Metabolic Responses to Heat Stress under Elevated Atmospheric CO₂ Concentration in a Cool-season Grass Species

Jingjin Yu

Horticulture College of Nanjing Agricultural University, Nanjing 210095, P.R. China; and the Department of Ecology, Evolution and Natural Resources, Rutgers University, New Brunswick, NJ 08901

Hongmei Du

School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 201101, P.R. China

Ming Xu¹

Department of Ecology, Evolution and Natural Resources, Rutgers University, New Brunswick, NJ 08901; and Key Laboratory of Ecosystem Network Observation and Modeling, Institute of Geographic Sciences and Natural Resources, Chinese Academy of Sciences, Beijing 100101, P.R. China

Bingru Huang¹

Department of Plant Biology and Pathology, Rutgers University, 59 Dudley Road, New Brunswick, NJ 08901

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ABSTRACT. Heat is a major factor limiting growth of C₃ grass species. Elevated CO₂ may mitigate the adverse effects of heat stress or enhance heat tolerance. The objective of this study was to determine metabolic changes associated with improvement of heat tolerance by elevated atmospheric CO₂ concentration in tall fescue (*Festuca arundinacea*). Plants (cv. Rembrandt) were exposed to ambient day/night temperature (25/20 °C) or heat stress (35/30 °C) and ambient CO₂ concentration (400 ± 10 μmol·mol⁻¹) or double ambient CO₂ concentration (800 ± 10 μmol·mol⁻¹) in growth chambers. Turf quality (TQ), shoot growth rate, and leaf electrolyte leakage results demonstrated that heat stress at ambient CO₂ concentration inhibits turf growth and reduces cell membrane stability, whereas heat-stressed plants under elevated CO₂ concentration exhibit improved TQ, shoot growth rate, and membrane stability. Plants exposed to heat stress under elevated CO₂ exhibited a significantly greater amount of several organic acids (shikimic acid, malonic acid, threonic acid, glyceric acid, galactaric acid, and citric acid), amino acids (serine, valine, and 5-oxoproline), and carbohydrates (sucrose and maltose) compared with heat-stressed plants at ambient CO₂. The increased production or maintenance of metabolites with important biological functions such as those involved in photosynthesis, respiration, and protein metabolism could play a role in elevated CO₂ mitigation of heat stress damage. Therefore, elevated CO₂ conditions may contribute to improved heat stress tolerance as exhibited by better TQ and shoot growth of heat-stressed plants. Practices to harness the power of CO₂ may be incorporated into turfgrass management for plant adaptation to increasing temperatures, particularly during summer months.

Heat stress is a major abiotic factor limiting growth of temperate plant species in many areas during summer months and may become a threat as global warming occurs [Fry and Huang, 2004; Intergovernmental Panel on Climate Change (IPCC), 2007]. Heat stress induces changes in various metabolites such as organic acids, amino acids, and carbohydrates, which have important functions involved in photosynthesis and respiration (Merewitz et al., 2012). These compounds are involved in vital metabolic functions within the plant such as regulating plant water relations, signaling, protein synthesis as well as stress defense (Sairam et al., 2000; Zobayed et al., 2005). CO₂ concentrations in the atmosphere are more than

100 μmol·mol⁻¹ higher since the beginning of the industrialization era and the concentration is predicted to rise at a rate of ≈2 μmol·mol⁻¹ per year (IPCC, 2007). Increasing evidence has shown that elevated CO₂ may promote plant growth and mitigate heat stress damage in various plant species, particularly C₃ species (Hamerlynck et al., 2000; Kirkham, 2011; Qaderi et al., 2006) including perennial turfgrass species such as tall fescue (Yu et al., 2012). Various studies report elevated CO₂ promotes shoot growth, root growth, and photosynthesis but inhibits dark respiration and photorespiration rates (González-Meler et al., 1996; Leakey et al., 2006; Reddy et al., 2010). However, the metabolites associated with the improvement in plant growth and changes in photosynthesis and respiration metabolic processes resulting from elevated CO₂, particularly under heat stress, are not well documented.

