Leaf Protein Abundance Associated with Improved Drought Tolerance by Elevated Carbon Dioxide in Creeping Bentgrass

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ABSTRACT. Elevated CO₂ may contribute toward plant tolerance to prolonged drought stress. The objective of this study was to investigate changes in protein abundance associated with mitigation of drought stress by elevated CO₂ in leaves of a cool-season grass species used as fine turfgrass. Plants of creeping bentgrass (*Agrostis stolonifera* cv. Penncross) were grown at either ambient CO₂ concentration (400 $\mu\text{L}\cdot\text{L}^{-1}$) or elevated CO₂ concentration (800 $\mu\text{L}\cdot\text{L}^{-1}$) for 35 days under well-irrigated and fertilized conditions and then subjected to drought stress for 21 days by withholding irrigation. Plants exposed to elevated CO₂ concentration maintained higher leaf water content, membrane stability, and visual turf quality (TQ) under drought stress compared with plants grown under ambient CO₂ conditions. The abundance of proteins involved in photosynthetic carbon fixation and assimilation, including chloroplastic glyceraldehyde phosphate dehydrogenase A (GAPDH-A) and ribulose 1,5-bisphosphate carboxylase (RuBisCO) decreased less and the abundance of proteins involved in respiratory metabolism (i.e., cytosolic GAPDH) increased less during drought due to elevated CO₂. The results suggest that elevated CO₂ lessened growth and physiological damages during drought by facilitating ribulose 1,5-bisphosphate regeneration and adenosine triphosphate (ATP) production in photosynthesis and downregulating factors contributing to respiratory metabolism.

Drought stress due to the lack of rainfall and declining availability of fresh water for irrigation is a primary factor limiting growth and productivity of many plant species. Within the context of global climate change, precipitation amounts and frequencies have changed drastically over the past century and many regions are now experiencing drought episodes more frequently (Solomon et al., 2007). Drought stress imposes many physiological limitations throughout the plant system encompassing damages at the biochemical, metabolic, and cellular levels (Aroca, 2012). Along with an increasing frequency of drought events, anthropogenic CO₂ emissions are driving a steady increase in atmospheric CO₂ concentrations of $\approx 2\text{--}3$ $\mu\text{L}\cdot\text{L}^{-1}$ per year and plants may therefore be exposed to prolonged drought stress under elevated CO₂ concentrations in the near future (Solomon et al., 2007). The effects of elevated CO₂ concentration on many aspects of plant development and function under nonstress conditions have been well-documented and generally positive effects of elevated CO₂ concentration on plant growth are reported in various plant species (Ainsworth et al., 2002; Ceulemans and Mousseau, 1994; Huang and Xu, 2015; Kirkham, 2011; Leakey et al., 2009; Peterson et al., 1999). Recent research has also demonstrated that elevated CO₂ may mitigate physiological damages due to abiotic stress, such as drought and heat, in various plant species including perennial grasses such as kentucky bluegrass (*Poa pratensis*) and tall fescue (*Festuca arundinacea*) (Lin and Wang, 2002; Qaderi et al., 2006; Wall et al., 2001; Yu et al., 2012). Despite the abundant knowledge regarding the positive

effects of elevated CO₂ on plant growth under nonstress or stress conditions, the underlying mechanisms by which elevated CO₂ attenuates the damaging effects of prolonged drought stress remain unclear and require further investigation.

Proteomic profiling of stress-responsive proteins by means of two-dimensional polyacrylamide gel electrophoresis separation and mass spectrometry (MS) identification has effectively described changes in proteomic abundance within various tissues of different plant species responding to abiotic stresses (Burgess and Huang, 2014; Ferreira et al., 2006; Huang et al., 2014; Jespersen et al., 2015; Kosová et al., 2011; Merewitz et al., 2011). In response to drought stress alone, the abundance of proteins involved in photosynthesis, membrane synthesis, cell wall loosening, cell turgor maintenance, and antioxidant defense decrease in drought-susceptible grasses and the decrease is less severe in drought-tolerant species or cultivars (Xu and Huang, 2010a, 2012a). Under elevated CO₂ alone, many of the proteins serving integral photosynthetic functions in the light reactions and light-independent reactions have decreased abundance but compensate with significantly higher enzyme activity or activation state (Yu et al., 2014). The majority of proteomic research related to drought stress has identified drought-responsive changes in protein abundance under ambient CO₂ concentration only while changes in protein abundance responding to the combined drought stress and elevated CO₂ are not well documented, although differential abundance of proteins affected by elevated CO₂ under drought stress from those under well-irrigated conditions could serve critical roles for CO₂ mitigation of drought damages. The main focus of the current study was on the analysis of CO₂-responsive proteins under well-irrigated or drought conditions or protein abundance altered by the combined CO₂ and drought stress.

Therefore, the objective of this study was to investigate changes in protein abundance responding to interactive effects

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of drought and CO₂ in leaves of creeping bentgrass, widely used as fine turfgrass, with a goal to suggest potential metabolic factors regulated by elevated CO₂ contributing to improved drought tolerance.

