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Differential proteomic responses to water stress induced by PEG in two creeping bentgrass cultivars differing in stress tolerance

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ABSTRACT

Protein metabolism and expression play important role in plant adaptation to water stress. The objectives of this study were to examine proteomic responses to water stress induced by polyethylene glycol (PEG) in creeping bentgrass (*Agrostis stolonifera* L.) leaves and to identify proteins associated with stress tolerance. Plants of two cultivars ('Penncross' and 'Penn-A4') differing in water stress tolerance were grown in sand irrigated daily with water (control) or PEG solution (osmotic potential of −0.66 MPa) to induce water stress, for 28 d in growth chambers. Shoot extension rate, relative water content and cell membrane stability were measured to compare drought tolerance between the two cultivars. All parameters maintained at a significantly higher level in 'Penn-A4' than in 'Penncross' under PEG treatment. After 28 d of water stress, proteins were extracted from leaves and separated by difference gel electrophoresis. Among 56 stress-responsive protein spots, 46 were identified using mass spectrometry. Some proteins involved in primary nitrogen and carbon metabolism were down-regulated by PEG-induced water stress in both cultivars. The abundance of antioxidant enzyme proteins (ascorbate peroxidase, catalase and glutathione-S-transferase) increased under water stress, particularly ascorbate peroxidase in 'Penn-A4'. The abundance levels of actins, UDP-sulfoquinovose synthase and glucan exohydrolase were greater in 'Penn-A4' than in 'Penncross' under PEG treatment. Our results suggest that proteins involved in membrane synthesis, cell wall loosening, cell turgor maintenance, and antioxidant defense may play roles in perennial grass adaptation to PEG-induced water stress.

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Introduction

Water deficit is a significant problem in agricultural production, including perennial grasses. Plant adaption to water stress may be accomplished through changes at the molecular, cellular, and physiological levels. Physiological studies have demonstrated that changes in water relation, nutrient uptake, hormonal metabolism, carbon metabolism, and antioxidant metabolism play important roles in drought tolerance (Bray, 1997). Transcriptomic studies have revealed that the expression of a wide range of genes is regulated in response to water deficit (Kreps et al., 2002; Seki et al., 2002; Shinozaki and Yamaguchi-Shinozaki, 2007). Study in *Arabidopsis* showed that 1008 mRNAs were up-regulated in response to water deficit (Kreps et al., 2002). Although RNA and DNA microarrays are powerful tools in the detection of gene expression, limited knowledge of stress-responsive protein expression remains a major gap in understanding biological functions of genes and the linkage between gene expression and physiological functions. Therefore, comprehensive profiling of stress-responsive proteins is important for further understanding the molecular mechanisms controlling plant drought tolerance.

Proteomics, the study of global changes in proteins, offers a powerful approach to discovering the genes and pathways that are crucial for stress responsiveness and tolerance. The identification and characterization of stress-responsive proteins and their corresponding genes has proven to be of immense practical values. Recently, proteomic-based technologies have been successfully applied to the systematic study of the proteomic responses in many plant species to a wide range of abiotic stresses, including water stress (Salekdeh et al., 2002; Plomion et al., 2006; Gazanchian et al., 2007; Hajheidari et al., 2007; Ingle et al., 2007; Kottapalli et al., 2009). These studies indicated that water stress altered the abundance of proteins involved in carbohydrate and energy metabolism, cellular detoxification, protein degradation and processing, signal transduction, and cell wall strengthening. Most of the previous work on water stress related proteomics was performed on annual crops (Salekdeh and Komatsu, 2007). However, very limited infor-

Abbreviations: APX, ascorbate peroxidase; DTT, dithiothreitol; EL, electrolyte leakage; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GST, glutathione-S-transferase; HSPs, heat shock proteins; IEF, isoelectric focusing; OEE, oxygen evolving enhancer; PEG, polyethylene glycol; PSII, photosystem II; RWC, relative water content; SHMT, serine hydroxymethyltransferase; TCA, trichloroacetic acid.

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mation is available on proteomic responses of perennial grasses to water stress. Perennial grasses may express stress-responsive proteins associated with long-term adaptation or stress survival, as perennial grasses must endure stress or persist through the stress period, unlike annual crops which produce seeds and may die in the case of severe drought (DaCosta and Huang, 2009). Thus, developing long-term adaptation mechanisms is critical for the survival of perennial grasses in water-limiting environments. A better understanding of proteomic responses to water stress in perennial grass species is vital for the development of breeding or biotechnology strategies to improve plant growth and productivity, and to reduce water use in areas with limited rainfall or irrigation. Investigation of stress-responsive proteins in tolerant cultivar in comparison to sensitive cultivar may identify specific proteins related to stress tolerance in grass. Fu et al. (2007) reported that drought tolerance of creeping bentgrass was improved by overexpression of LEA3 genes encoding dehydrin proteins. The objective of this study was to identify proteins responsive to polyethylene glycol (PEG)-induced water stress in perennial grass by comparing proteomic responses to water stress between two cultivars of creeping bentgrass (*Agrostis stolonifera*), a grass species widely used as a forage and turf in cool-climatic regions.