Metabolic profiling is an effective and quantitative method to elucidate mechanisms of abiotic stress tolerance, including heat stress (Kaplan et al., 2004; Mayer et al., 1990). Mayer et al. (1990) reported an increase in the abundance of γ-aminobutyric

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¹Corresponding author. E-mail: huang@aesop.rutgers.edu, mingxu@crssa.rutgers.edu.

acid (GABA), β -alanine, alanine, and proline in cowpea (*Vigna unguiculata*) as a result of heat shock under ambient CO₂ conditions. Du et al. (2011) reported that heat stress at ambient CO₂ induced significant accumulation of multiple metabolites, including threonic acid, galacturonic acid, gluconic acid, succinic acid, proline, aspartic acid, serine, valine, methyl-maleic acid, fructose, galactose, xylose, glucose, mannose, and sucrose in kentucky bluegrass (*Poa pratensis*). Most of the previous studies examined metabolic changes in response to heat stress without consideration of the interaction with CO₂. Very few studies investigated changes in metabolite accumulation in response to heat stress under elevated CO₂ conditions despite some studies that reported the positive effects of elevated CO₂ on plant growth under detrimental environments such as water dehydration and fertilizer deficiency (Kirkham, 2011; Lavola and Julkunen-Tiitto, 1994).

Tall fescue is a widely used cool-season, C₃ turfgrass species. This species exhibits good drought avoidance traits such as deep rooting characteristics but has limited high temperature tolerance (Fry and Huang, 2004). Doubling ambient CO₂ concentration improved tall fescue tolerance to the combined stress of heat and drought by enhancing plant water status, cellular membrane stability, and photosynthesis capacity and by suppressing carbon consumption through lowering respiration rate (Yu et al., 2012). Understanding metabolic changes associated with mitigation of heat stress injury by increasing CO₂ will provide further insight into the interactive effects of heat stress and elevated CO₂ in plant species. In addition, it has great potential for future use in the development of novel management practices. For instance, products aimed to enhance the CO₂ in the gas layers surrounding plants or CO₂ fertilization could be used as management strategies of turfgrasses or other plant species. Therefore, the objective of this study was to examine differential changes in metabolite accumulation of tall fescue in response to heat stress under elevated CO₂ with an aim to further understand metabolic mechanisms for elevated CO₂ enhancement of heat tolerance in C₃ perennial grass species.

Materials and Methods

PLANT MATERIALS AND GROWTH CONDITIONS. Sod pieces of tall fescue (cv. Rembrandt) plants were collected from the research farm at Rutgers University in Adelphia, NJ, and transplanted into plastic tubes (10 cm diameter and 60 cm long) filled with a mixture of fine sand and soil (fine-loamy-mixed mesic typic Hapludult) (1:1, v/v). Plants were maintained in a greenhouse with an average temperature regime of 21/16 °C (day/night) and 810 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetically active radiation (PAR) in natural sun light and 65% relative humidity for 70 d (May to June 2011) to establish canopy and roots. During this establishment period, plants were watered every other day and fertilized once weekly with half-strength Hoagland's solution (Hoagland and Arnon, 1950). Plants were cut once a week to maintain a copy height of 5 to 6 cm. After establishment, plants were moved to growth chambers with the temperature set at 25/18 °C (day/night), 70% relative humidity, PAR of 650 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and a 12-h photoperiod.

EXPERIMENTAL DESIGN AND TREATMENTS. The experiment consisted of two factors (two CO₂ concentrations and two temperatures), which were arranged in a complete, randomized block design with four replicates for each treatment. The CO₂ treatments included ambient CO₂ ($400 \pm 10 \mu\text{mol}\cdot\text{mol}^{-1}$) and

elevated CO₂ ($800 \pm 10 \mu\text{mol}\cdot\text{mol}^{-1}$). Temperature was controlled at two levels: 25/20 °C (day/night, optimal temperature control) and 35/30 °C (day/night, heat stress). Plants were well watered to maintain soil water content at the field capacity (through irrigating plants until drainage ceased). Plants were grown at two CO₂ levels in the growth chambers for 70 d before imposition of temperature treatments.

