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Proteins and Metabolites Regulated by Trinexapac-ethyl in Relation to Drought Tolerance in Kentucky Bluegrass

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Abstract Plants have developed various mechanisms in adaptation to water deficit stress, including growth retardant to reduce water loss. Previous studies reported that plants treated with a growth inhibitor, trinexapac-ethyl (TE), had improved drought tolerance. The objective of this study was to determine alterations in proteins and metabolite accumulation associated with drought tolerance improvement in a perennial grass species, Kentucky bluegrass (*Poa pratensis*), induced by TE application. Plants were treated with TE [1.95 ml l^{-1} (v:v); a.i. TE = 0.113%] through foliar spray for 14 days, and then subjected to drought stress by withholding irrigation for 15 days in growth chambers. TE-treated plants exhibited significantly higher relative water content and photosynthetic capacity and lower membrane leakage than nontreated plants under drought stress, suggesting TE-enhanced drought tolerance in Kentucky bluegrass. Physiological improvement in drought tolerance through TE application was associated with the increased accumulation of various proteins and metabolites, including ferritin, catalase, glutathione-S-transferase, Rubisco, heat shock protein 70, and chaperonin 81, as well as fatty acids (palmitic acid, α-linolenic acid, linoleic acid, and octadecanoic acid). Our results suggest that TE may regulate metabolic processes for antioxidant defense, protective protein synthesis, photorespiration, and fatty acid synthesis, and thereby contribute to better drought tolerance in Kentucky bluegrass.

Keywords Grass · Growth inhibitor · Metabolites · Proteome · Water deficit

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Introduction

Drought stress is one of the major abiotic stresses limiting plant growth in arid and semiarid environments. Improving drought tolerance is critical for plant growth and productivity in water-limited environments. Drought tolerance may be achieved by genetic selection for elite germplasm and manipulation of physiological processes or modulation of associated metabolic processes through external factors such as application of plant growth regulators (Vettakkorumakankav and others 1999).

A plant growth inhibitor, trinexapac-ethyl (TE), inhibits gibberellin (GA) synthesis by blocking the transformation of metabolically inactive GA_{20} to metabolically active GA_1. TE has been used typically to control shoot growth in various plant species (King and others 1997; McCarty and others 2004; Pannacci and others 2004). Plants treated with TE exhibited dwarf shoots with darker green leaves due to increased mesophyll cell density and chlorophyll concentration (Ervin and Koski 2001; Heckman and others 2005; McCullough and others 2006). However, under stressful environments such as drought, heat, freeze, or shade, GA inhibitors such as TE, ancymidol, or paclobutrazol were found to help maintain better plant growth in various plant species, suggesting that plant growth inhibitors modulating GA synthesis may not only control plant growth but also promote stress tolerance (Fletcher and Hofstra 1990; Pinhero and Fletcher 1994; Qian 1998; Vettakkorumakankav and others 1999; Jaleel and others 2007). However, the mechanisms of how TE may affect stress tolerance are not well understood. Improved drought tolerance in TE-treated plants has been associated with reduction in water use rate due to shoot growth reduction and increases in osmotic adjustment due to the accumulation of inorganic solutes and soluble sugars (McCann and Huang 2007; Bian and others

2009). The combined treatment of TE and abscisic acid enhanced the expression of dehydrin (15-kDa protein) in wheat seeds (*Triticum aestivum* L., 'Bet Hashita') associated with dehydration tolerance (Korol and Klein 2002). TE-induced drought tolerance could result from the altered modulation of metabolic processes controlling stress tolerance traits, such as the synthesis of proteins and metabolites that regulate photosynthesis, control water loss and cellular hydration, and protect cellular structures and functions from oxidative damage (Nilsen and Orcutt 1996). However, the above-proposed mechanisms have not been well documented, and no detailed studies have been conducted to elucidate TE-induced changes in proteomic and metabolic profiles and their potential contribution to drought tolerance. Knowledge of proteins and metabolites associated with TE-induced stress tolerance is important for a more thorough understanding of the mechanisms of TE-regulated drought tolerance.

The objectives of this study were to identify proteins and metabolites altered by TE application under drought stress and to determine the proteins and metabolites associated with TE-induced drought tolerance in a perennial grass species, Kentucky bluegrass (*Poa pratensis*), through the combined approaches of physiological analysis and profiling of the proteome and metabonome. Effects of TE on physiological responses to drought stress were evaluated by assessing leaf net photosynthetic rate, stomatal conductance, transpiration rate, cell membrane stability, and relative water content. Proteomic analysis was performed using two-dimensional electrophoresis and mass spectrometry. Metabolites were analyzed using gas chromatography–mass spectrometry.

Materials and Methods

Plant Materials, Growing Conditions, and Treatments

Sods of Kentucky bluegrass (*Poa pratensis* L. 'Baron') collected from field plots in the turfgrass research farm at Rutgers University, New Brunswick, NJ, were planted in pots filled with a mixture of soil and sand (1:1 v:v). Following a 4-week period of plant establishment in a greenhouse, plants were moved to a growth chamber for treatments. The growth chamber conditions were set at temperatures of 20/15°C (day/night), 75% relative humidity, and 600 μmol m^{-2} s^{-1} of photosynthetically active radiation. Plants were watered every 2 days and fertilized once a week with half-strength Hoagland's nutrient solution (Hoagland and Arnon 1950).

After 1-week acclimation to the growth chamber conditions, plants were divided into two groups. The first group was sprayed with TE [1.95 ml l^{-1} (v:v); a.i. TE = 0.113% dissolved in water with pH of 6.5] and the second group was sprayed with water. Two weeks later, plants of the first group were sprayed with TE again. One day after the last TE application, drought stress was imposed by withholding watering, while the well-watered control plants were watered every 2 days during the experimental period. The experiment consisted of three treatments: (1) well-watered control plants without TE application, (2) drought stress with TE application, and (3) drought without TE application. Each treatment had six replicates (six pots of plants).

