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Proteomic and phosphoproteomic analysis reveals the response and defense mechanism in leaves of diploid wheat *T. monococcum* under salt stress and recovery

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ABSTRACT

Salinity is a major abiotic stress factor affecting crops production and productivity. *Triticum monococcum* is closely related to *Triticum urartu* (A^UA^U), which is used as a model plant of wheat A genome study. Here, salt stress induced dynamic proteome and phosphoproteome profiling was focused. The *T. monococcum* seedlings were initially treated with different concentrations of NaCl ranging from 80 to 320 mM for 48 h followed by a recovery process for 48 h prior to proteomic and phosphoproteomic analysis. As a result, a total of 81 spots corresponding to salt stress and recovery were identified by MALDI-TOF/TOF-MS from 2-DE gels. These proteins were mainly involved in regulatory, stress defense, protein folding/assembly/degradation, photosynthesis, carbohydrate metabolism, energy production and transportation, protein metabolism, and cell structure. Pro-Q Diamond staining was used to detect the phosphoproteins. Finally, 20 spots with different phosphorylation levels during salt treatment or recovery compared with controls were identified. A set of potential salt stress response and defense biomarkers was identified, such as cp31BHv, betaine-aldehyde dehydrogenase, leucine aminopeptidase 2, Cu/Zn superoxide dismutase, and 2-Cys peroxiredoxin BAS1, which could lead to a better understanding of the molecular basis of salt response and defense in food crops.

Biological significance: Soil salinity reduces the yield of the major crops, which is one of the severest problems in irrigated agriculture worldwide. However, how crops response and defense during different levels of salt treatment and recovery processes is still unclear, especially at the post-translational modification level. *T. monococcum* is a useful model for common wheat. Thus, proteomic and phosphoproteomic analyses of *T. monococcum* leaves were performed in our study, which provided novel insights into the underlying salt response and defense mechanisms in wheat and other crops.

1. Introduction

Salinity is a major environmental stress constraining crop growth and productivity [1]. Irrigation water that contains trace amounts of sodium chloride (NaCl) is a common effect on salt accumulation in arable soils [2,3]. Once Na^+ and Cl^- are taken up in large amounts by roots, increased salt concentrations cause ion toxicity, hyperosmotic stress and oxidative damage, impairing metabolic processes and decreasing photosynthetic efficiency [3,4]. Hence, improved salt tolerance of crops has also become an urgent task to reduce the spread of secondary salinity and sustain increases in food production worldwide [5].

Actually, plants have evolved sophisticated mechanisms to cope with salinity stress. The salt signal is primarily perceived through roots, which rapidly respond and transmit signals to the shoot for appropriate changes in function, regulating the transcription and translation of intracellular genes associated with stress response, ultimately generating a series of physiological and biochemical responses in plants [6]. Recently, many studies have provided a solid basis for studying salt tolerance mechanisms. In *Arabidopsis thaliana*, two salt overly sensitive (SOS) genes, *SOS1* and *SOS2*, were cloned, whose encoding proteins are required for Na^+ sequestration and extrusion to maintain intracellular Na^+/K^+ homeostasis [7]. Such types of genes also include GhNHX1 in tobacco [8] and SKC1 in rice [9]. In addition, plenty of pivotal salt-responsive genes are also involved in multiple biological processes, such as 5′-triphosphate (GTP)-binding protein (G protein), heat shock proteins (HSPs) and glutamate dehydrogenase (GDH) involved in signaling, protein turnover and amino acid metabolism, respectively [10]. Knowledge about these mechanisms is also crucial for continued

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development of rational breeding and transgenic strategies to improve stress tolerance in crops [11].

Proteomics has to date been 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, and widely used to study the molecular mechanisms of plant organs response to salt stress in different crops, such as rice, wheat, barley, and maize [11]. Several comparative proteomic studies have been performed in wheat under salt stress [1,12–14]. Gao et al. identified 83 differentially expressed spots from wheat leaves under four different concentrations (1.0%, 1.5%, 2.0%, and 2.5%) of NaCl, including several salt stress-stimulated proteins, such as H (+)-ATPases, glutathione S-transferase, ferritin and triosephosphate isomerase [12]. Forty-nine salt-responsive proteins were identified from seedling roots of two wheat cultivars under 200 mM salt treatment for 24 h. These proteins are mainly involved in signal transduction, transcription and translation, transporting, chaperones, proteolysis and detoxification [1]. Our previous work showed that 41 root salt-responsive proteins were shared by salt-tolerant (Jing-411) and salt sensitive (Chinese Spring) wheat cultivars subjected to a range of salt stress concentrations (0.5%, 1.5% and 2.5%) for 2 days, and interestingly more salt-responsive proteins were only found in salt-tolerant wheat cultivar (Jing-411) [13]. There studies provide many salt stress biomarkers for further elucidating the mechanisms of salt response and defense in crops. However, the information on proteome changes in response to salt stress has been fragmentary [15]. Typical studies only focus on comparison analysis of non-stressed (control) and stressed plants, while very few studies in this area have taken into account the dynamic processes of the plant response to salt stress that depend on stress intensity and duration. In addition, very few proteomic studies were performed for investigating the mechanism of recovery process in crops after removing salt stress.