The treatment set-up and assignment in growth chambers followed the same designed as described in Yu et al. (2012). Four chambers were maintained at the ambient CO₂ level with two of them set to elevated temperature (35/30 °C) and the other two set at the optimal temperatures (25/20 °C). Following the ambient CO₂ treatment, the same four growth chambers were set to the elevated CO₂ level with two at elevated temperature and two at the optimal temperature. Plants were relocated among the different chambers once per week to minimize confounding effects of environmental variation between different chambers. The concentration of CO₂ inside each growth chamber was maintained with an automated, open-chamber CO₂ control system connected to a gas tank containing 100% CO₂ (Airgas, Radnor, PA) (Yu et al., 2012). The CO₂ levels were continuously monitored through an infrared gas analyzer (LI-820; LI-COR, Lincoln, NE) and controlled using an automatic system consisting of a programmable logic controller unit, solenoid valves, and a laptop computer with monitoring software accurate to within 10 $\mu\text{mol}\cdot\text{mol}^{-1}$ of the target levels (400 and 800 $\mu\text{mol}\cdot\text{mol}^{-1}$).

GROWTH AND CELL MEMBRANE STABILITY ANALYSIS. Plant growth was evaluated by rating of TQ and shoot vertical growth rate. TQ is a widely used parameter evaluating overall plant performance (Turgeon, 1996). TQ was visually rated on a scale from 1 (completely dead plants) to 9 (green and dense canopy). Shoot vertical growth rate (expressed as millimeters per day) was calculated as the difference in average canopy height at 3-d intervals and in which canopy height was measured with a floating disk ruler method as the vertical distance from a paper disk placed on the turf canopy and the base of the shoot (Ervin and Zhang, 2007; Sharrow, 1984).

Cellular level of heat damage or tolerance was evaluated as cell membrane stability of leaves, which was determined by measuring electrolyte leakage (EL) (Blum and Ebercon, 1981). For EL analysis, 0.2 g of fresh leaves was placed in test tubes containing 20 mL deionized water and then shaken for 24 h under room temperature to measure the initial conductivity of the solution (C_{initial}) with a conductivity meter (YSI Instrument, Yellow Springs, OH). Leaves were then killed by autoclaving at 140 °C for ≈ 20 min and to measure the conductivity of killed tissues (C_{max}) after which samples were shaken for another 24 h. The percent EL was calculated as the ratio of C_{initial} to $C_{\text{max}} \times 100$ (Blum and Ebercon, 1981).

EXTRACTION AND DERIVATIZATION OF METABOLITES AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY ANALYSIS. The procedure was conducted following the method used by Du et al. (2011). Leaf tissue samples of 28-d treatment were harvested and immediately frozen in liquid nitrogen and stored at –80 °C for metabolic profiling. The extraction protocol was modified from Rizhsky et al. (2004) and Roessner et al. (2000). For each sample, frozen leaves were ground to a fine powder with liquid nitrogen, and then 25 mg leaf tissue powders were transferred into 10-mL microcentrifuge tubes, and they were extracted in 1.4 mL of 80% (v/v) aqueous methanol for 2 h at 23 °C. Ribitol solution of 10 μL (2 mg·mL^{–1} water) was added as an internal

standard before incubation. Then, extraction was done in a water bath at 70 °C for 15 min. Tubes were centrifuged for 30 min at 9660 g_n and the supernatant was decanted to new culture tubes, and 1.4 mL of water and 0.75 mL of chloroform were added. The mixture was vortexed thoroughly and centrifuged for 5 min at 5025 g_n and then 2 mL of the polar phase (methanol/water) was decanted into 1.5-mL high-performance liquid chromatography vials and dried in a benchtop centrifugal concentrator (Centrивap; Labconco Corp., Kansas City, MO). The dried polar phase was methoximated with 80 μ L of 20 mg·mL⁻¹ methoxyamine hydrochloride at 30 °C for 90 min and was trimethylsilylated with 80 μ L N-methy-N-(trimethylsilyl) trifluoroacetamide (with 1% trimethylchlorosilane) for 60 min at 70 °C.

