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Identification of alkali-responsive proteins from early seedling stage of two contrasting *Medicago* species by iTRAQ-based quantitative proteomic analysis

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ABSTRACT

Saline-alkaline stress is one of the primary abiotic stresses that limits crop yields worldwide. The early seedling stage of plants is the most vulnerable stage to stress conditions. In this study, the physiological and phenotypic changes induced by alkali treatments (Na_2CO_3 and $NaHCO_3$ mixtures) were analyzed for alfalfa (*Medicago sativa* L. cv. Zhongmu-3) and barrel medic (*Medicago truncatula* line R108) seedlings. As expected, Zhongmu-3, which is alkali tolerant, and R108, which is alkali sensitive, responded differently to alkaline stress. To characterize the protein expression profiles of these two *Medicago* species in response to alkaline stress, an iTRAQ-based quantitative proteomic analysis was applied to detect alkali-responsive proteins. A total of 467 differentially changed alkali-responsive proteins were identified from Zhongmu-3 and R108. Compared with their levels in untreated control seedlings, the abundance of 349 proteins increased and 38 proteins decreased in alkali-treated Zhongmu-3 seedlings, whereas 142 proteins increased and 35 proteins decreased in R108 seedlings. Zhongmu-3 and R108 shared 97 common differentially changed proteins, but a large percentage of them showed different change patterns between Zhongmu-3 and R108. Subsequent functional annotation indicated these proteins influenced diverse processes, such as catalytic activity, signaling, and antioxidant activity. The transcript levels of genes encoding 10 differentially changed proteins were determined by quantitative PCR. The data provide new insights into the regulatory mechanisms responsible for alkali stress responses in leguminous plants and have potential implications for breeding of alkaline-resistant alfalfa and other crops.

1. Introduction

Soil alkalization is one of the most serious abiotic factors limiting agricultural production. In nature, alkaline stress often co-occurs with saline stress. More than 10% of the world's arable land has been affected by salinity or alkalinity or both, and more than 50% of this area is alkalinized (FAO, http://www.fao.org) (Krasensky and Jonak, 2012; Li et al., 2010; Sekmen et al., 2012). Alkaline and saline cause distinct kinds of stresses on plants, and plants may respond differently to each. Alkaline stress is caused mainly by high concentrations of alkaline salts (Na_2CO_3 or $NaHCO_3$) that damage plants via high pH, ion toxicity, osmotic stress, excessive levels of reactive oxygen species, nutritional deficiency, and other adverse conditions (Lin et al., 2017; Yang et al., 2007; Zhang et al., 2012). Compared with saline stress, alkaline stress is a more diversified form of stress that also creates high pH stress. The pH of alkaline affected soil is generally higher than 8.5 (Wang et al., 2009). High pH environments surrounding plant roots inhibit ion uptake and disrupt ion homeostasis in cells, which then negatively affects the growth and photosynthesis of plants (Wang et al., 2012). Alkaline tolerance of different plants implies the presence of a complex tolerance mechanism (Paz et al., 2014). Many previous studies have focused on plant salt stress responses, but alkaline stress responses have received less attention. In recent years, some studies on the effects of alkaline or mixed salt-alkaline stresses have been reported on a few plant species, such as soybean (Fan et al., 2013), *Arabidopsis* (Zhu et al., 2014; Chen et al., 2015), *Lotus tenuis* (Paz et al., 2014), *Medicago ruthenica* (Guan

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et al., 2009), tomato (Zhang et al., 2015), maize (Abdel and Tran, 2016), flax (Yu et al., 2014) and rice (Zhang et al., 2017; Wei et al., 2015). However, alkaline stress tolerance mechanisms of plants have not been studied intensively. Thus, improving the understanding of the basic mechanisms of plant responses to alkaline stress is urgently needed and will be helpful in enhancing and breeding alkali-tolerant crops through biotechnology.

Alfalfa (*Medicago sativa*), a perennial tetraploid (2n = 4× = 32) forage crop, is cultivated widely all over the world for its high protein content, good quality, and high yield. Barrel medic (*Medicago truncatula*), a small annual diploid legume (2n = 16), has been used as a model leguminous plant because of its small sequenced genome. Additionally, it is self-fertile and amenable to genetic transformation. Some alfalfa cultivars, such as Zhongmu-1 and Zhaodong, are resistant to salinity and alkalinity stresses and are widely cultivated in saline-alkaline arable land in North China (An et al., 2016; Long et al., 2016). Barrel medic is a glycophyte and sensitive to saline and alkaline stresses (Long et al., 2016). Plants differ considerably in their tolerance to salinity, as reflected by their different growth responses. A number of genes and their protein products, rather than individual genes or proteins, were reported to be involved in plant alkaline stress responses (DuanMu et al., 2015; Fan et al., 2013; Zhang et al., 2015). Hence, a more comprehensive understanding of alkaline stress tolerance mechanisms in plants may be obtained using transcriptome and proteome analyses.

Zhongmu-3, an upgrade alfalfa cultivar of Zhongmu-1, can germinate and grow at high pH in the alkaline soil of North China, indicating Zhongmu-3 has developed distinctive molecular and physiological mechanisms to adapt to alkaline stress conditions. Therefore, Zhongmu-3 is a candidate for screening alkaline stress resistance genes and for studying the molecular mechanisms of alkaline stress tolerance. In contrast, line R108 (*Medicago truncatula*) is more sensitive to alkaline than Zhongmu-3, so it can be used as a negative candidate for alkaline stress analysis. Seed germination and early seedling are the initial and most important stages in the plant life cycle. High alkali levels have serious impacts on seed germination. Alkaline tolerance at the early seedling stage was found to be significantly correlated with alkaline tolerance during germination (Guan et al., 2009). Proteomics-based technologies are powerful tools for studying protein abundance and modification. In the present study, an iTRAQ-based quantitative proteomic analysis was used to examine the proteomic changes in early seedling of Zhongmu-3 and R108 under alkaline stress. Physiological changes in the seedlings were also measured. Two alkaline salts ($NaHCO_3$ and Na_2CO_3) were used in mixtures to mimic alkaline stress conditions. Our results provide a valuable data resource for the increased understanding of plant alkaline tolerance mechanisms, as well as for genetic improvement of alkaline stress tolerance of alfalfa.

