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Chilling-responsive mechanisms in halophyte *Puccinellia tenuiflora* seedlings revealed from proteomics analysis

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ABSTRACT

Alkali grass (*Puccinellia tenuiflora*), a monocotyledonous perennial halophyte species, is a good pasture with great nutritional value for livestocks. It can thrive under low temperature in the saline-alkali soil of Songnen plain in northeastern China. In the present study, the chilling-responsive mechanism in *P. tenuiflora* leaves was investigated using physiological and proteomic approaches. After treatment of 10 °C for 10 and 20 days, photosynthesis, biomass, contents of osmolytes and antioxidants, and activities of reactive oxygen species scavenging enzymes were analyzed in leaves of 20-day-old seedlings. Besides, 89 chilling-responsive proteins were revealed from proteomic analysis. All the results highlighted that the growth of seedlings was inhibited due to chilling-decreased enzymes in photosynthesis, carbohydrate metabolism, and energy supplying. The accumulation of osmolytes (i.e., proline, soluble sugar, and glycine betaine) and enhancement of ascorbate–glutathione cycle and glutathione peroxidase/glutathione S-transferase pathway in leaves could minimize oxidative damage of membrane and other molecules under the chilling conditions. In addition, protein synthesis and turnover in cytoplasm and chloroplast were altered to cope with the chilling stress. This study provides valuable information for understanding the chilling-responsive and cross-tolerant mechanisms in monocotyledonous halophyte plant species.

1. Introduction

Chilling (0–15 °C) is one of the most crucial environmental factors limiting plant geographic distribution, growth, and development [1,2]. Chilling stress could damage chloroplast and plasma membrane (PM) structures, increase production of reactive oxygen species (ROS), and influence cellular metabolism. Some plant species have evolved the ability to enhance their tolerance in response to chilling stress [3]. Gene expression evidences reveal that chilling tolerance is not entirely constitutive, but at least part of it is developed during exposure to chilling conditions [3,4].

The acquired chilling tolerance involves reprogramming at various levels of gene, protein, and metabolism. Previous full-genome transcript profiling, in combination with mutational and transgenic analyses have revealed a number of genes in the complicated chilling-responsive molecular network [4]. However, the mRNA level does not always correlate well with protein level. The pattern changes of protein abundance generated from high-throughput proteomics can provide exhaustive information for understanding the molecular mechanisms of chilling response [5]. Proteomic approaches have been applied into investigation of chilling tolerance in model plant *Arabidopsis thaliana* and major cereal crops produced worldwide, such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*) [6]. These studies elucidated that photosynthesis, carbohydrate and energy metabolism, ROS scavenging, redox adjustment, cell wall remodeling, cytoskeletal rearrangement, and defense/detoxification were widely altered to cope with chilling stress [6–8]. Therefore, proteomic analysis of total proteins could provide new insights into the chilling response. However, little is known about the molecular mechanism in certain halophyte species.

Alkali grass (*Puccinellia tenuiflora*), a monocotyledonous perennial halophyte species in Gramineae, is widely distributed in the northeastern and northwestern China. *P. tenuiflora* has great nutritional value for livestocks because its seedlings have soft stem and large amount of leaves, which contain abundant crude proteins, less crude ash and crude fiber [9]. The plants can survive in the extreme saline-alkali soil (pH range of 9–10) of Songnen plain in northeastern China, and it is also characterized by chilling tolerance. In April, *P. tenuiflora* becomes green under low temperature (usually lower than 10 °C) and grows well under prevernal chilling conditions in the Songnen plain [9]. The plant has developed an efficient stress tolerance system, and thus is a potential source of genetic determinants for salinity and chilling tolerance. The physiological mechanisms of salinity tolerance have been widely studied using genomic, transcriptomic, and proteomic

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approaches [10–19]. In contrast, its chilling-tolerant characteristics are poorly understood. More importantly, the plants might have developed cross-tolerance mechanisms to cope with diverse stresses. It is known that some salinity-responsive genes/proteins in Arabidopsis were also involved in cold tolerance [20]. However, whether these saline- and/or alkali- responsive genes/proteins in *P. tenuiflora* seedlings were also involved in chilling tolerance is not known [21]. Investigation of the chilling-responsive proteins is crucial for rational engineering of the plants for enhanced stress tolerance and high yield. In the present study, we analyzed the temporal changes of total proteins in *P. tenuiflora* leaves after 10 °C treatment for 10 days and 20 days. The chilling-responsive physiological and proteomic features were highlighted.

2. Experimental procedures

2.1. Plant growth and chilling treatment

Seeds of *P. tenuiflora* (Turcz.) scribn. et Merr. were sown on vermiculite and grown in Hoagland solution under fluorescent light of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (13 h light/11 h dark) in a controlled environment chamber (25 °C and about 75% relative humidity) for 20 days [18]. The plants for chilling treatment were transferred to a chamber at 10 °C for 10 days and 20 days, respectively. The plants of control were still grown under 25 °C. Leaves of control and chilling-stressed plants were harvested and used fresh or immediately frozen in liquid nitrogen and stored at −80 °C. At least three biological replicates for each treatment and control were conducted for all the experiments. About ten fresh leaves (0.2 g) from more than three plant seedlings were used as a replicate for physiological analyses. About fifty fresh leaves (1.0 g) from over fifteen plants were taken as a replicate for proteomics analyses. The workflow of experimental design was shown in Supplementary Fig. S1 in the online version at http://dx.doi.org/10.1016/j.jprot.2016.04.038.

