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Root protein metabolism in association with improved root growth and drought tolerance by elevated carbon dioxide in creeping bentgrass

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ABSTRACT

Atmospheric carbon dioxide (CO_2) concentration has been increasing and is predicted to further increase in the future along with the climatic changes which may increase evaporative demand on plants. Elevated CO_2 concentration has a positive effect on plant growth and tolerance to drought stress with regard to above-ground plant organs but limited information is available describing effects of elevated CO_2 concentration on root growth and the subsequent impact on plant responses to drought stress. The specific proteins and metabolic pathways controlling root functions regulated by CO_2 that may contribute to improved root growth and drought stress damages are not well understood. In this study with creeping bentgrass (*Agrostis stolonifera* cv. Penncross), a widely-used perennial grass for forage and turf, elevated CO_2 concentration (800 $\mu L\,L^{-1}$) promoted root proliferation compared to the ambient CO_2 concentration (400 $\mu L\,L^{-1}$). Under drought stress, roots developed under elevated CO_2 concentration were able to maintain improved membrane integrity as demonstrated by lower electrolyte leakage. Proteins were extracted from roots of creeping bentgrass exposed to both elevated and ambient CO_2 concentration under well-watered and drought stress conditions. Drought- and CO_2-responsive proteins were separated with two-dimensional electrophoresis and identified using mass spectrometry. Root proteins were mainly classified into the following functional categories: cellular growth, energy, metabolism, and defence. The improved root growth and mitigation of drought stress in creeping bentgrass under elevated CO_2 could be mainly associated with alteration of proteins governing primary metabolism involving nitrogen metabolism (glutamine synthetase), energy metabolism involving respiration (glyceraldehyde-3-phosphate dehydrogenase), and stress defence by strengthening antioxidant metabolism (ascorbate peroxidase, superoxide dismutase, and catalase) and chaperone protection (HSP81-1).

1. Introduction

Atmospheric carbon dioxide (CO_2) levels have risen by 69 $\mu L\,L^{-1}$ between years 1958 and 2008 (Dlugokencky, 2008) and recent environmental trends suggest the rate of increase will continue to hasten over the next century (Houghton et al., 2001). Water availability for irrigation of plants is also becoming limited which may increase frequency and severity of drought stress adversely affecting plant growth and productivity (Cattivelli et al., 2008). Numerous studies have demonstrated that elevated CO_2 may promote plant growth and mitigate damages from abiotic stresses, including drought stress, which has been largely attributed to increases in net photosynthetic rate of leaves and improved water and nutrient use efficiency (Kirkham, 2011). Most of the previous studies focused on responses of the above-ground parts (leaves and shoots) to elevated CO_2, such as increased tillering (Hocking and Meyer, 1991), leaf area, and total shoot biomass (Wand et al., 1999). Limited information is available regarding effects of elevated CO_2 on root growth and subsequent

Abbreviations: ACN, Acetonitrile; AGC, Automatic gain control; APX, Ascorbate peroxidase; ATP, Adenosine triphosphate; CAT, Catalase; CBB, Coomassie Brilliant Blue G-250; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate; Ci, Initial conductance; Cmax, Final conductance; CoA, Coenzyme A; CO_2, Carbon dioxide; EF, Elongation factor; EL, Electrolyte leakage; FNR, Ferredoxin NADP-reductase; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GS, Glutamine synthetase; HSP, Heat shock protein; IPG, Immobilized pH-gradient; IT, Ion transfer time; LSD, Least significant difference; MS, Mass spectrometry; NADP, Nicotinamide adenine dinucleotide phosphate; NCBI, National Center for Biotechnology Information; PAR, Photosynthetic active radiation; PSM, Peptide spectral match; ROS, Reactive oxygen species; RPLC, Reverse phase liquid chromatography; SDS, Sodium dodecyl sulfate; SOD, Superoxide dismutase; SWC, Soil water content; TCA, Tricarboxylic acid; TEMED, Tetramethylethylenediamine; WUE, Water-use efficiency.

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impact on plant adaptation to drought stress, although root systems control water uptake capacity and play key roles in a plant's ability to avoid drought stress. Previous studies found positive effects of elevated CO_2 on root formation and root elongation under non-stress conditions (Taylor et al., 1994; Pritchard et al., 2000; Kirkham, 2011). Here we investigated proteins that are involved in root growth responses and mitigation of drought stress by elevated CO_2.

Proteomic profiling is a powerful tool for the identification and quantification of proteins involved in various metabolic processes controlling plant growth and has been successfully utilized to reveal a wide array of proteins and associated metabolic processes in shoots regulating plant growth and responses to drought stress alone (Xu and Huang, 2010; Merewitz et al., 2011) or elevated CO_2 alone (Bae and Sicher, 2004; Bokhari et al., 2007; Yu et al., 2014). These studies demonstrate that the majority of stress-responsive or CO_2-reponsive proteins in leaves are related to photosynthesis, carbon metabolism, protein synthesis, energy pathways, and stress defence. Drought-responsive proteins in roots are mainly involved in respiration metabolism and energy production functions (Xu and Huang, 2010; Merewitz et al., 2011) though root proteomic responses to elevated CO_2 and interacting CO_2 and drought stress are unknown. Understanding protein responses to elevated CO_2, particularly under drought stress is critically important for unraveling the mechanisms underlying improved root growth and drought tolerance under the scenarios with increasing CO_2 concentration and climate changes. The objective of this project was to identify proteins and metabolic processes involved in root-growth responses to elevated CO_2 concentration under well-watered and drought stress conditions for creeping bentgrass plants.