The gas chromatography–mass spectrometry (GC-MS) analysis was modified from Qiu et al. (2007). The derivatized extracts were analyzed with a gas chromatograph coupled with a mass spectrometer (TurboMass-Autosystem XL; PerkinElmer, Waltham, MA). A 1- μ L extract aliquot of the extracts was injected into a capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m, DB-5MS; Agilent J&W Scientific, Folsom, CA). The inlet temperature was set at 260 °C. After a 6.5-min solvent delay, initial GC oven temperature was set at 60 °C; 1 min after injection, the GC oven temperature was raised to 280 °C with 5 °C·min⁻¹ and finally held at 280 °C for 15 min. The injection temperature was set to 280 °C and the ion source temperature was adjusted to 200 °C. Helium was used as the carrier gas with a constant flow rate set at 1 mL·min⁻¹. The measurements were made with electron impact ionization (70 eV) in the full scan mode [with a mass to charge ratio (m/z) of 30 to 550]. The metabolites detected were identified by Turbomass 4.1.1 software (PerkinElmer) coupled with commercially available compound libraries (NIST 2005; PerkinElmer) and Wiley 7.0 (John Wiley & Sons, Hoboken, NJ). For GC-MS results, compounds were identified based on retention time and comparison with reference spectra in mass spectral libraries. Peaks areas of compounds were integrated with the Genesis Algorithm program (New Light Industries, Spokane, WA).

STATISTICAL ANALYSIS. Data were analyzed using SAS (Version 9.0; SAS Institute, Cary, NC). The analysis of variance with a fixed model was used to determine treatments effects. When a particular F test was significant, the means were tested with a least significance difference test at a confidence level of 0.05.

Results and Discussion

PHYSIOLOGICAL RESPONSES TO HEAT STRESS AND ELEVATED CO₂ CONCENTRATION. TQ (Fig. 1A) and shoot vertical growth rate (Fig. 1B) declined under heat stress and under ambient CO₂ conditions, but plants exposed to the high CO₂ concentration had significantly higher TQ and shoot growth rate under heat stress than those plants at ambient CO₂ concentration. Leaf EL increased with heat stress at ambient CO₂ conditions, but the increase was to a significantly less extent under elevated CO₂ conditions (Fig. 1C). These results demonstrate that elevated CO₂ suppresses heat inhibition of turf growth and damage to cellular membranes. These results are consistent with the positive effects of elevated CO₂ on tall fescue tolerance to the combined stress of heat and drought observed in our previous study (Yu et al., 2012). It was noted that elevated CO₂ concentration decreased TQ and increased EL significantly after 14 d of

Fig. 1. Changes in turf quality (TQ) (A), shoot growth rate (mm·d⁻¹) (B), and electrolyte leakage of leaves (EL) (C) of tall fescue in response to heat stress and elevated CO₂ concentrations. TQ was visually rated on a scale from 1 (completely dead plants) to 9 (green and dense canopy). The treatment symbols are 25 to 800 for optimal temperature, and elevated CO₂, 25 to 400 for optimal temperature, and ambient CO₂, 35 to 800 for heat stress, and elevated CO₂, 35 to 400 for heat stress, and ambient CO₂. Vertical bars indicate least significant difference values for the comparison between treatments at a given day of treatment based on $P \leq 0.05$.

treatment compared with plants grown at ambient CO₂ conditions, indicating that higher CO₂ under unstressed conditions for a long time was an additional stress to plants (Yu et al., 2012).

METABOLIC RESPONSES TO HEAT STRESS AND ELEVATED CO₂. A total of 41 metabolites responding to elevated CO₂ and heat stress were identified by GC-MS (Table 1). Among the 41 metabolites, organic acids, amino acids, and carbohydrates accounted for 37%, 27%, and 17% of the total amount of metabolites, respectively (Table 1).