Materials and Methods

PLANT MATERIAL AND GROWTH CONDITIONS. Thirty uniform-size tillers from creeping bentgrass (cv. Penncross) plants were transplanted from the Rutgers University turfgrass research farm (New Brunswick, NJ) into each pot (10 cm diameter and 40 cm depth) filled with fritted clay medium (Profile Products, Deerfield, IL) on 10 May 2014 and plants were maintained in controlled-environment growth chambers (Environmental Growth Chambers, Chagrin Falls, OH) set to 21/18 °C (day/night), 650 μmol·m⁻²·s⁻¹ photosynthetically active radiation, 60% relative humidity, and 14-h photoperiod for 7 d to allow plant acclimation to growth chamber conditions before exposing plants to CO₂ treatments on 18 May 2014.

TREATMENTS AND EXPERIMENTAL DESIGN. Twenty pots (4 treatments × 5 replicates) of plants were established for 35 d (18 May to 21 June 2014) at ambient (400 μL·L⁻¹) or elevated (800 μL·L⁻¹) CO₂ concentration under well-irrigated conditions with excess water draining from pot bases and fertilized twice per week with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). Following establishment under either CO₂ concentration, all plants were irrigated to pot capacity on 22 June 2014 (0-d drought stress) and subjected to drought stress for 21 d (23 June to 13 July 2014) by withholding irrigation until volumetric soil water content (SWC) decreased to 7.0% or irrigated to maintain SWC at the pot capacity (≈29%) as the nonstress control. During 21-d drought stress, plants were continually exposed to either ambient or elevated CO₂ concentration.

The ambient and elevated CO₂ concentrations within growth chambers were maintained through an automatic CO₂ controlling system connected to a source tank containing 100% research-grade CO₂ following the method described in Yu et al. (2012). CO₂ concentrations inside the chambers were continuously monitored and recorded using an infrared gas analyzer (LI-820; LI-COR, Lincoln, NE) connected to a computer data logger. The CO₂ concentration was maintained using an automatic controlling system consisting of a programmable logic controller unit, solenoid valves, and a laptop computer with software capable of monitoring and maintaining CO₂ concentration within 10 μL·L⁻¹ of the ambient or elevated target levels.

The experiment was arranged in a split-plot design with CO₂ treatment (ambient or elevated) as the main plot and irrigation treatment (well irrigated or drought stress) as the sub-plot. Each CO₂ treatment was performed in four different growth chambers and five replicate pots of well-irrigated or drought treatments were randomly placed inside each growth chamber. All plants were relocated between the four growth chambers every 3 d to avoid possible confounding effects of unique growth chamber environmental variations from occurring.

SOIL WATER STATUS AND PHYSIOLOGICAL ANALYSIS. The SWC was monitored daily using a time reflectometer (Trase System 1; Soilmoisture Equipment Corp., Santa Barbara, CA). Three buriable waveguide probes, each measuring 30 cm in length, were inserted into the root zone and SWC was measured in

drought-stressed and well-irrigated treatments (Topp et al., 1980).

Leaf relative water content (RWC) was measured to indicate leaf hydration status following 21 d of drought treatment. About 0.2 g leaf tissue of second and third fully expanded leaves was collected and fresh weight (FW) measured using a mass balance immediately after harvesting. Leaves were then wrapped in tissue paper, submerged in deionized water for 12 h at 4 °C, removed from water, blotted dry, and again weighed to measure turgid weight (TW). Leaves were then dried in an oven at 80 °C for 3 d, weighed to determine dry weight (DW) and RWC (percent) calculated using the formula [(FW – DW)/(TW – DW)] × 100 (Barrs and Weatherley, 1962).

Leaf membrane stability was estimated by measuring cellular electrolyte leakage (EL) following 21 d of drought treatment. About 0.2 g leaf tissue of second and third fully expanded leaves was collected, rinsed with deionized water to remove external solutes, and placed in a test tube containing 30 mL deionized water. Tubes were agitated on a conical flask shaker for 12 h and the initial conductance (C_i) of incubation solution measured using a conductivity meter (YSI, Yellow Springs, OH). Leaf tissue was then killed by autoclaving at 121 °C for 20 min, cooled to room temperature, agitated for 12 h, and the maximal conductance (C_{max}) of incubation solution was measured. Leaf EL (percent) was calculated using the formula (C_i/C_{max}) × 100 (Blum and Ebercon, 1981).

Visual evaluation of TQ was performed to indicate overall turfgrass performance on a scale of 1 to 9 with 1 being brown and dead turf, 6 being the minimum acceptable quality level, and 9 being green and healthy turf. TQ ratings were based on parameters such as canopy uniformity, density, and color (Beard, 1973).