2. Materials and methods

2.1. Plant material and growth conditions

Individual tillers (20 per pot) (without roots) of creeping bentgrass (*Agrostis stolonifera* cv. Penncross) were collected from a single stock plant and transplanted into pots (10 cm diameter $\times$ 40 cm depth) filled with fritted clay medium (Profile Products, Deerfield, IL). Plants were maintained in a controlled-climate growth chambers (Environmental Growth Chamber, Chagrin Falls, Ohio, USA) set to 21/18 °C (day/night), 650 $\mu mol\, m^{-2}\, s^{-1}$ photosynthetic active radiation (PAR), 60% relative humidity, and 14 h photoperiod for a week to allow plant acclimation to the growth chamber conditions prior to exposing plants to CO_2 treatments.

2.2. Treatments and experimental design

Plants were initially established for 35 d (March 1 to April 4, 2013) at ambient (400 $\mu L\, L^{-1}$) or elevated (800 $\mu L\, L^{-1}$) CO_2 concentration under well-watered conditions and fertilized twice per week with half-strength Hoagland's solution (Hoagland and Arnon, 1950). Following 35 d of plant establishment (formation of new roots) under either of the two CO_2 treatments, plants were subjected to drought stress for 20 d (April 5 to April 24, 2013) by withholding irrigation. Soil water content was monitored daily using the time domain reflectometry method (Topp et al., 1980) (Trase Soil Moisture Equipment, Santa Barbara, CA).

The ambient and elevated CO_2 concentrations within chambers were maintained through an automatic CO_2 controlling system connected to the CO_2 source-tank containing 100% research-grade CO_2 following the method described in Yu et al., (2012a). CO_2 concentrations inside the chambers were continuously monitored using an infrared gas analyzer (Li-820, LICOR, Inc., Lincoln, NE) connected to a computer data logger. The CO_2 concentration was maintained using an automatic controlling system consisting of a programmable logic controller unit, solenoid valves, and a laptop computer with a software capable of monitoring and maintaining CO_2 concentration within 10 $\mu L\, L^{-1}$ of the ambient or elevated target levels.

The experiment was arranged in a split-plot design with CO_2 treatment as the main plot and water treatment as the sub-plot with four replicates for each treatment. The ambient or CO_2 treatments were applied concurrently and each treatment was imposed in four different growth chambers. Plants were relocated between the chambers every 3 d to avoid possible confounding effects of unique growth chamber environmental variations from occurring.

2.3. Shoot growth and root growth analysis

Plants in four pots (replicates) from ambient or elevated CO_2 treatment which were either well-watered or drought-stressed were destructively sampled for shoot and root analysis parameters at 20 d of drought stress. Shoots were severed from roots, and roots were washed free of fritted clay, stained in 1% crystal violet solution, and scanned with a digital scanner (Epson Expression 1680, U.S. Epson, Inc., Long Beach, CA) to generate root images. Images were then analyzed with WinRHIZO Basic V.2002 software (Regent Instruments Inc., Quebec, QC, Canada) for root length, surface area, and diameter. All tissues (shoots and roots) were dried in an oven at 80 °C for 7 d. Shoot and root dry weight was measured, and root to shoot biomass ratio was calculated.

2.4. Root physiological analysis

Root electrolyte leakage (EL) was measured on four replicated samples from each treatment following 20 d of drought stress to evaluate root cellular membrane stability (Blum and Ebercon, 1981). Fresh root tissue was collected, rinsed with deionized water to remove external solutes, and placed in a test tube containing 30 mL deionized water. Tubes were agitated in a conical flask shaker for 12 h and the initial conductance (Ci) of immersion liquid measured using a conductivity meter (YSI Model 32, Yellow Springs, OH). Root samples were then autoclaved at 121 °C for 20 min and again shaken for 12 h. The maximal conductance (Cmax) of autoclaved immersion liquid was then measured and electrolyte leakage calculated ($EL(\%) = (Ci/Cmax) \times 100$). Roots were then dried in an oven at 80 °C for 7 d and the dry weight was incorporated into growth analysis parameters for each plant.

2.5. Root protein extraction and separation

Proteins were extracted from roots in four replicated samples from each treatment collected at 20 d of drought stress using the acetone/trichloroacetic acid (TCA) protein extraction method (Xu et al., 2008) with modifications. Roots were washed free of fritted clay, immediately frozen in liquid nitrogen, and stored at −80 °C for further analysis. Approximately 3 g of root tissues were homogenized and incubated in 35 mL ice-cold precipitation solution (10% TCA, 0.07% 2-mercaptoethanol in acetone) for 12 h at −20 °C. Precipitated proteins were pelleted through centrifugation at 11,600 $\times g$ at 4 °C and washed three times with rinse solution (0.07% 2-mercaptoethanol in acetone) to remove pigments and lipids yielding colorless supernatant. The pellet was vacuum-dried and suspended and sonicated in 8 mL resolubilization solution (8 M urea, 2 M thiourea, 1% CHAPS, 1% dithiothreitol, 1% IPG buffer (GE Healthcare), in deionized water) and centrifuged at 11,600 $\times g$ to separate insoluble tissue from soluble proteins. The supernatant was then used for determination of protein concentration according to Bradford (1976) using a commercial dye

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