Physiological Measurements

Plant responses to drought stress were evaluated by measuring leaf net photosynthetic rate (P_n), stomatal conductance (g_s), transpiration (Tr), electrolyte leakage (EL), relative water content (RWC), and photochemical efficiency (F_v/F_m). All measurements were made on fully expanded leaves. Leaf P_n, g_s, and Tr were measured using a portable infrared gas analyzer (Li-6400, LICOR, Inc., Lincoln, NE). The analyzer was set at a 500-μmol s^{-1} flow rate, leaf temperature of 23°C, and 60 ± 5% relative humidity, and a LED external light source provided a photosynthetic photon flux density of 600 μmol m^{-2} s^{-1}. 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 90°C. TW was determined as the weight of leaves after having soaked in distilled water for 24 h. Leaf photochemical efficiency was determined using a fluorometer (ADC BioScientific, Hoddedson, UK), which measured the variable to maximum fluorescence ratio (F_v/F_m) in the nonenergized state following dark adaptation for 30 min. For EL analysis, about 0.2 g FW of leaves was placed in a test tube containing 30 ml of distilled deionized water. Test tubes were shaken for 17–18 h and the initial conductance (C_i) was measured with a YSI Model 32 Conductivity Meter (Yellow Spring, OH). Leaves were then killed at 120°C for 30 min, and the maximal conductance of killed tissue (C_{max}) was measured. The relative EL was calculated as 100 × C_i/C_{max}.

Protein Extraction

A previously described protein extraction protocol using acetone/trichloroacetic acid (TCA) precipitation was used for protein extraction (Xu and others 2008). At 0, 10, and 15 days of drought treatment, leaves were harvested, immediately frozen in liquid nitrogen, and then stored at − 80°C prior to analysis. 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 until the supernatant was colorless. The pellet was vacuum-dried, resuspended in resolubilization solution (8 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, 1% pharmalyte), 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 bovine serum albumin (BSA) as a standard.

Two-dimensional Gel Electrophoresis and Protein Identification

An IPGPhor apparatus (GE Healthcare, Piscataway, NJ) was used for isoelectric focusing (IEF) with immobilized pH gradient (IPG) strips (pH 3.0–10.0, linear gradient, 13 cm). The IPG strips were rehydrated for 14 h at 20°C with 250 µl rehydration buffer (8 M urea, 2 M thiourea, 2% w/v CHAPS, 1% v/v IPG buffer, 1% DTT, and 0.002% bromophenol blue) containing 300 µg proteins. The voltage settings for IEF were 500 V for 1 h, 1,000 V for 1 h, and 8,000 V to a total 56.50 kVh. Following IEF, the proteins in the strips were denatured with equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 1% DTT) and then incubated with the same buffer containing 2.5% iodoacetamide instead of DTT for 20 min. The second-dimension electrophoresis was performed on a 12.5% gel using a Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare). Gels were stained with colloidal Coomassie brilliant blue G-250 to detect total proteins (Newsholme and others 2000).

Gel images were analyzed with SameSpots software (Nonlinear, Newcastle upon Tyne, UK). Image analysis included the following procedures: spot detection, spot measurement, background subtraction, and spot matching. Only spots that were detected on all three replicate gels were further analyzed. 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. Data were subjected to analysis of variance to test for the effects of drought and TE. Means were separated by the least significance difference test ($p \leq 0.05$).

Gel spots were excised and washed with 30% ACN in 50 mM ammonium bicarbonate prior to DTT reduction and iodoacetamide alkylation. Trypsin was used for digestion at 37°C overnight. The resulting peptides were extracted with 30 µl of 1% trifluoracetic acid (TFA) followed by C_{18} Ziptip desalting. For mass spectrometry (MS) analysis, the peptides were mixed with 7 mg ml^{-1} α-cyano-4-hydroxycinnamic acid matrix in a 1:1 ratio and spotted onto a matrix-assisted laser desorption/ionization (MALDI) plate. The peptides were analyzed on a 4800 MALDI TOF/TOF analyzer (Applied Biosystems, Foster City, CA). Mass spectra (*m/z* 880–3,200) were acquired in positive-ion reflector mode. The 25 most intense ions were selected for subsequent MS/MS sequencing analysis in 1-kV mode. Protein identification was performed by searching the combined MS and MS/MS spectra against the green plant NCBI database using a local MASCOT search engine (ver. 1.9) on a GPS (ver. 3.5, ABI) server. Proteins containing at least two peptides with confidence interval (CI) values no less than 95% was considered identified.

Metabolite Analysis

Metabolic profiling was performed following the procedure described by Du and others (2011). Frozen leaves were ground to fine powder in liquid nitrogen. Leaf tissue powders were transferred into a 10-ml microcentrifuge tube and extracted in 1.4 ml of 80% (v/v) aqueous methanol for 2 h at 500×*g*. A total of 100 µl of ribitol solution (2 mg ml^{-1} water) was added as an internal standard prior to incubation. Then, extraction was done in a water bath at 70°C for 15 min. The extraction solution was centrifuged for 30 min at 14,000×*g*, the supernatant was decanted to a new tube, 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 5,000×*g*. The 300-µl polar phase (methanol/water) was decanted into 1.5-ml HPLC vials and dried overnight in a benchtop centrifugal concentrator (Labconco Corporation, Kansas City, MO). Before methoximation, the polar phase was dried with nitrogen gas. The dried polar phase was methoximated with 80 µl of 20 mg ml^{-1} methoxyamine hydrochloride at 30°C for 90 min and trimethylsilylated with 40 µl of MSTFA (with 1% TMCS) for 30 min at 70°C.