PROTEIN EXTRACTION, SEPARATION, QUANTIFICATION, AND IDENTIFICATION. Protein extraction and separation were performed using the acetone/trichloroacetic acid extraction and two-dimensional gel electrophoresis method of Xu et al. (2008). Following 21 d of drought stress, second and third fully expanded leaves were collected, immediately frozen in liquid nitrogen, and stored at –80 °C for protein analysis. About 0.4 g leaf tissue was ground to powder in liquid nitrogen and further homogenized in 4 mL ice-cold precipitation solution (10% trichloroacetic acid, 0.07% 2-mercaptoethanol in acetone) for 12 h at –20 °C. Precipitated leaf tissue was centrifuged at 11,600 g for 15 min at 4 °C, the supernatant was removed and the remaining pellet was washed three times with rinse solution (0.07% 2-mercaptoethanol in acetone). The remaining pellet was vacuum dried at room temperature and resuspended in 2 mL resolubilization solution {8 M urea, 2 M thiourea, 1% 3 [(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 1% dithiothreitol, 1% 3/10 biolytes}. Aliquots of the resulting protein solution were then used to determine protein concentration according to Bradford (1976) using a commercial dye reagent (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin as the standard. Immobiline DryStrips (pH 3–10, linear gradient, 13 cm; GE Healthcare, Piscataway, NJ) were rehydrated with 250 μg resolubilized protein and loaded onto an IPGphor apparatus (GE Healthcare) for first dimension separation. The voltage settings for first dimension isoelectric focusing were 50 V for 14 h, 500 V for 1 h, 1000 V for 1 h, 5000 V for 1 h, and 8000 V to a total of 80 kVh. Following first dimension focusing, strips were denatured in 10 mL equilibration buffer [50 mM Tris–Base (pH 8.7), 6 M urea, 30% glycerol,

2% sodium dodecyl sulfate, 0.002% bromophenol blue, and 1% dithiothreitol] for 20 min and incubated again in the same buffer with dithiothreitol replaced with 2.5% iodoacetamide. An electrophoresis unit (Hoefer SE 600 Ruby; GE Healthcare) was used to perform second dimension electrophoresis on a 12.5% sodium dodecyl sulfate–polyacrylamide gel {42% monomer solution (30% acrylamide and 0.8% N,N'-Methylenebiscacrylamide), 25% resolving gel buffer [1.5 M Tris-Base and 6 N hydrochloric acid (pH 8.8)], 0.01% sodium dodecyl sulfate, 3.4 ppm tetramethylethylenediamine, and 50 ppm ammonium persulfate}. Voltage settings for second dimension electrophoresis were 5 mA per gel for 30 min followed by 20 mA per gel for 6.5 h. Gels were stained with colloidal Coomassie Brilliant Blue G-250 stain (Neuhoff et al., 1988) and scanned on a Typhoon FLA 9500 (GE Healthcare) to generate digital gel images. Gel images were analyzed using SameSpots software (version 4.5; Nonlinear USA, Durham, NC) and protein volumes were normalized as a percentage of total protein volume to correct for variability during staining. Proteins with probability values less than or equal to 0.05 were chosen for further identification by reversed-phase liquid chromatography (RPLC).

Proteins chosen for identification were manually excised from gels and washed with 30% acetonitrile in 50 mM ammonium bicarbonate solution before dithiothreitol reduction and iodoacetamide alkylation. Trypsin was used for digestion at 37 °C overnight. The resulting peptides were extracted with 30 µL 1% trifluoroacetic acid followed by C₁₈ ziptip desalting to simultaneously remove salts and concentrate the peptides. Peptides were further fractionated by RPLC on a LC system (Ultimate 3000; Dionex, Sunnyvale, CA) coupled to a mass spectrometer (Q-Exactive; Thermo Fisher Scientific, Waltham, MA) with a nano-electrospray ionization source (Thermo Fisher Scientific). Source ionization parameters included a 2.2-kV spray voltage, 275 °C capillary temperature, and 50.0 s-lens. Full-scan MS mode [300–1650 m/z (mass-to-charge ratio)] was operated at a resolution of 70,000, automatic gain control (AGC) target was 1 × 10⁶, and maximum ion transfer time (IT) was 500 ms. MS/MS parameters for selected ions included 17,500 resolution, 5 × 10⁴ AGC, 250 ms IT, 4.0 m/z isolation window, 25.0 normalized collision energy, 5.0% underfill ratio, and a 30 s dynamic exclusion.

Raw files were analyzed using the Proteome Discoverer software platform (version 1.3, Thermo Fisher Scientific) with Mascot (2.4.1) search engine against the Green plant protein sequences (1,474,035 entries) of nonredundant National Center for Biotechnology Information protein database. Mascot parameters included trypsin, two missed cleavages, 10 ppm precursor mass tolerance, 0.1 Da fragment mass tolerance, as well as methionine oxidation and cysteine carbamidomethylation dynamic modifications with decoy search option for Mascot engaged. Proteins with 100% peptide spectral match were considered to be present throughout the majority of analyzed proteins.

STATISTICAL ANALYSIS. The effects of CO₂ level, irrigation regimen, and their interactions on physiological parameters, protein abundance, and relative protein accumulation were determined by analysis of variance according to the general linear model procedure of SAS (version 9.2; SAS Institute, Cary, NC). Differences between treatment means were separated by Fisher's protected least significance difference test ($\alpha = 0.05$).