ORGANIC ACID ACCUMULATION. A total of 15 organic acids is identified and the data for those acids responsive to CO₂ or heat stress are presented in Figure 2. Significant interactive effects between CO₂ concentration and temperature were detected and different organic acids exhibited differential responses to elevated CO₂ or heat stress. Biological functions of metabolites responsive to heat stress under either ambient CO₂ or elevated CO₂ conditions are discussed in the context of CO₂ mitigation of heat stress damages in tall fescue.

Table 1. List of 41 identified metabolites in leaves of tall fescue exposed to elevated CO₂ and heat stress for 28 d.

No.	Compound	Retention time (min)	Derivative ^z	m/z for quantification ^y
1	Cyclohexanol	6.982	O-TMS	172
2	Pyruvic acid	8.053	MEOX1, O-TMS	189
3	Lactic acid	8.229	O,O-TMS	234
4	Glycolic acid	8.67	O,O-TMS	220
5	Alanine	9.316	N, O-TMS	233
6	Oxalic acid	10.347	O, O-TMS	234
7	Phosphoric acid monomethyl ester	11.357	O,O-TMS	256
8	Malonic acid	12.075	O,O-TMS	248
9	Valine	12.286	N, O-TMS	261
10	Urea	13.265	N, N-TMS	204
11	Ethanolamine	13.624	N, N, O-TMS	277
12	Glycerol	13.905	O, O,O-TMS	308
13	Threonine	14.463	O,O-TMS	263
14	Glycine	14.695	N, N, O-TMS	291
15	Fumaric acid	14.764	O,O-TMS	260
16	Succinic acid	15.074	O,O-TMS	262
17	Glyceric acid	15.407	O, O,O-TMS	322
18	Serine	16.195	N, O, O-TMS	321
19	3,4-dihydroxy-2(3H)-furanone	16.562	O,O-TMS	262
20	Aspartic acid	18.976	N, O, O-TMS	349
21	Malic acid	19.524	O, O,O-TMS	350
22	5-Oxoproline	20.262	N, O-TMS	273
23	γ -aminobutyric acid	20.411	N, O-TMS	319
24	Threonic acid	21.179	O, O,O, O-TMS	424
25	6-hydroxy-2-aminohexanoic acid	22.611	N, O, O-TMS	363
26	Putrescine	25.015	N-4TMS	376
27	Shikimic acid	26.808	O-4TMS	462
28	Citric acid	26.922	O-4TMS	480
29	Fructose	27.911	MEOX1, O-5TMS	569
30	Galactose	28.262	MEOX1, O-5TMS	569
31	Glucose	28.402	MEOX1, O-5TMS	569
32	Mannitol	28.972	O-6TMS	614
33	<i>p</i> -Hydroxycinnamic acid	29.402	O, O-TMS	308
34	Galactaric acid	30.534	O-6TMS	642
35	Gluconic acid	30.626	O-6TMS	628
36	Palmitic acid	31.449	O-TMS	328
37	Myo-inositol	32.023	O-6TMS	612
38	Maltose	39.201	O-8TMS	918
39	Sucrose	41.253	O-8TMS	918
40	Melibiose	45.891	O-8TMS	918
41	Floridoside	48.498	O-6TMS	686

^zTMS = trimethylsilyl; MEOX = methoxime derivative(s).^ym/z = mass to charge ratio.

Under ambient CO₂ concentration, heat stress caused a significant decline in the abundance of a majority of the organic acids identified in this study, including oxalic acid (68%), shikimic acid (71%), malonic acid (68%), threonic acid (55%), glyceric acid (71%), and galactaric acid (85%), whereas the abundance of citric acid increased; other organic acids such as pyruvic acid and malic acid did not change under heat stress (data not shown) (Fig. 2). Under elevated CO₂ concentration, heat-stressed plants exhibited a significantly higher abundance of shikimic acid (2.9-fold), malonic acid (2.8-fold), threonic acid (90%), glyceric acid (1.5-fold), galactaric acid (1.3-fold), and citric acid (1.7-fold) than plants exposed to the control temperature (Fig. 2).