The derived extracts were analyzed with a PerkinElmer gas chromatograph coupled with a TurboMass-Autosystem XL mass spectrometer (PerkinElmer, Waltham, MA). A 1-µl aliquot of extract was injected into a DB-5MS capillary column (30 m × 0.25 mm × 0.25 µm, Agilent J&W Scientific, Folsom, CA). The inlet temperature was set at 260°C. After a 5-min solvent delay, initial gas chromatograph (GC) oven temperature was set at 80°C; 2 min after injection, the GC oven temperature was raised to 280°C at 5°C min^{-1}, and finally held at 280°C for 13 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 (*m/z* 30–550). The metabolites were identified using Turbomass 4.1.1 software coupled with commercially available compound libraries

(NIST 2005, PerkinElmer). Hierarchical clustering was performed using Cluster/TreeView 2.11 software based on the Pearson correlation coefficient (Eisen and others 1998).

Results and Discussion

Physiological Responses to Drought Stress

Leaf RWC was maintained at 90% under well-watered conditions during the experiment (Fig. 1a). At 5 days of drought treatment, leaf RWC was unchanged from the control level in TE-treated plants but declined to 80% in nontreated plants. At 10 and 15 days of drought treatment, leaf RWC decreased to 61 and 26% in TE-treated plants and 34 and 19% in nontreated plants, respectively. TE-treated plants had significantly higher leaf RWC than nontreated plants under drought stress, which could be associated with reduction in water lost by evapotranspiration from the plant canopy (McCann and Huang 2007) and/or increases in osmotic adjustment due to TE application (Bian and others 2009). Leaf EL increased to 26 and 67% in TE-treated plants and 39 and 87% in nontreated plants at 10 and 15 days of drought stress, respectively, which were significantly lower in TE-treated than in nontreated plants. These results suggest that TE treatment could help maintain higher cellular hydration, as shown by higher RWC and less damage to cellular membranes exhibited by lower EL in Kentucky bluegrass, which is in agreement with previous reports on creeping bentgrass (McCann and Huang 2007; Bian and others 2009).

Responses of leaf photochemical reactions and gas exchange to drought stress were also affected by TE treatment. Leaf F_v/F_m decreased under drought stress, but TE-treated plants had significantly higher F_v/F_m than nontreated plants at 10 and 15 days (Fig. 2). Beginning at 5 days of drought treatment, P_n was significantly higher in TE-treated plants than in nontreated plants. By 10 days of drought stress, P_n decreased to zero in nontreated plants and to 2.6 μmol CO_2 m^{-2} s^{-1} in TE-treated plants (Fig. 2). Single-leaf Tr and g_s exhibited the same patterns of responses to TE treatment as P_n under drought stress, which were significantly higher in TE-treated than in nontreated plants (Fig. 2). These results suggest that TE-treated plants are able to maintain more efficient photochemical reactions and carbon fixation associated with less restriction of gas exchange through stomatal regulation.

Effects of TE Application on Proteomic Responses to Different Levels of Water Deficit

As described above, leaf RWC was 61 and 26% in TE-treated plants and 34 and 19% in nontreated plants at 10 and 15 days of drought treatment, respectively. Previous studies demonstrated that permanent physiological and cellular damage occurred when RWC was decreased to below 25% in Kentucky bluegrass and when RWC and F_v/F_m values could not be returned to prestressed levels upon rewatering (Huang and Wang 2005). Hu and others (2010) reported that upon rewatering, two Kentucky bluegrass cultivars ('Midnight' and 'Brilliant') fully recovered in RWC and F_v/F_m when leaf RWC dropped to 53 and 39%, respectively, after drought treatment. Therefore, in this study, the 19–26% RWC range represented a severe level of leaf water deficit and 34–61% RWC indicated a moderate level of leaf water deficit in Kentucky bluegrass. The functions of proteins responsive to TE application under a moderate level of water deficit at 10 days of drought stress or a severe level of water deficit at 15 days of drought stress are discussed below.

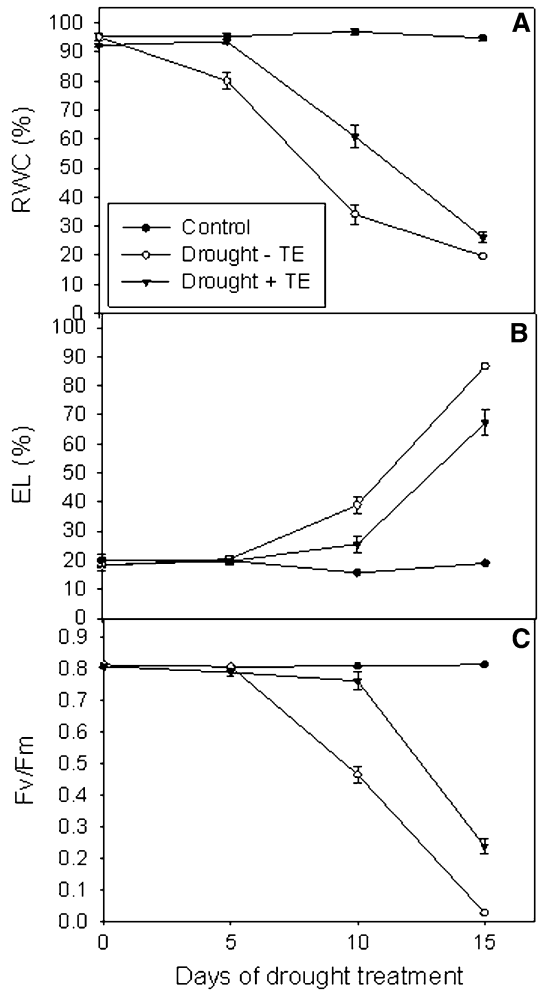

Fig. 1 Changes in RWC (**a**), electrolyte leakage (**b**), and photochemical efficiency (**c**) under drought stress with or without trinexapac-ethyl (TE) foliar application. *Each bar* is the mean ± SE ($n = 6$) for each treatment