2.2. Measurements of biomass and relative water content (RWC)

The shoot length and fresh weight (FW) of seedlings were measured immediately at 10 days and 20 days after chilling (DAC). Dry weight (DW) of seedlings/leaves was obtained after oven-dried at 105 °C for 15 min, followed by being dried at 60 °C until to a constant weight. For measurement of RWC, 0.2 g FW of leaves were floated on deionized water for 24 h, and the turgid weight (TW) was quickly measured. RWC was calculated as follows: RWC = [(FW − DW)/(TW − DW)]*100% [22].

2.3. Photosynthesis and chlorophyll contents measurement

Net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO_2 concentration (Ci), and transpiration rate (Tr) were measured at 10:00 am in 10 DAC and 20 DAC using a portable photosynthesis system LICOR 6400 (LI-COR Inc., USA) [18]. Chlorophyll contents were determined by 80% (v/v) acetone extraction [22].

2.4. Determination of malondialdehyde (MDA) content, relative electrolyte leakage (REL),total soluble sugar, proline, and glycine betaine contents

The MDA content and REL were determined according to previous methods [22]. Contents of proline and total soluble sugar were determined using ninhydrin reaction and a sulfuric acid-anthrone method according to Li et al. [23]. Glycine betaine content was determined using reinecke salt as described by Tan[24].

2.5. Measurements of ROS and antioxidant substance, and enzyme activity assay

Generation rate of O_2^- was determined according to the method of Tan [24]. The content of H_2O_2 was determined according to the method of Ibrahim and Jaafar [25].

For the activity assays of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), glutathione peroxidase (GPX), glutathione S-transferase (GST), and glycolate oxidase (GO), 0.2 g of leaves were homogenized on ice in 3 mL of 50 mM phosphate buffer (pH 7.8). The activities of SOD, POD, CAT, APX, GR, GST, and GO were measured according to our previous methods [18]. The activities of DHAR, MDHAR, and GPX were assayed according to the method of Suo et al. [26]. In addition, contents of ascorbate (AsA), dehydroascorbate (DHA), reduced glutathione (GSH), and oxidized glutathione (GSSG) were determined according to the method of Tan [24]. In all the enzymatic preparations, protein content was determined using the method of Bradford [27].

2.6. Protein sample preparation, 2-DE, and protein abundance analysis

Total protein from leaves under different treatment conditions was extracted according to the method of Wang et al. [22]. The protein pellets were dissolved in a lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 4% proteinase inhibitor cocktail, 2% (v/v) pH 4–7 ampholytes). Protein concentration was determined using a Quant-kit (GE Healthcare, USA) according to manufacturer's instructions. BSA was used as the standard. The protein concentration was also evaluated by 12.5% SDS-PAGE using a Mini-PROTEAN Tetra Cell (1.0 mm, 165-8001, Bio-Rad, USA). When loading 20, 40, 60, and 80 μg proteins for each lane, the abundance of each protein band was equally increased to the maximum with the increasing loading quantity.

The protein samples were separated and visualized using 2-DE approaches according to Dai et al. [28]. The experiments were repeated three times using protein samples independently prepared from different treatments. Protein samples were separated on 24 cm IPG strips (pH 4–7 linear gradient) through IEF in the first dimension. About 1.3 mg protein was loaded for each gel. The IEF program was applied as follows: 30 V for 8 h, 50 V for 4 h, 100 V for 1 h, 300 V for 1 h, 600 V for 1 h, 1000 V for 1 h, and 8000 V for 10 h. The temperature was kept at 20 °C. The equilibrated strips were loaded on the 12.5% SDS-PAGE gels using an Ettan DALT Six Electrophoresis Unit (GE Healthcare). Gels were stained by CBB. Images were acquired by scanning each CBB-stained gel using an ImageScanner III (GE Healthcare) at a resolution of 300 dpi and 16-bit grayscale pixel depth. The gel images were analyzed with ImageMaster 2D software (version 5.0, GE Healthcare). After protein spot detection, quantification, and background subtraction, a normalized percentage of spot volume (vol%) was generated by dividing the volume of each spot by the total volume of all spots in a gel using ImageMaster 2D software according to a method described by Dai et al. [28]. Comparisons and statistical analysis were performed using the normalized vol% values from three biological replicates among different treatments. The significance of differentially abundant proteins was determined by *t*-test. These proteins displaying consistent abundance changes among three biological replicates with at least 1.5-fold changes and a p value smaller than 0.05 were considered as chilling-responsive proteins.

2.7. Protein identification by ESI-Q-TOF MS/MS and database searching

The differentially abundant spots were excised from the 2-DE gels and digested with trypsin according to the method of Dai et al. [28]. MS/MS was acquired on an ESI-Q-TOF MS (QSTAR XL, AB Sciex, USA). A Mass Standards Kit (AB Sciex) and a standard BSA digest (Sigma-