Results and Discussion

A characteristic response of drought-susceptible plants is a steady decline in cellular water content concurrent with dysfunction and eventual failure of cellular membranes, along with numerous other metabolic and biochemical changes (Kopp and Jiang, 2013). Drought stress caused significant reduction in RWC under both ambient and elevated CO₂ concentrations (Fig. 1A) when SWC decreased to $\approx 7.0\%$ following 21 d of drought stress (Fig. 2). Drought-induced reduction in RWC was more pronounced (by 42%) under ambient CO₂ concentration than that under elevated CO₂ concentration (by 28%) following 21 d of drought stress. Elevated CO₂ treatment led to significantly higher (by 19%) RWC compared with the ambient CO₂ treatment following drought stress whereas no significant changes in RWC were observed with elevated CO₂ under well-irrigated conditions (Fig. 1A). Maintaining adequate water content within cells or delaying cellular dehydration during stress periods in CO₂-enriched plants may be due to the effects of elevated CO₂ on the induction of stomatal closure restricting transpirational water loss and enhanced osmotic adjustment due to the accumulation of solutes, such as soluble sugars, as well as enhanced root growth for water uptake (Leakey et al., 2009; Yu et al., 2015). Maintenance of photosynthetic processes has also been associated with improved RWC during drought stress in other cool-season turfgrass species, such as tall fescue (Yu et al., 2012). Moreover, elevated CO₂ has been shown to enhance root growth in creeping bentgrass (Burgess and Huang, 2014), which could access more water from soil and contribute to the improvement of leaf water status under drought stress.

Cellular membrane stability evaluated by quantifying ion leakage is a commonly used indicator for cell integrity and viability in various plant tissues (Jambunathan, 2010). Leaf EL significantly increased with drought stress under either ambient or elevated CO₂ concentration following 21-d drought treatment (Fig. 1B). Drought-induced increase in EL was greater (by 51%) under ambient CO₂ concentration than under elevated CO₂ concentration (by 43%) following drought stress. Elevated CO₂ treatment led to significantly lower (by 18%) EL compared with the ambient CO₂ treatment following drought stress whereas no significant changes in EL were observed with elevated CO₂ under well-irrigated conditions (Fig. 1B). The results demonstrate that elevated CO₂ treatment effectively mitigated drought-induced cellular membrane deterioration or facilitated maintenance of membrane integrity. Similar results of elevated CO₂ effects on membrane stability of drought-stressed plants have been reported in tall fescue (Yu et al., 2012). Despite elevated CO₂ promoting osmotic adjustment and stomatal constriction with secondary effects on membrane status, the mechanisms by which elevated CO₂ contributes directly to cellular membrane stability remain largely unknown, though it was suggested that elevated CO₂ treatment increases leaf antioxidant content to reduce reactive oxygen species (ROS) content in spring wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) cultivars (Lin and Wang, 2002; Pérez-López et al., 2009). Whether elevated CO₂ conditions stimulate cool-season turfgrass species to use similar antioxidant mechanisms mitigating ROS accumulation thereby delaying significant membrane damages during stress periods is not yet known and deserves further investigation.

Fig. 1. Changes in (A) leaf relative water content, (B) leaf electrolyte leakage, and (C) turf quality of creeping bentgrass plants exposed to ambient ($400 \mu\text{L}\cdot\text{L}^{-1}$) or elevated ($800 \mu\text{L}\cdot\text{L}^{-1}$) CO_2 concentration for 35 d followed by well-irrigated or drought-stress conditions for 21 d. Turf quality is rated on a scale of 1–9 (1 = brown and dead turf, 6 = minimum acceptable quality level, and 9 = green and healthy turf). Vertical lines atop bars represent SE of five replicates for each treatment and different letters atop bars indicate significant differences between treatments exist according to Fisher's least significant difference test ($\alpha = 0.05$).

Fig. 2. Soil volumetric water content of creeping bentgrass exposed to ambient ($400 \mu\text{L}\cdot\text{L}^{-1}$) or elevated ($800 \mu\text{L}\cdot\text{L}^{-1}$) CO_2 concentration for 35 d followed by well-irrigated or drought-stress conditions for 21 d. Vertical lines atop bars represent least significant difference values where significant differences between treatments exist according to Fisher's least significant difference test ($\alpha = 0.05$).

Visual evaluation of turfgrass quality is a subjective criteria commonly used to evaluate overall turfgrass performance based on visual characteristics including canopy density, leaf color, and uniformity (Beard, 1973). Visual TQ displayed significant decrease with drought stress under either ambient or elevated CO_2 concentration following 21-d drought treatment (Fig. 1C). Drought-induced reduction in TQ was more pronounced (by 38%) under ambient CO_2 concentration than that under elevated CO_2 concentration (by 28%) following 21 d of drought stress. Elevated CO_2 treatment led to significantly higher (by 13%) TQ compared with the ambient CO_2 treatment following drought stress whereas no significant changes in TQ were observed with elevated CO_2 under well-irrigated conditions (Fig. 1C). The promotive effects of elevated CO_2 on TQ corresponded to enhanced RWC and decreased EL during drought stress, both of which are strongly correlated to TQ for creeping bentgrass during drought stress in turfgrasses (Jespersen et al., 2013; Sun et al., 2013). The improved growth and physiological characteristics favoring plant tolerance to drought stress as affected by elevated CO_2 concentration could be associated with changes in abundance for specific proteins involved in several major metabolic processes, as discussed below.