Citric acid is a key metabolite in the respiration pathway for energy production (Benkeblia et al., 2007). Increases in citric

acid content during heat stress under either ambient or elevated CO₂ indicate that heat stress could stimulate respiration rates leading to more citric acid production or maintenance. Heat-induced accumulation of citric acid was also found in other turfgrass species [hybrid bermudagrass (*Cynodon transvaalensis* × *C. dactylon*) and kentucky bluegrass (Du et al., 2011)]. Elevated CO₂ may suppress heat-enhanced respiration, because plants exposed to elevated CO₂ had a lower abundance of citric acid than plants with ambient CO₂ under heat stress. Previous studies suggest that increasing atmospheric CO₂ concentration inhibited dark respiration in some plant species (González-Meler et al., 1996; Hamilton et al., 2001). Ziska and Bunce (1993) separated total respiration required for growth and maintenance and found a significant effect of elevated CO₂

Fig. 2. Changes in organic acid accumulation of tall fescue leaves in response to 28 d of heat stress under ambient or elevated CO₂ concentrations. The treatment symbols are 25 and 35 °C for optimal temperature and heat stress and 400 and 800 for ambient and elevated CO₂ concentrations, respectively. Bars with the same lowercase letter are not significantly different based on least significant difference test at $P \leq 0.05$.

on both components, causing a significant reduction in growth respiration at 20, 25, and 30 °C in alfalfa (*Medicago sativa*) and at 15 and 25 °C in *Dactylus glomerata*.

The majority of the organic acids (oxalic acid, shikimic acid, malonic acid, threonic acid, glyceric acid, and galactaric acid) responsive to heat or elevated CO₂ found in this study are involved in several metabolic pathways, in particular stress defense pathways. Oxalic acid is known to be involved in antioxidant stress defense (Ding et al., 2007; Jiang et al., 2008; Zhang et al., 2001). Exogenous application of oxalic acid improved heat tolerance of pepper (*Capsicum annuum*) (Zhang et al., 2001) and alfalfa by enhancing chlorophyll accumulation and increasing antioxidant enzyme activities that was inhibited by heat stress (Jiang et al., 2008). Its accumulation has also been associated with drought tolerance in creeping bentgrass [*Agrostis stolonifera* (Merewitz et al., 2012)] and cold tolerance in arabidopsis [*Arabidopsis thaliana* (Korn et al., 2010)]. Shikimic acid is involved in the production of polyphenol flavonoid compounds such as anthocyanins, which have antioxidant properties to suppress heat-induced oxidative damage (Shao et al., 2007). Xu and Huang (2012) reported a decrease in shikimic acid resulting from drought stress in kentucky bluegrass. Little is known about the direct effects of elevated CO₂ on shikimic acid, but studies have shown an increase in secondary metabolites derived from the shikimic acid pathway such as

tannins in response to enriched CO₂ (Lindroth et al., 2001; Peñuelas and Estiarte, 1998). Malonic acid is a dicarboxylic acid and malonate is its ionized form. Malonate acts as a major competitive inhibitor of succinate dehydrogenase involved in the tricarboxylic acid cycle of respiration (Li and Copeland, 2000). Malonate also has been associated with osmotic adjustment and stress defensive system (Lecoeur et al., 1992; Li and Copeland, 2000). Threonic acid is a metabolic product of ascorbic acid and also correlated with glyceric acid synthesis (Helsper and Loewus, 1982). Threonic acid and glyceric acid have been reported to be sensitive to heat stress in many species, including arabidopsis (Kaplan et al., 2004), hybrid bermudagrass, and kentucky bluegrass (Du et al., 2011). Galactaric acid is derived from galacturonic acid by galacturonic acid oxidase, which has been found to stimulate the oxidation of indole acetic acid by peroxidase (Pressey, 1991) and acts as a substrate for galacturonic acid reductase leading to the synthesis of ascorbic acid. Sanchez et al. (2008) reported that galactaric acid was decreased by salinity in arabidopsis. The decline in the abundance of these organic acids under heat stress suggested that heat stress mainly weakened the stress defense mechanisms, whereas

the increases or maintenance of the abundance of those organic acids in plants exposed to elevated CO₂ under heat stress could contribute to the improvement in heat tolerance by enhancing or maintaining more active oxidative defense mechanisms. However, direct mechanisms of CO₂ mitigation of heat damage involving these organic acids are yet to be determined.