2. Materials and methods

2.1. Plant material, growth conditions, and stress treatments

Two *Medicago* species, alfalfa (*M. sativa*) cultivar Zhongmu-3 (ZM-3) and barrel medic (*M. truncatula*) line R108 were used in this study. Zhongmu-3 is a saline-alkaline resistant alfalfa cultivar and barrel medic is more sensitive to abiotic stresses than alfalfa (Long et al., 2016). Zhongmu-3 and R108 seeds were surface-sterilized in 75% ethanol for 10 min and then washed three times with double-distilled water. About 80 seeds were germinated in a Petri dish (φ = 15 cm) containing 10 mL of 0, 5, 10, or 15 mM alkali solution ($NaHCO_3$:Na_2CO_3 = 1:1). The pHs of the alkali solutions were similar (pH 9.9–10.1). All the seeds were placed in a growth chamber at 25 °C with relative humidity 70% under a 16-h light/8-h dark cycle. The germination rate was calculated for three biological replicates after 3 days. Then, the germinated seedlings were frozen in liquid nitrogen and stored at −80 °C for protein and RNA extraction. The seedlings treated with the 5 mM (treated samples) and 0 mM (control samples) alkali solutions were used for the iTRAQ analysis. The Zhongmu-3 treated and control samples were called ZM-3-A and ZM-3-0, respectively, and the R108 treated and control samples were called R108-A and R108-0, respectively.

2.2. Physiological analysis

After germinating for 3 days, the physiological characteristics of the ZM-3-A, ZM-3-0, R108-A, and R108-0 seedlings were analyzed. Root and hypocotyl lengths of five seedlings per biological replicate were measured, and fresh weights were determined for 10 seedlings per biological replicate. The analyzed samples were then ground to a fine powder in liquid nitrogen using a mortar and pestle. The samples were subsequently examined by enzyme-linked immunosorbent assay (ELISA) (Nanjing Jiancheng Bioengineering, China) using an RT-6100 Microplate Reader (Rayto, USA) to measure the concentrations of peroxidase (POD), superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH), melatonin (MT), and abscisic acid (ABA).

2.3. Protein and RNA extraction

For each sample (12 in total), approximately 1 g of 3-day-old seedlings were frozen in liquid nitrogen and ground with a mortar and pestle. The ground samples were mixed with lysis buffer [1 mL/0.1 g; 7 M urea, 2 M thiourea, 4% CHAPS, and 1% protease inhibitor cocktail] (Roche, USA) in centrifuge tubes. The solutions were ultrasonicated and then incubated at room temperature for 30 min before being centrifuged at 15,000 × *g* for 1 h at 4 °C. The supernatants were collected, precipitated in four volumes of 10% (w/v) trichloroacetic acid/acetone solution, and stored at −20 °C overnight. The samples were centrifuged at 15,000 × g for 10 min at 4 °C and the pellets were rinsed three times with ice-cold acetone. The washed pellets were air-dried, dissolved in lysis buffer, and analyzed with a Bradford assay to measure protein concentration (Bradford, 1976). Total RNA was extracted from the samples using a Quick RNA Isolation Kit (Huayueyang, China) according to the manufacturer's instructions, then dissolved in RNase-free double-distilled H_2O. The RNA quality was analyzed with a NanoDrop 2000 spectrophotometer (Thermo, USA), after which cDNA was synthesized using a PrimeScript Reverse Transcriptase Kit (Takara, Japan) for subsequent quantitative PCR.

2.4. Digestion, iTRAQ labelling, and liquid chromatography with tandem mass spectrometry (LC–MS/MS)

For each sample, 200 μg protein dissolved in lysis buffer was reduced with 25 mM DTT, alkylated with 50 mM iodoacetamide, and subjected to centrifugal ultrafiltration (12,000 × *g* for 20 min) through a 10-kDa cut-off filter (Millipore, USA). The filters were washed three times with 100 μL dissolution buffer (20 mM triethylammonium bicarbonate), followed by centrifugation at 12,000 × *g* for 20 min A 50-μL aliquot of dissolution buffer (20 mM triethylammonium bicarbonate) containing 4 μg trypsin was added to each filter, which was then incubated at 37 °C for 12 h (filter-aided sample preparation method) (Wisniewski et al., 2011). The peptides were collected by centrifugation at 12,000 × *g* for 10 min. Finally, the filters were washed with 50 μL dissolution buffer (20 mM triethylammonium bicarbonate). The collected peptide-containing dissolution buffers were combined. Peptides digested from 100 μg protein for each sample in dissolution buffer were labelled with isobaric tags from an iTRAQ Reagent-8plex Multiplex Kit (AB Sciex, USA) according to the manufacturer's recommended procedure (114, 113, 117, and 116 for three biological replicates of R08-0, R08-A, ZM-3-0, and ZM-3-A, respectively, and 119 for the pooled R08-0, R08-A, ZM-3-0, ZM-3-A sample). The labelled peptide mixtures were then pooled and dried by vacuum centrifugation. Then, the labelled peptides were fractionated using a strong cation-exchange