As discussed in the introduction, most proteomic research related to drought stress has identified drought-responsive changes in protein abundance under ambient CO_2 concentration only. Our study focused on the analysis of CO_2 -responsive changes in protein abundance under well-irrigated or drought conditions or protein abundance altered by the combined CO_2 and drought stress. Over 300 proteins were detected on each two-dimensional gel in leaves of creeping bentgrass (Fig. 3) and 37 proteins successfully identified by RPLC-MS exhibited differential abundance (upregulated or downregulated) in response to elevated CO_2 concentration under well-irrigated or drought-stress conditions. Those proteins were categorized into

Fig. 3. An example represents two-dimensional electrophoresis gel of creeping bentgrass leaf soluble proteins stained with Coomassie Brilliant Blue. Numbers of highlighted proteins correspond with proteins numbers in Table 1 for proteins exhibiting significant changes in abundance following exposure to ambient ($400 \mu\text{L}\cdot\text{L}^{-1}$) or elevated ($800 \mu\text{L}\cdot\text{L}^{-1}$) CO_2 concentration for 35 d followed by well-irrigated or drought-stress conditions for 21 d.

five functional categories according to the criteria set forth by Bevan et al. (1998): energy production, stress defense, metabolism, protein destination and storage, and protein synthesis (Table 1). Among the 37 identified proteins, 67.6%, 8.1%, 16.2%, 5.4%, and 2.7% served functions in energy production, stress defense, metabolism, protein destination and storage, and protein synthesis functions, respectively (Fig. 4). A total of 18 proteins (1–6, 11, 17, 18, 23, 24, 26, 30–33, 36, and 37) were upregulated and 19 proteins (7–10, 12–16, 19–22, 25, 27–29, 34, and 35) were downregulated by elevated CO_2 under well-irrigated conditions (Figs. 5, 6A and B).

Elevated CO_2 upregulated 20 proteins (1, 2, 4–7, 10, 11, 14, 19, 23–25, 30–33, and 35–37) and downregulated 17 proteins (3, 8, 9, 12, 13, 15–18, 20–22, 26–29, and 34) under drought-stress conditions while 20 proteins (1, 4–7, 10–12, 14, 23–25, and 30–37) were upregulated and 17 proteins (2, 3, 8, 9, 13, 15–22, and 26–29) were downregulated under ambient CO_2 concentration following drought-stress treatment (Figs. 5, 6A and B). Moreover, the fold change in abundance was significantly different between elevated CO_2 well-irrigated and elevated CO_2 drought-stress treatments for 24 proteins (2, 3, 7, 8, 10, 13–15, 17–26, 29, 30, 32, and 35–37), between ambient CO_2 drought stress and elevated CO_2 drought-stress treatments for 22 proteins (1, 2, 4, 8–13, 15, 16, 19, 21, 27–29, and 32–37), and among all three treatments for 11 proteins (2, 8, 10, 13, 15, 19, 21, 29, 32, 35, and 36) (Figs. 5, 6A and B). The biological functions of those proteins with upregulated or downregulated abundance by elevated CO_2 are discussed, with an emphasis on several notable proteins regulated by elevated CO_2 , which may contribute to CO_2 mitigation of drought-stress damages in creeping bentgrass.

The majority of soluble proteins responding to elevated CO_2 with or without drought stress were involved in energy metabolism in creeping bentgrass leaves. Elevated CO_2 increased the abundance of several major proteins involved in the Calvin–Benson cycle including fructose biphosphate aldolase precursor (FBA), chloroplastic GAPDH-A, and chloroplastic sedoheptulose biphosphatase precursor (SBPase) under well-irrigated and drought conditions. The drought-induced decrease in RuBisCO abundance was less severe due to elevated CO_2 compared with ambient CO_2 . The drought-induced increase in cytosolic GAPDH abundance contributing to respiratory glycolytic breakdown of glucose was less severe due to elevated CO_2 compared with ambient CO_2 . Our results suggest that the enhanced drought tolerance observed in creeping bentgrass under drought stress may be in part due to changes in abundance for those proteins regulating key energy functions within leaf tissues and are discussed in detail below.

FBA catalyzes the sixth reaction of the Calvin–Benson cycle converting fructose 1,6-bisphosphate to glyceraldehyde 3-phosphate yielding dihydroxyacetone phosphate and ATP and has been shown to have direct effects on the regeneration rate ribulose-1,5-bisphosphate (RuBP) for carbon fixation (Iwaki et al., 1991; Taiz and Zeiger, 2010). Research describing changes in FBA abundance responding to abiotic stresses is limited, although Abbasi and Komatsu (2004) reported FBA abundance was upregulated in rice (*Oryza sativa*) leaf sheaths during salinity stress, while gene transcript level analysis revealed differential responses of eight FBA genes in *Arabidopsis thaliana* shoots responding to chilling, heat, or drought stress (Lu et al., 2012). Leaf FBA content at the transcript and protein level significantly increased due to elevated CO_2 treatment for rice under nonstress conditions and tall fescue under heat-stress conditions (Fukayama et al., 2009; Yu et al., 2014). In this study, FBA abundance increased by 1.0- and 1.1-fold due to elevated CO_2 under well-irrigated conditions and drought stress, respectively, but increased by 2.3-fold due to drought stress under ambient CO_2 concentration. The regulation of FBA abundance under elevated CO_2 suggested that elevated CO_2 could sustain constant ATP production and RuBP regeneration rates supporting plant growth during drought periods.