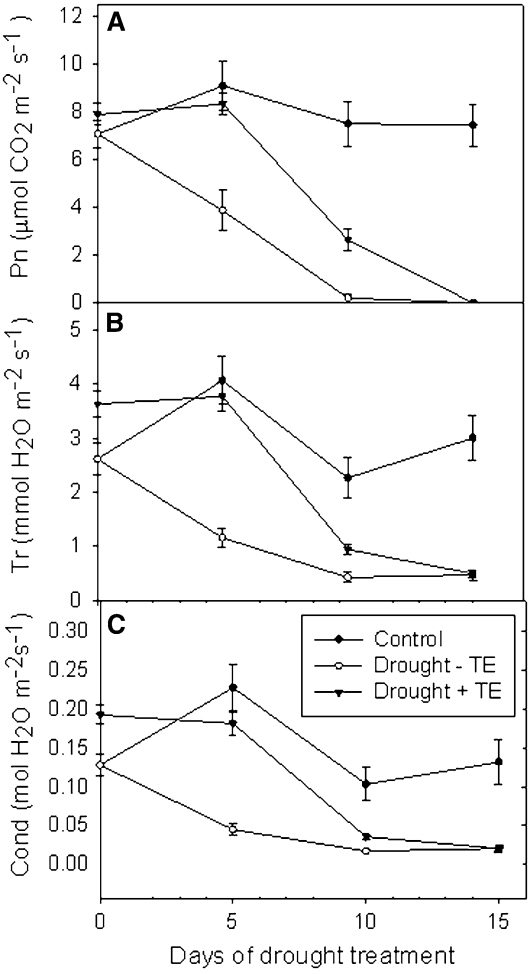

Fig. 2 Changes in leaf net photosynthetic rate (**a**), transpiration (**b**), and stomatal conductance (**c**) under drought stress with or without trinexapac-ethyl (TE) foliar application. *Each bar* is the mean ± SE ($n = 6$) for each treatment

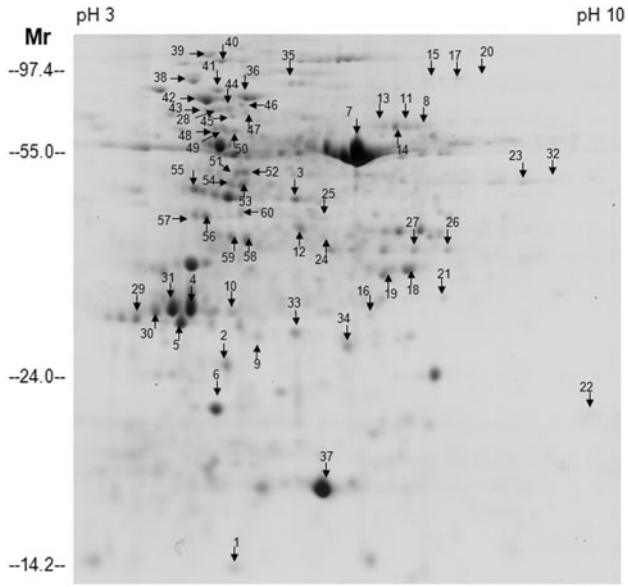

Fig. 3 Coomassie-stained 2-DE gel of separated proteins from leaves. Proteins were separated in the first dimension on a IPG strip (pH 3.0–10.0) and in the second dimension on a 12.5% gel. The *numbered spots* were subjected to mass spectrometric analysis

Two-dimensional gel electrophoresis was used to detect protein spots responsive to TE or drought stress at 10 and 15 days. A representative gel image is shown in Fig. 3. A total of 60 responsive spots were further analyzed (Figs. 4, 5). Fifty-eight protein spots that exhibited differential responses to TE treatment under moderate or severe water deficit levels were identified by mass spectrometry (Table 1).

Leaf Protein Upregulation in Response to Water Deficit as Affected by TE Application

Under moderate water deficit, the abundance of 13 spots (spots 8, 11, 13, 14, 21, 25, 39–42, and 48–50) increased in TE-treated plants, whereas in nontreated plants the abundance of 10 spots (spots 13, 15, 25, 39–42, and 48–50) increased (Table 1; Fig. 5). The abundance level of catalase (spots 8, 11, 13, 14), metalloendopeptidase (spot 39), and cell division cycle protein 48 (CDC48, spot 40) increased at 10 days of drought stress in both TE-treated and nontreated plants but was significantly higher in TE-treated plants than in nontreated plants. Catalase converts H_2O_2 into water and oxygen and is found predominantly in the peroxisome. Metalloendopeptidase is a proteolytic peptidase that breaks peptide bonds of nonterminal amino acids. CDC48, a member of the hexameric ATPases associated with diverse cellular activities (Frohlich and others 1991), is a cytosolic chaperone required for endoplasmic reticulum (ER)-associated protein degradation and participates in the fusion of ER membranes during cell cycle progression (Latterich and others 1995; Rabinovich and others 2002). It appears that TE treatment could help protect plants from moderate water deficit by accumulating more proteins involved in active oxygen species (AOS) scavenging, specific peptide degradation, and cell cycle progression.