AMINO ACID ACCUMULATION. A total of 11 amino acids was identified and the data for those amino acids responsive to CO₂ or heat stress are presented in Figure 3. Amino acids exhibited differential responses to elevated CO₂ or heat stress and significant interactive effects between CO₂ concentration and temperature were detected.

Under ambient CO₂ concentration, the abundance of alanine and serine was 48% and 58% lower, respectively, at heat stress than at the control temperature condition, but GABA abundance increased 2.3-fold under heat stress; valine and 5-oxoproline did not change in response to heat stress (Fig. 3). Under elevated CO₂ concentration, the abundance of serine (3.8-fold), valine (96%), and 5-oxoproline (1.5 fold) of heat-stressed plants were significantly higher than that of the control plants, whereas GABA abundance was lower (70%) in heat-stressed plants than in the control plants; alanine maintained the abundance at the equivalent level under heat stress and the control temperature (Fig. 3).

Alanine and serine are constituents of many proteins and involved in various metabolic processes (Bourguignon et al.,

1999). The decline in alanine and serine abundance under heat stress at ambient CO₂ and the accumulation of serine and maintenance of alanine under elevated CO₂ for plants exposed to heat stress could be reflective of CO₂ mitigation of the heat inhibitory effects on protein synthesis and metabolisms involving these two important amino acids. Valine is also used for synthesizing proteins and secondary metabolites (Singh, 1999).

Tschaplinski et al. (1995) reported significant increases in valine accumulation under elevated CO₂ concentration in drought-stressed plants. Valine accumulation was reported to be associated with improved drought stress tolerance (Merewitz et al., 2012) and with heat shock (Kaplan et al., 2004). The increased accumulation of valine in heat-stressed plants under elevated CO₂ in tall fescue in this study could be associated with the CO₂-induced improvement in heat tolerance, although the specific mechanisms are not clear.

Fig. 3. Changes in amino acid accumulation of tall fescue leaves in response to 28 d of heat stress under ambient or elevated CO₂ concentrations. The treatment symbols are 25 and 35 °C for optimal temperature and heat stress and 400 and 800 for ambient and elevated CO₂ concentrations, respectively. GABA = γ-aminobutyric acid. Bars with the same lowercase letter are not significantly different based on least significant difference test at $P \leq 0.05$.

Fig. 4. Changes in carbohydrate accumulation of tall fescue leaves in response to 28 d of heat stress under ambient or elevated CO₂ concentrations. The treatment symbols are 25 and 35 °C for optimal temperature and heat stress and 400 and 800 for ambient and elevated CO₂ concentrations, respectively. Bars with the same lowercase letter are not significantly different based on least significant difference test at $P \leq 0.05$.

Pyroglutamic acid or 5-oxoproline is a precursor to the synthesis of glutamate (Ohkama-Ohtsu et al., 2008). Conversion of 5-oxoproline to glutamate is required in the regeneration of glutathione, which is known as an effective antioxidant. Increased 5-oxoproline production may be involved in a strengthened antioxidant system (Marrs, 1996). The accumulation of 5-oxoproline that resulted from elevated CO₂ under heat stress indicated the positive CO₂ effects on promoting antioxidant metabolism in tall fescue under heat stress.