Chloroplastic GAPDH is composed of A and B subunits, which catalyze the nicotinamide adenine dinucleotide phosphate-consuming reduction of 1,3-bisphosphoglycerate to glyceraldehyde 3-phosphate during the reduction phase of the Calvin–Benson cycle (Sparla et al., 2005; Taiz and Zeiger, 2010). In a manner similar to FBA limitation, plants with decreased chloroplastic GAPDH abundance may experience reduced CO_2 assimilation due to a reduction in RuBP regeneration, which can then cause subsequent declines in photosynthetic rates and net biomass accumulation (Price et al., 1995). Abiotic stresses such as drought cause significant decreases in chloroplastic GAPDH abundance whereas plants with increased stress tolerance typically exhibit greater abundance of chloroplastic

Table 1. Creeping bentgrass leaf soluble proteins differentially accumulated following exposure to ambient (400 $\mu\text{L}\cdot\text{L}^{-1}$) or elevated (800 $\mu\text{L}\cdot\text{L}^{-1}$) CO_2 concentration for 35 d followed by well-irrigated or drought-stress conditions for 21 d. Spot numbers correspond to highlighted proteins in Fig. 3.

Functional category	Spot no.	Protein name	Abbreviation	Accession no.	MW/PI
Energy	1	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	GAPDH	120668	33.2/6.20
	2	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic	GAPDH-A	115458768	42.7/7.62
	3	Fructose 1,6-bisphosphate aldolase, cytoplasmic isozyme	FBA (cyt)	115468886	37.7/7.57
	4	Fructose 1,6-bisphosphate aldolase precursor	FBA	8272480	41.9/9.01
	5	Triosephosphate isomerase, cytosolic	TIM	2507469	26.79/5.39
	6	Cytoplasmic aldolase	Cyt. ald.	218157	38.7/6.56
	7	Phosphoglycerate mutase	PGAM	32400802	29.6/5.43
	8	Rubisco activase beta form precursor	RCA β	32481063	47.1/7.57
	9	Ribulose 1,5-bisphosphate carboxylase	RuBisCO	1488586	45.3/6.28
	10	Ribulose-5-phosphate 3-epimerase, chloroplast precursor	R5P3E	2833386	30.3/8.23
	11	Sedoheptulose-1,7-bisphosphatase, chloroplast precursor	SBPase	1173347	42/6.04
	12	Ferredoxin-NADP+ reductase, chloroplastic isozyme	FNR	115443657	38.7/7.98
	13	Crystal structure complex between ferredoxin and ferredoxin NADP+ reductase	FDI-FNR	13096165	35.3/7.01
	14	ATP synthase F1 sector beta subunit	ATPF1 β	50401827	53.8/5.17
	15	ATP synthase CF1 alpha subunit	ATPCF1 α	14017569	55.3/6.11
	16	ATP synthase alpha subunit	ATP α	51556908	55.5/6.03
	17	Oxygen-evolving complex protein 1	OEE1	739292	26.5/5.13
	18	Oxygen-evolving enhancer protein 2, chloroplast precursor	OEE2	131394	27.3/8.84
	19	HCF136 photosystem II stability/assembly factor, chloroplast precursor	HCF136	75252730	45.4/9.02
	20	PSI type III chlorophyll a/b-binding protein	PSI a/b	430947	29.1/8.61
	21	Light-harvesting complex I	LHC1	544700	24.2/8.11
	22	Carbonic anhydrase, chloroplast precursor	CAH	729003	35.1/8.93
	23	Malate dehydrogenase	MDH	10798652	35.4/5.91
	24	Dihydrolipoyl dehydrogenase-1, mitochondrial isoform	DLD	115436320	52.6/7.21
	25	Glycerate dehydrogenase HPR, peroxisomal	HPR	115443619	42/6.56
Stress defense	26	Thioredoxin peroxidase	TPX	3328221	28.1/6.34
	27	Catalase-1	CAT	2493543	56.8/6.52
	28	Heat shock protein 70	HSP70	1143427	75.4/5.15
Metabolism	29	Alpha-glucan phosphorylase, H-isozyme	AGP	14916632	93.6/7.27
	30	Glutamate-ammonia ligase, chloroplast precursor	GAL	121340	47.1/5.11
	31	Aspartate carbamoyltransferase	ACT	21535795	42.6/6.06
	32	Phosphoribulokinase	PRK	21839	45/5.84
	33	Aminomethyltransferase, mitochondrial	AMT	115460656	43.9/8.53
	34	Methionine synthase	MS	50897038	84.5/5.68
	35	PFTF precursor, FTSH-like	PFTF	52075838	72.5/5.54
Protein destination and storage	36	Protein disulfide isomerase 2 precursor	PDIM	13925726	56.4/5.03
Protein synthesis	37	Tu translational elongation factor	EF-Tu	17225494	50.4/6.19

MW = molecular weight in kilodaltons; PI = isoelectric point; NADP = nicotinamide adenine dinucleotide phosphate; ATP = adenosine triphosphate; HCF = high chlorophyll fluorescence; PSI = photosystem I.