The abundance of several other proteins also increased at 10 days of drought stress but did not differ between TE-treated and nontreated plants; these included aldo/keto reductase (AKR, spot 25), enolase (spots 48 and 50), heat shock protein 70 (HSP 70, spots 41 and 42), and 60-kDa chaperonin (spot 43). AKR encompasses a large superfamily of oxidoreductases that catalyze the NADPH-dependent reduction of a wide variety of carbonyl compounds such as steroid hormones, sugars, aldehydes, ketones, and monosaccharides (Hyndman and others 2003). Enolase, also known as phosphopyruvate dehydratase, is a metalloenzyme responsible for catalyzing the conversion of 2-phosphoglycerate to phosphoenolpyruvate, the penultimate step of glycolysis. The upregulation of AKR and

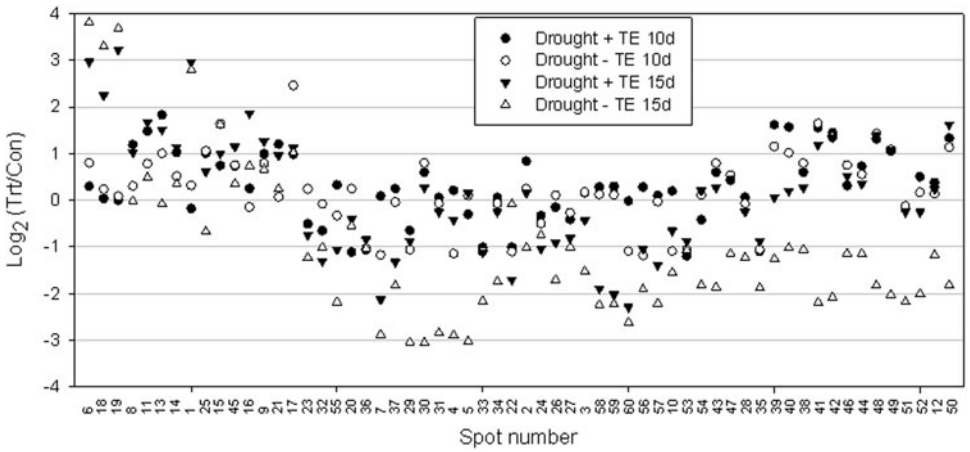

Fig. 4 Abundance ratio of drought and TE-responsive protein spots in TE-treated and nontreated plants. The ratio is expressed as log 2 (abundance in stressed plants/abundance in control plants)

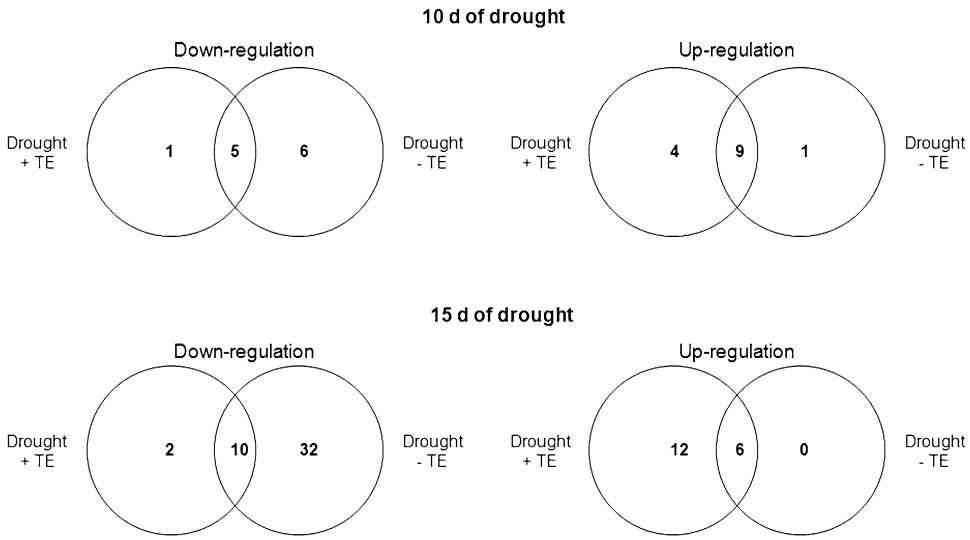

Fig. 5 Venn diagram illustrating the expression patterns of drought and TE-responsive proteins

enolase indicated that secondary metabolism and carbohydrate metabolism were enhanced under moderate water deficit at 10 days of drought stress. It is well known that HSP and chaperonins can prevent and reverse incorrect protein interactions, avoid aggregation of incorrectly folded proteins, and facilitate correct folding of proteins (Georgopoulos and Welch 1993). The increases in the abundance of HSP 70 and 60-kDa chaperonin at 10 days of drought stress may reflect adaptive mechanisms for drought stress, which is consistent with other studies (Wang and others 2004; Xu and Huang 2010). However, the lack of difference in the accumulation of AKR, enolase, HSP 70, and 60-kDa chaperonin at 10 days of drought stress between TE-treated and nontreated plants suggested that these proteins may not contribute to the differential physiological effects of these two treatments under moderate water deficit.

Under a severe level of water deficit, the abundance of 18 spots (spots 1, 6, 8, 9, 11, 13–19, 41, 42, 45, and 48–50) increased in TE-treated plants, whereas in nontreated plants only 6 spots (spots 1, 6, 15, and 17–19) were upregulated (Table 1; Fig. 5). Some spots were upregulated only in TE-treated plants; they were identified as catalase-1 (spots 8, 11, 13, and 14), glutathione-S-transferase (GST, spot 16), HSP 70 (spots 41 and 42), chaperonin (spot 49), enolase (spots 48 and 50), and ferritin (spot 9). Catalase and GST are enzymes involved in AOS scavenging (Noctor and Foyer 1998). GST has functions in the conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles (Edwards and others 2000). Hajheidari and others (2007) reported that drought stress increased GST abundance in a tolerant cultivar of sugar beet (*Beta vulgaris*) whereas it decreased in a sensitive cultivar. Ferritin plays an important role in protecting cells against oxidative stress by storing excess free Fe in a safe and bioavailable form, and its synthesis is induced in response to oxidative stress (Briat 1996). Other studies have shown that inhibited GA biosynthesis could

Table 1 Protein spots differently accumulated under water deficit in TE-treated and nontreated grasses

