.0) for 11 min, and 35–80% buffer B for 1 min. The system was then maintained in 80% buffer B for 3 min before equilibrating with buffer A for 10 min prior to the next injection. Elution was monitored by measuring absorbance at 214 nm, and fractions were collected every 1 min.