Materials and methods

Plant materials and water stress treatments

Plants of creeping bentgrass 'Penncross' and 'Penn-A4' were collected from field plots in the turfgrass research farm at Rutgers University, New Brunswick, NJ. Previous studies reported that 'Penn-A4' had superior drought resistance relative to 'Penncross' (McCann and Huang, 2008). All plants were propagated vegetatively in plastic pots (15 cm deep and 15 cm in diameter) filled with washed, fine sand. During the plant establishment period, plants were watered daily until water drained from the bottom of the pots and fertilized twice a week with full-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). Plants were maintained in a greenhouse for 30 d and then moved to a growth chamber set at 20/15 °C (day/night temperature), 75% relative humidity, 600 μmol m^{-2} s^{-1} of photosynthetically active radiation, and 12-h photoperiod. Plants were allowed to acclimate to the growth chamber conditions for 7 d before treatments were imposed.

The plants were irrigated daily with water (control) or with *polyethylene glycol* (PEG) 8000 solution to induce water stress for 28 d. Water stress was obtained by adding PEG solution with osmotic potential of −0.15 and −0.30 MPa twice a day for 2-d intervals at each concentration, and followed by adding PEG solution with osmotic potential of −0.66 MPa twice a day for 28 d. Each treatment for each cultivar was replicated in four pots. At each time of watering, sufficient water or PEG solution was applied until the sand medium was fully saturated. During the treatment period, plants were fertilized once a week with full-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950).

Physiological measurements

Physiological responses to water stress were evaluated by measuring shoot extension rate, leaf electrolyte leakage (EL) and relative water content (RWC) during the treatment period according to the method of Jiang and Huang (2002). For EL analysis, about 0.2 g (fresh weight) of leaves was placed in test tube containing 30 mL of distilled, deionized H_2O. Test tubes were shaken for 17–18 h at 23 °C, and the initial conductance (C_i) was measured with a conductivity meter (YSI Instrument, Yellow Spring, OH). Leaves then were killed at 120 °C for 30 min, and the conductance of killed tissue (C_{max}) was measured. The relative EL was calculated as $100 \times C_i/C_{max}$. RWC was calculated using the formula: 100[(FW − DW)/(TW − DW)] where FW is fresh weight, TW is turgid weight, and DW is dry weight following oven-drying leaf samples for 72 h at 80 °C. Shoot extension rate (mm d^{-1}) was measured as change in shoot height within each week of treatment.

Protein extraction and labeling

Protein extraction was performed following a protocol using acetone/trichloroacetic acid (TCA) precipitation as described previously (Xu et al., 2008). At 28 d of treatment, leaves were harvested and immediately frozen in liquid nitrogen, and then stored at −80 °C prior to analysis. Three independent samples were harvested from each treatment. About 0.5 g of leaf samples were homogenized and incubated with 10 mL of precipitation solution (10% TCA and 0.07% 2-mercaptoethanol in acetone) for 2 h at −20 °C. The precipitated proteins were pelleted and washed with ice-cold acetone containing 0.07% 2-mercaptoethanol to remove pigments and lipids until the supernatant was colorless. The pellet was vacuum-dried, resuspended in resolubilization solution (30 mM Tris–HCl pH 8.5; 7 M urea; 2 M thiourea; 4% CHAPS) and sonicated to extract proteins. Insoluble tissue was removed by centrifugation at 21,000 g for 15 min. Protein concentration was determined according to Bradford (1976) using a commercial dye reagent (Bio-Rad Laboratories, Hercules, CA) with BSA as a standard.

Proteins from plants of the control and PEG treatment were labeled respectively with Cy3 and Cy5 CyDye DIGE Fluor minimal dyes (GE Healthcare, Amersham, UK) as described previously (Xu et al., 2010). The dye: protein labeling ratio is deliberately kept low so that only proteins containing a single dye molecule are visualized on the gel and variants containing more than one dye molecule are avoided (Tonge et al., 2001). Cyanine dyes were reconstituted in 99.8% anhydrous dimethylformamide and added to labeling reactions in a ratio of 100 pmol CyDye: 25 μg protein. Protein labeling was achieved by incubation on ice for 30 min in the dark. The reaction was quenched by the addition of 10 mM lysine followed by incubation on ice for 10 min. In addition, a pooled internal standard composed of equal quantity of protein from all the experimental samples was labeled with Cy2. For each gel, 25 μg of each sample was mixed with 25 μg protein of the pooled internal standard.

Two-dimensional electrophoresis

The Immobiline DryStrips (13 cm pH 3–10 linear GE Healthcare) were passively rehydrated with the labeled samples for 1 h. The voltage settings for isoelectric focusing (IEF) were 50 V for 12 hrs, 500 V for 1 hr, 1000 V for 1 hr, and 8000 V to a total 80 kVh. Following IEF, the protein in the strips was denatured with equilibration buffer (50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate, 0.002% bromophenol blue, 1% dithiothreitol/DTT) and then incubated with the same buffer containing 2.5% iodoacetamide instead of DTT for 20 min at room temperature. The second dimension electrophoresis was performed on a 12.5% gel using a Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare, Piscataway, NJ). Labeled proteins were visualized using the Typhoon 9410 imager (GE Healthcare, Piscataway, NJ). The Cy2 images were scanned using a 488 nm laser and an emission filter of 520 nm BP (band pass) 40. Cy3 images were scanned using a 532 nm laser and an emission filter of 580 nm BP 30. Cy5 images were scanned using a 633 nm laser and a 670 nm BP 30 emission filter. The narrow BP emission filters ensure that there is negligible cross-talk between fluorescence channels. Images were analyzed using SameSpots software (Nonlinear). To correct the variability due to staining, the spot volumes were normalized as a percentage of the total volume of all spots on the gel. The detailed procedure