ID	Protein name [species]	MO	PM	AccN	10 days		15 days	
					D + TE	D − TE	D + TE	D − TE
6	Rubisco large subunit (fragment) [*Raddia brasiliensis*]	436	5	gil57283830	ns	ns	↑***	↑***
18	Rubisco large subunit (fragment) [*Psathyrostachys fragilis* subsp. *fragilis*]	952	9	gil31087917	ns	ns	↑***	↑***
19	Rubisco large subunit [*Psathyrostachys fragilis* subsp. *fragilis*]	991	9	gil31087917	ns	ns	↑***	↑***
8	Catalase-1 [*Oryza sativa* (*japonica* cultivar-group)]	478	6	gil2493543	↑*	ns	↑*	ns
11	Catalase-1 [*Oryza sativa* (*japonica* cultivar-group)]	571	6	gil2493543	↑*	ns	↑**	ns
13	Catalase-1 [*Oryza sativa* (*japonica* cultivar-group)]	566	7	gil2493543	↑**	↑*	↑**	ns
14	Catalase-1 [*Oryza sativa* (*japonica* cultivar-group)]	554	8	gil2493543	↑*	ns	↑*	ns
1	Chlorophyll a/b binding protein [*Oryza sativa*]	117	2	gil3126854	ns	ns	↑***	↑***
25	Aldo/keto reductase family protein [*Arabidopsis thaliana*]	269	2	gil42571931	↑*	↑*	ns	ns
15	Cell wall beta-glucosidase [*Secale cereale*] (beta-D-glucan exohydrolase)	102	2	gil46451431	ns	↑**	↑*	↑**
45	V-type proton ATPase	270	2	gil1352830	ns	ns	↑*	ns
16	Glutathione-S-transferase, I subunit [*Hordeum vulgare* subsp. *vulgare*]	220	2	gil21212950	ns	ns	↑**	ns
9	Ferritin [*Triticum aestivum*]	426	5	gil58221595	ns	ns	↑*	ns
21	Unidentified				↑*	ns	ns	ns
17	Unidentified				ns	ns	↑*	↑*
23	Aminomethyl transferase [*Oryza sativa* (*japonica* cultivar-group)]	189	2	gil115460656	ns	ns	ns	↓*
32	Aminomethyl transferase [*Oryza sativa* (*japonica* cultivar-group)]	286	3	gil115460656	ns	ns	↓*	↓*
55	Glutamine synthetase1 precursor [*Zea mays*]	224	2	gil162459403	ns	ns	↓*	↓**
20	Beta-D-glucan exohydrolase, isoenzyme [*Triticum aestivum*]	119	2	gil1203832	↓*	ns	ns	ns
36	Transketolase [*Oryza sativa* (*japonica* group)]	340	4	gil28190676	↓*	↓*	ns	↓*
7	Rubisco large subunit [*Psathyrostachys fragilis* subsp. *fragilis*]	1,020	12	gil61378666	ns	↓*	↓**	↓***
37	Rubisco small subunit [*Avena clauda*]	672	8	gil6573202	ns	ns	↓*	↓**
29	Chlorophyll a/b binding protein of LHCII	127	2	gil14423661	ns	↓*	ns	↓***
30	Chlorophyll a/b binding protein	136	2	gil14423661	ns	ns	ns	↓***
31	Chlorophyll a/b binding protein	262	3	gil115825	ns	ns	ns	↓***
4	Chlorophyll binding protein [*Arabidopsis thaliana*]	177	2	gil15220615	ns	↓*	ns	↓***
5	Chlorophyll a/b binding protein [*Amaranthus hypochondriacus*]	339	4		ns	ns	ns	↓***
33	Oxygen-evolving enhancer protein 2	274	4	gil131394	↓*	↓*	↓*	↓***
34	Oxygen-evolving enhancer protein 2	434	4	gil131394	ns	ns	ns	↓**
22	PSI reaction center subunit IV, chloroplast [*Arabidopsis thaliana*]	91	2	gil131176	↓*	↓*	↓**	ns
2	Light-harvesting complex I; LHC I [*Hordeum vulgare*]	372	4	gil544700	ns	ns	ns	↓*
24	Chloroplast ferredoxin-NADP + oxidoreductase precursor [*Capsicum annuum*]	150	2	gil6899972	ns	ns	↓*	ns
26	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic	444	4	gil120661	ns	ns	ns	↓**
27	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic	497	5	gil120661	ns	ns	ns	↓*