GABA is a non-protein amino acid, serving as a signaling molecule and regulating numerous stress response mechanisms such as the carbon/nitrogen balance, osmotic potential, free radical scavenging, and pH regulation (Bouche and Fromm, 2004; Kinnersley and Turano, 2000). In the present study, the level of GABA significantly increased under heat stress for tall fescue exposed to ambient CO₂, but under elevated CO₂, heat-stressed plants had lower GABA abundance than the control plants. Studies conducted under ambient CO₂ conditions reported rapid accumulation of GABA after heat shock in arabidopsis (Rizhsky et al., 2004) or heat stress in cowpea (Mayer et al., 1990) as well as drought stress (Bor et al., 2009; Raggi, 1994). Tschaplinski et al. (1995) reported an increase in GABA caused by elevated CO₂ in sugar maple (*Acer saccharum*) under ambient temperature. Studies comparing GABA content in plants differing in drought tolerance reported a negative relationship of GABA with drought tolerance (Kinnersley and Turano, 2000). However, little is known about the interactive effects of CO₂ and temperature on GABA production.

CARBOHYDRATE ACCUMULATION.

A total of seven carbohydrates is identified and the data for five carbohydrates responsive to CO₂ or heat

stress are presented in Figure 4. Significant interactive effects between CO₂ concentration and temperature on different carbohydrates were detected.

Under ambient CO₂ concentration, heat-stressed plants had significantly greater abundance of sucrose than the control plants, but under elevated CO₂, sucrose abundance did not change with temperature (Fig. 4). Heat caused reduction in the abundance of maltose under ambient CO₂ but did not have effects under elevated CO₂. The abundance of three monosaccharides (fructose, glucose, and galactose) was unaffected by heat stress under ambient CO₂, but under elevated CO₂, heat-stressed plants had significantly lower contents of each sugar than the control plants. The abundance of fructose, glucose, and galactose increased with elevated CO₂ under the control temperature but not under heat stress (Fig. 4).

Monosaccharides such as glucose, fructose, and galactose have important functions such as serving as energy sources and osmoregulators, whereas disaccharides such as sucrose and maltose are the main forms of carbohydrates for transport and storage in plants (Kaplan et al., 2004; Merewitz et al., 2012; Urbonaviciute et al., 2006). Previous studies with CO₂ effects on carbohydrates examined plants exposed to elevated CO₂ under normal temperature and found unchanged sucrose content but increased content of fructose and glucose by doubling ambient CO₂ concentration such as in radish [*Raphanus sativus* (Urbonaviciute et al., 2006)], scots pine [*Pinus sylvestris* (Jach and Ceulemans, 1999)], and silver birch [*Betula pendula* (Lavola and Julkunen-Tiitto, 1994)]. Those results were similar to the results with sucrose, fructose, and glucose in tall fescue in this study. The increased accumulation of fructose and glucose as primary assimilates from photosynthesis is consistent with the enhanced photosynthetic rate by elevated CO₂ in tall fescue, as reported in a previous study (Yu et al., 2012). Little is known of the interactive effects of CO₂ and heat stress on carbohydrate accumulation. In this study, tall fescue plants exposed to heat stress had significantly lower contents of fructose, glucose, and galactose under elevated CO₂ than plants exposed to normal temperature, whereas the abundance of sucrose and maltose was not responsive to heat stress under elevated CO₂. The maintenance of disaccharides (sucrose and maltose) under heat stress in plants with elevated CO₂ may help to maintain carbohydrate storage and supply for continued plant growth. However, how the decline in the monosaccharides (fructose, glucose, and galactose) may be involved in the interactive effects of heat and elevated CO₂ is not clear, and they may be reflective of more active metabolic activities that are needed for continued consumption of soluble sugars under heat stress in plants exposed to elevated CO₂.

Conclusions

Exposure of plants to elevated CO₂ improved growth and physiological activities of tall fescue under heat stress and altered responses of various metabolites to heat stress. Elevated CO₂ led to increased production of several organic acids (shikimic acid, malonic acid, threonic acid, glyceric acid, galactaric acid, and citric acid) and amino acids (serine, valine, and 5-oxoproline) as well as the maintenance of production of two disaccharide (sucrose and maltose) under heat stress. Those metabolites could play roles in elevated CO₂ mitigation of heat stress damage in tall fescue and thus could contribute to improved heat tolerance.

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