GAPDH compared with stress-sensitive plants (Merewitz et al., 2011; Xu and Huang, 2010a, 2010b). Reports on elevated CO_2 regulation of chloroplastic GAPDH abundance are limited and vary across different plant species and different stress conditions. Yu et al. (2014) reported that the abundance of

chloroplastic GAPDH decreased due to elevated CO_2 under nonstress conditions but did not change due to elevated CO_2 following heat-stress treatment in tall fescue. Furthermore, chloroplastic GAPDH enzyme activity was either significantly increased or relatively unchanged due to elevated CO_2 under

Fig. 4. Functional classification and percent of proteins with differential responses in abundance in creeping bentgrass leaves following exposure to ambient ($400 \mu\text{L}\cdot\text{L}^{-1}$) or elevated ($800 \mu\text{L}\cdot\text{L}^{-1}$) CO_2 concentration for 35 d followed by well-irrigated or drought-stress conditions for 21 d.

nonstress conditions in other plant species (Haake et al., 1999; Ribeiro et al., 2012; Zhang et al., 2005). In this study, chloroplastic GAPDH abundance increased by 2.2- and 1.7-fold due to elevated CO_2 under well-irrigated conditions and drought stress, respectively, and was significantly greater during drought stress under elevated CO_2 compared with ambient CO_2 concentration (Fig. 6A). The observed changes in GAPDH abundance indicated that elevated CO_2 treatment effectively mitigated the decrease in chloroplastic GAPDH abundance upon prolonged drought stress. The ability of elevated CO_2 to alleviate the decline in chloroplastic GAPDH abundance may aid in RuBP regeneration and a continuation of CO_2 assimilation driving photosynthesis during drought-stress periods.

RuBisCO is the most abundant soluble protein responsible for catalyzing the first step of the Calvin–Benson cycle, reacting CO_2 and water with RuBP yielding 3-phosphoglycerate (Cleland et al., 1998). Abiotic stresses such as drought impose metabolic limitations on photosynthesis by reducing net abundance of large and small RuBisCO subunits concurrent with inhibition of RuBisCO activation state, among many other biochemical changes (Huang et al., 2014; Shi et al., 2013). Grass species displaying improved tolerance to various abiotic stresses by means of endogenous or exogenous modification typically maintain sufficient RuBisCO abundance or mitigate the extent of decline in RuBisCO abundance (Jespersen et al., 2015; Xu and Huang, 2012b, 2010c; Zhao et al., 2011). It is well accepted that a key component of photosynthetic acclimation to long-term elevated CO_2 treatment is a substantial decrease in RuBisCO both at the transcript and protein level associated with disproportionate resource allocation favoring RuBP regeneration (Bowes, 1991; Feng et al., 2014; Ziska and Teramura, 1992). The stress-mitigating effects of elevated CO_2 facilitated by changes to photosynthetic constituents have been well described for a number of plant species under various abiotic stresses (Alonso et al., 2009; Huang and Xu, 2015). Vu et al. (1998) reported that the decline in RuBisCO abundance and activity was significantly less for drought-stressed rice

plants grown at elevated CO_2 compared with those at ambient CO_2 and similar effects were also reported with regard to RuBisCO enzyme activity in sugarcane (*Saccharum officinarum*) under drought stress (Vu and Allen, 2009). In this study, RuBisCO abundance decreased by 1.1- and 1.5-fold due to elevated CO_2 under well-irrigated conditions and drought stress, respectively, and the decrease was significantly less during drought stress under elevated CO_2 compared with ambient CO_2 concentration (Fig. 6A). Decreased RuBisCO abundance by elevated CO_2 may lead to suppression of photorespiration in cool-season plants (Ehleringer et al., 1991), which could help to avoid inefficient oxygenation of RuBP.

Chloroplastic SBPase is the second bisphosphatase enzyme of the Calvin–Benson cycle, the first being fructose 1,6-bisphosphatase, and is responsible for catalyzing sedoheptulose 1,7-bisphosphate dephosphorylation to sedoheptulose-7-phosphate during the regeneration phase (Raines et al., 1999). Research using transgenic antisense tobacco (*Nicotiana tabacum*) lines has suggested a direct link between SBPase content and capacity for RuBP regeneration and subsequent carbon assimilation (Harrison et al., 1998, 2001). Despite the crucial role of SBPase in plant energy metabolism, there exists far less information regarding effects of abiotic stresses on SBPase abundance. In a review by Kosov et al. (2011), it was reported that SBPase abundance was downregulated in rice subjected to ozone stress but upregulated in poplar (*Populus euphratica*) tolerant of heavy metal hyperaccumulation. Transgenic rice overexpressing and accumulating SBPase prevented RuBisCO activase sequestration thereby maintaining sufficient RuBisCO activation for improved tolerance to heat stress (Feng et al., 2007). More specifically, SBPase abundance is significantly downregulated in creeping bentgrass and kentucky bluegrass upon drought stress but the downregulation was significantly less for drought-tolerant cultivars of each species (Xu and Huang 2010a, 2010c). Similar effects were also noted in creeping bentgrass lines expressing differential tolerance to heat stress (Xu and Huang, 2010b). Given that SBPase is another limiting factor of RuBP regeneration, tobacco plants overexpressing SBPase displayed increased photosynthesis and biomass accumulation when grown at elevated CO_2 compared with plants at ambient CO_2 (Rosenthal et al., 2011). However, the direct effects of elevated CO_2 on SBPase protein abundance and transcript level are variable based on plant species and possible interacting stress effects. SBPase transcript level increased in rice plants exposed to elevated CO_2 across varied nitrogen fertility regimens, but decreased due to high CO_2 when soil temperature was increased above the optimal range (Fukayama et al., 2009, 2011). Protein abundance and transcript levels of SBPase remained unaffected in durum wheat (*Triticum durum*) exposed to elevated CO_2 under nonstress conditions and similar results were also reported for perennial ryegrass (*Lolium perenne*) protein abundance (Aranjuelo et al., 2013; Nie et al., 1995; Rogers et al., 1998). In this study, SBPase protein abundance increased by 1.1- and 1.0-fold due to elevated CO_2 under well-irrigated conditions and drought stress, respectively (Fig. 6A). The response in SBPase abundance was similar to that of FBA, likely because condensation of SBPase and fructose 1,6-bisphosphatase are both catalyzed by FBA in the Calvin–Benson cycle (Lu et al., 2012). The results suggest that ATP production and RuBP regeneration rates may be supported by