Table 1 continued

ID	Protein name [species]	MO	PM	AccN	10 days		15 days	
					D + TE	D − TE	D + TE	D − TE
3	Glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic	565	6	gil120663	ns	ns	ns	↓**
58	Fructose 1,6-bisphosphate aldolase precursor [*Avena sativa*]	360	4	gil8272480	ns	ns	↓**	↓***
59	Fructose 1,6-bisphosphate aldolase precursor [*Avena sativa*]	445	4	gil8272480	ns	ns	↓***	↓***
60	Fructose-1,6-bisphosphatase [*Pisum sativum*]	251	2	gil5305145	ns	↓*	↓***	↓***
56	Phosphoribulokinase [*Triticum aestivum*]	642	8	gil21839	ns	↓*	↓*	↓***
57	Phosphoribulokinase, chloroplastic				ns	ns	↓*	↓***
10	Triosephosphat-isomerase [*Triticum aestivum*]	597	6	gil11124572	ns	↓*	ns	↓**
53	Actin [*Setaria italica*]	761	7	gil9965319	↓*	↓*	ns	↓*
54	Actin [*Hordeum vulgare*]	420	4	gil24496452	ns	ns	ns	↓**
43	FtsH-like protein [*Nicotiana tabacum*]	451	5	gil4325041	ns	ns	ns	↓**
47	FtsH protease [*Arabidopsis thaliana*]	238	2	gil18402995	ns	ns	ns	↓*
28	FtsH protein [*Oryza sativa (japonica cultivar-group)*]	327	5	gil52075838	ns	ns	ns	↓*
35	FtsH protease [*Arabidopsis thaliana*]	542	4	gil18423214	↓*	↓*	ns	↓**
39	Metalloendopeptidase [*Arabidopsis thaliana*]	139	2	gil22331173	↑**	↑*	ns	↓*
40	Cell division cycle protein 48 [*Arabidopsis thaliana*]	693	5	gil15231775	↑**	↑*	ns	↓*
38	Heat shock protein 81-1	691	8	gil158513648	ns	ns	ns	↓*
41	Heat shock protein 70	1230	12	gil162457723	↑**	↑**	↑**	↓***
42	Heat shock protein 70	659	6	gil6911551	↑**	↑**	↑**	↓***
46	Heat shock protein 70				ns	ns	ns	↓*
44	Vacuolar proton-ATPase [*Hordeum vulgare* subsp. *vulgare*]	694	6	gil11527563	ns	ns	ns	↓*
48	Enolase [*Oryza sativa* (*japonica* cultivar-group)]	340	2	gil90110845	↑*	↑**	↑*	↓**
49	60-kDa chaperonin				↑*	↑*	↑*	↓***
51	Cytosolic monodehydroascorbate reductase				ns	ns	ns	↓***
52	Chloroplast translational elongation factor Tu [*Oryza sativa*]	426	4	gil6525065	ns	ns	ns	↓***
12	Os07g0513000 [*Oryza sativa* (*japonica* cultivar-group)]	291	4	gil115472339	ns	ns	ns	↓*
50	Enolase	734	6	gil90110845	↑*	↑*	↑**	↓**

ID spot ID (Fig. 5), *MO* MOWSE score, *PM* the number of unique peptides matched, *AccN* accession number, ↓ spot intensity decreased; ↑ spot intensity increased

* $0.05 > p \geq 0.01$; ** $0.01 > p \geq 0.001$; *** $0.001 > p$

increase superoxide dismutase activity in nonstressed grass (Zhang and Schmidt 2000) and *Narcissus tazetta* (Chen and Ziv 2004). The upregulation of ferritin, catalase, and GST in this study indicated that TE treatment enhanced the accumulation of these antioxidant proteins, which could protect plants from drought-induced oxidative damage. The upregulation of HSP 70 and chaperonin 81 in TE-treated plants at 15 days of drought stress indicated that the accumulation of protective proteins could be associated with improved tolerance of TE-treated plants to severe water deficit.

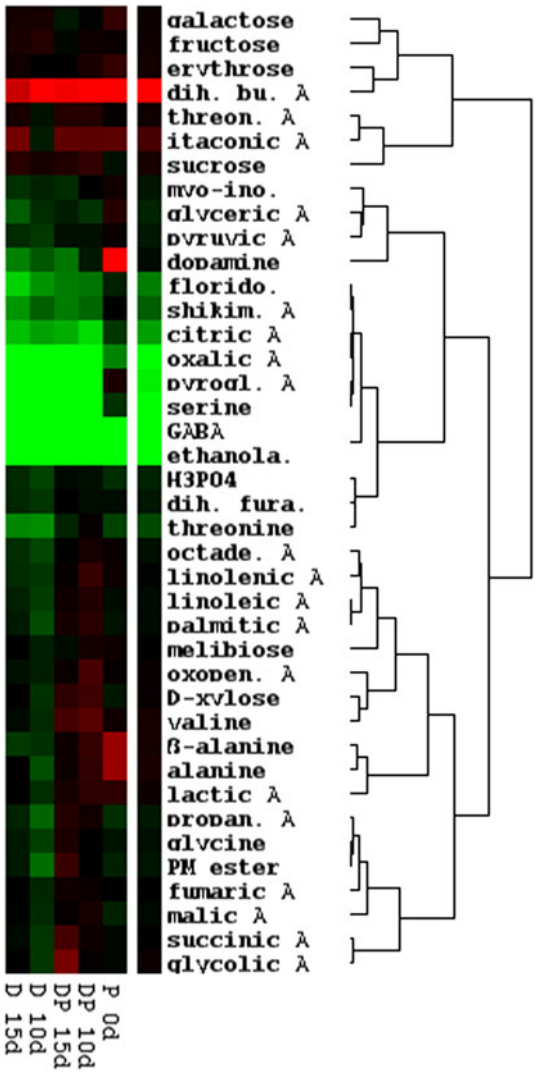

Fig. 6 Hierarchical display of responses of 40 metabolites to drought stress and TE treatment. The values of abundance difference divided by the abundance in the control were applied as input data. The dendrogram and colored image were produced as described by Eisen and others (1998). *Green* indicates downregulation, *red* indicates upregulation, and *dark* indicates no change

Leaf Protein Downregulation in Response to Water Deficit as Affected by TE Application

Under moderate water deficit at 10 days of drought, 11 spots exhibited declines in protein abundance in nontreated plants whereas 6 spots showed lower abundance in TE-treated plants, compared to the well-watered control plants. Several proteins, ribulose bisphosphate carboxylase/oxygenase (Rubisco) large subunit (spot 7), chlorophyll a/b binding protein (CAB, spot 4), fructose-1,6-bisphosphatase (spot 60), phosphoribulokinase (spot 56), and triosephosphate-isomerase (spot 10), were downregulated under moderate water deficit only in nontreated plants, but did not change in TE-treated plants. Rubisco is the primary enzyme in photosynthetic carbon fixation. Chlorophylls bound to CAB form chlorophyll–protein complexes that are involved in harvesting light energy and transferring it to photochemical reaction centers (Taiz and Zeiger 2002). Phosphoribulokinase, fructose-1,6-bisphosphatase, and triosephosphate-isomerase are enzymes involved in RuBP regeneration, carbon fixation, and reduction of photosynthesis. There are contradictory reports showing stimulatory (Yuan and Xu 2001; Ashraf and others 2002), inhibitory (Dijkstra and others 1990), or no effects (Biemelt and others 2004) of GA on photosynthetic rate. The results in this study suggest that TE treatment could have alleviated drought damage to photosynthetic metabolism, including light harvesting, carbon fixation and reduction, and RuBP regeneration under moderate water deficit. However, the observed change in protein abundance is not necessarily a direct effect of GA inhibition because long-term treatment may affect many aspects of plant growth. Actually, previous studies indicate that improved drought tolerance in TE-treated plants has been associated with the reduction in water loss due to shoot growth reduction and increases in osmotic adjustment (McCann and Huang 2007; Bian and others 2009).