Protein phosphorylation, known as a common post-translational modification (PTM), is related to the regulation of diverse processes, including metabolism, transcription/translation, protein degradation, homeostasis, cellular communication and communication. Recently, large-scale phosphoproteomic analyses have been performed to elucidate the mechanisms towards biological processes and stress resistance in common wheat [16,17]. However, underlying knowledge about phosphorylation modification under other abiotic stress in wheat remains unclear.

Domestication of crop species and centuries of cultivation have improved production yields at the expense of crop germplasm diversity [18]. Einkorn wheat, one of three cereal crops domesticated prior to 7000 BCE, contributed to the Neolithic Revolution [19]. Domesticated subspecies, *T. monococcum* L. ssp. *monococcum* ($2n = 2\times = 14$, A^mA^m) is closely related to *Triticum urartu* (A^UA^U), a progenitor of the A genome of hexaploid wheat (*Triticum aestivum*) (AABBDD) [20]. However, 5.6 Gb of genome size in comparison to approximately 17 Gb genome size of common wheat endows that the diploid *T. monococcum* has gradually been recognized as an attractive model for exploitation of useful traits [21], discovery of novel genes, such as the genes resistance to salt, chilling, stem rust, and powdery mildew [22–25]. However, studies on the salt stress are still insufficient and unsystematic. In this study, a comprehensive proteomic and phosphoproteomic analysis was performed in *T. monococcum* under salt stress and recovery, which widens the knowledge of salt stress response and defense mechanisms in crops.

2. Materials and methods

2.1. Plant materials and NaCl treatments

Plump seeds of *T. monococcum* were surfacely sterilized by 5% sodium hypochlorite for 5 min, and rinsed 4 times in sterile distilled water. Seeds were submerged in water for 12 h at room temperature, and then transferred to wet filter paper to germinate at room temperature (22–25 °C) for 24 h. The uniformly germinated seeds were selected to grow in plastic pots containing Hoagland solution that was changed every two days. At the three leaf stage, the plants were treated by NaCl with five different concentrations, 0 mM, 80 mM, 160 mM, 240 mM, and 320 mM. After treatment for 48 h, half of the plant leaves was harvested for analyses. Part of the sampled leaves were used to measure the physiological parameters immediately after leaf sampling and the remaining leaves were kept frozen in −80 °C for later use. The remaining half of the seedlings were rinsed two times (each time 2 min) with distilled water, then transferred to Hoagland solution without NaCl to recover for 48 h. After recovery, the leaves were collected and analyzed as description above.

2.2. Physiological parameters measurement

The harvested leaves mentioned above were used to measure the physiological parameters. Chlorophyll (chlorophyll a and b) content and relative water content (RWC) were measured according to Lv et al. [26]. The photosynthetic activity was evaluated via chlorophyll fluorescence determinations of the photochemical yield (Fv/Fm), which represents the maximum quantum yield of PSII, using a portable fluorometer (FluorPen FP 100; Photon Systems Instruments; Drasov, Czech Republic) after a 30 min dark adaptation. Soluble sugars and proline contents were measured according to Dubois et al. [27] and Troll and Lindsley [28]. Glycine betaine (GB) content, malondialdehyde (MDA) content, and peroxidase (POD) enzyme activity were measured by using the kits (Cat. nos. XG6, EY2, and FY3) supplied by Suzhou Keming science and technology co., Ltd. (China). Superoxide dismutase (SOD) enzyme activity was measured by using the kit from the Nanjing Jiancheng Bioengineering Institute of Jiangsu Province, China (Cat. no. A001-3). Three biological replicates were used to minimize experimental error. Statistical significances of the differences were determined by Student's *t*-test by using SPSS 17.0 software.

2.3. Protein extraction and two-dimensional gel electrophoresis (2-DE)

Leaf total protein was extracted according to the method previously described [16]. Three biological replicates were conducted for 2-DE and each sample were performed three technical replicates. Each sample including 1 mg total protein in 360 μL rehydration buffer (7 M urea, 2 M thiourea, 2% w/v CHAPS, 0.2% bromophenol blue) containing 65 mM DTT and 0.5% IPG buffer (pH 4–7) (GE Healthcare) was loaded onto a 18 cm, pH 4–7 linear gradient IPG strip (GE Healthcare). The separation of proteins in the first and second dimension was carried out as reported before [26]. After electrophoresis, all gels were stained with Coomassie brilliant blue (CBB). Image analysis was performed with ImageMaster 2D Platinum Software Version 7.0 (Amersham Biosciences). The abundance of each spot was estimated by the percentage volume (%vol). Only those with significant and biological reproducible changes (abundance variation at least 2-fold, one way ANOVA, $p < 0.05$) were considered to be differentially accumulated protein (DAP) spots.

2.4. Protein identification using MALDI-TOF/TOF-MS

The selected protein spots were manually excised from the 2-DE gels and digested with trypsin as described in Lv et al. [26]. Identification of the spots was performed by using matrix-assisted laser desorption/ionisation time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF-MS) as previously described [26]. The MS together with MS/MS spectra were searched against the NCBI wheat protein database (77,037 entries, as described by Lv et al. [16]) using software MASCOT version 2.1 (Matrix Science) with the following parameter settings: trypsin cleavage, one missed cleavage allowed, carbamidomethylation set as fixed modification, oxidation of methionines allowed as variable modification, peptide mass tolerance set to 100 ppm and fragment tolerance set to ±0.3 Da. All searches were evaluated based on the

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