Fig. 5. Examples of creeping bentgrass leaf soluble proteins with differential responses in abundance following exposure ambient ($400 \mu\text{L}\cdot\text{L}^{-1}$) or elevated ($800 \mu\text{L}\cdot\text{L}^{-1}$) CO_2 concentration for 35 d followed by well-irrigated or drought-stress conditions for 21 d. GAPDH = cytosolic glyceraldehyde-3-phosphate dehydrogenase; LHC1 = light-harvesting complex I; MDH = malate dehydrogenase; OEE2 = oxygen-evolving enhancer protein 2, chloroplast precursor.

increased FBA abundance and further sustain plant growth during drought periods.

Cytosolic GAPDH catalyzes the sixth step of glycolysis by oxidizing aldehyde to carboxylic acid releasing energy to phosphorylate glyceraldehyde 3-phosphate into 1,3-bisphosphoglycerate in the presence of nicotinamide adenine dinucleotide and inorganic phosphate (Ramzan et al., 2013; Taiz and Zeiger, 2010). Abiotic stresses including heat, drought, or anoxic conditions have been shown to significantly increase cytosolic GAPDH abundance, which maintains glycolytic breakdown of carbohydrates for energy production and suppresses the production of inhibitory ROS (Chang et al., 2000; Ferreira et al., 2006; Xu and Huang, 2012b; Yang et al., 1993). However, increasing or maintaining the abundance of glycolytic chemical constituents driving respiration can promote whole-plant stress tolerance only when photosynthetic carbon input equals or exceeds respiratory carbon consumption (Song et al., 2014). A review of plant proteomic changes under abiotic stresses showed that excessive abundance of cytosolic GAPDH is typically associated with susceptibility to abiotic stresses such as drought, waterlogging, and hypoxia/anoxia

(Kosová et al., 2011). In this study, cytosolic GAPDH protein abundance increased by 3.2- and 3.4-fold due to elevated CO_2 under well-irrigated conditions and drought stress, respectively, and the increase was significantly less under drought due to elevated CO_2 compared with ambient CO_2 concentration (Figs. 5 and 6A). Elevated CO_2 effectively maintained an increased abundance of cytosolic GAPDH content under drought-stress conditions, which may sustain energy production while avoiding a potential excess of cytosolic GAPDH abundance observed during drought stress under ambient CO_2 concentrations.

In summary, this study suggests beneficial effects of elevated CO_2 for lessening the drought damages and improving physiological functions in creeping bentgrass potentially facilitated by changes in protein abundance supporting energy metabolism of leaves in creeping bentgrass. Elevated CO_2 improved creeping bentgrass growth by maintaining leaf hydration and membrane integrity, which may be in part a result of changes in abundance for proteins of the Calvin-Benson cycle including FBA precursor, chloroplastic GAPDH-A, RuBisCO, and chloroplastic SBPase precursor.

Fig. 6. (A and B) Fold changes in soluble protein abundance in creeping bentgrass leaves following exposure to ambient ($400 \mu\text{L}\cdot\text{L}^{-1}$) or elevated ($800 \mu\text{L}\cdot\text{L}^{-1}$) CO_2 concentration for 35 d followed by well-irrigated or drought-stress conditions for 21 d relative to ambient CO_2 well-irrigated control treatment. Positive data indicate increases in protein abundance or upregulation and negative data indicate decreases in protein abundance or downregulation. Only those proteins who had significantly altered accumulations compared with ambient CO_2 well-irrigated control treatment are represented in the figure ($\alpha = 0.05$). Vertical lines atop bars represent SE of five replicates for each treatment and asterisks atop bars indicate significant differences between treatments exist according to Fisher's least significant difference test ($\alpha = 0.05$). Protein abbreviations correspond with respective protein information provided in Table 1.

Elevated CO₂ also decreased cytosolic GAPDH abundance during drought, which may have downstream effects on certain aspects of plant respiration. Further research is needed to confirm the biological functions and associated molecular factors of CO₂-responsive proteins identified in this study contributing to improved drought tolerance in cool-season grass species.

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