Under severe water deficit at 15 days of drought, the abundance of 12 spots (spots 7, 22, 24, 32, 33, 37, and 55–60) decreased in TE-treated plants, whereas in nontreated plants 42 spots (spots 2–5, 7, 10, 12, 23, 26–44, and 46–60) exhibited a decline in abundance (Table 1; Fig. 5). Two spots were downregulated only in TE-treated plants; these were identified as PSI reaction center subunit IV (spot 22) and chloroplast ferredoxin-NADP + oxidoreductase (FNR, spot 24). Both proteins are important components of light reaction complexes in photosynthesis. The downregulation of these proteins may reflect reduction in photosynthetic ATP production under severe water deficit, as FNR and PSI reaction center proteins are involved in the generation of transmembrane proton electrochemical potential and ATP synthesis (Cramer and others 1996). The control of energy production, when carbon fixation is

inhibited under severe water deficit, may prevent AOS accumulation which is associated with excessive energy production (Carvalho 2008). Many other proteins were downregulated only in nontreated plants, including those involved in photosynthesis (CAB of LHCII, oxygen-evolving enhancer protein, and light-harvesting complex I), carbohydrate metabolism (transketolase, glyceraldehyde-3-phosphate dehydrogenase, fructose 1,6-bisphosphate [FBP] aldolase, triosephosphate isomerase, and enolase), protein storage and destination (FtsH protein, metalloendopeptidase, and HSP 70), protein synthesis (elongation factor Tu), AOS defense (cytosolic monodehydroascorbate reductase), cell structure (actin), and transport (vacuolar proton-ATPase).

Some proteins were downregulated in both TE-treated and nontreated plants, but TE-treated plants had higher abundance levels at 15 days of drought stress, including Rubisco large and small subunits (spots 7 and 37), FBP aldolase (spots 58–60), phosphoribulokinase (spots 56 and 57), and P protein of glycine decarboxylase (GDC, spot 32). Rubisco, FBP aldolase, and phosphoribulokinase are enzymes that control carbon fixation or regeneration of ribulose-1,5-bisphosphate in the Calvin cycle of photosynthesis. GDC cooperates with serine hydroxymethyltransferase to mediate photorespiratory glycine–serine interconversion, salvaging photorespiratory glycine for regeneration of C3 units that can re-enter the Calvin cycle (Kisaki and others 1971; Oliver 1994). The less severe decline or lack of changes in the abundance of the above-mentioned proteins in TE-treated plants suggests that TE treatment could have provided protection to photosynthesis, carbon metabolism, protein synthesis, and maintenance of AOS scavenging metabolism under severe water deficit.

TE Effects on Metabolite Responses to Drought Stress

To explore metabolite response to TE and water deficit, the relative abundance of 40 metabolites was analyzed by GC–MS. Analyses of hierarchical clustering showed that drought-responsive metabolites with or without TE treatment could be divided into three groups based on their response pattern at 10 and 15 days of drought (Fig. 6). Group I includes metabolites increased under drought stress only in TE-treated plants and/or decreased only in nontreated plants. Group II are metabolites that deceased in abundance level under drought stress in both TE-treated and nontreated plants. Group III includes metabolites that increased under drought stress in both TE-treated and nontreated plants (Fig. 6).

Group I metabolites include some fatty acids such as palmitic acid (16:0), α-linolenic acid (18:3), linoleic acid (18:2), and octadecanoic acid (18:0). Fatty acid unsaturation level has been positively associated with membrane fluidity, which is important for proper cellular metabolism and function under drought stress conditions (Hoekstra and others 2001). High levels of unsaturated fatty acids, particularly linolenic acids and linoleic acids, are positively correlated with drought tolerance in Kentucky bluegrass (Xu and others 2011). Palmitic acid, one of the most common saturated fatty acids, is the first fatty acid produced during lipogenesis and from which longer fatty acids can be produced. Octadecanoic acid is a major constituent of phospholipids in membranes and plays roles in energy dissipation with antioxidant effects (Bernardi and others 2002). Previous studies showed that free fatty acids can regulate the alternative oxidase, the plant mitochondrial ATP-sensitive potassium channel (PmitoKATP), and the plant mitochondrial uncoupling protein (PUMP), all of which are energy-dissipating systems in plant mitochondria found to prevent AOS production (Vanlerberghe and McIntosh 1997; Jezek and others 1998; Kowaltowski and others 1998; Pastore and others 1999). Linoleic acid can activate PUMP in plant mitochondria and reduce hydrogen peroxide production (Kowaltowski and others 1998; Pastore and others 2000). The increased abundance of unsaturated fatty acids and saturated fatty acids in TE-treated plants could help maintain cellular membrane stability and provide antioxidant protection from drought damage, respectively.

Some metabolites in group I are involved in photorespiration. In photorespiration, glycolate is converted to glycine and also produces H_2O_2, which is catalyzed by catalase, whereas glycine is converted to serine by GDC, and other byproducts are used to synthesize glutamate, which is a precursor for GABA and pyroglutamic acid biosynthesis (Taiz and Zeiger 2002). In nontreated plants, the content of glycolate decreased under drought stress, which could result in low levels of glycine and serine, whereas reduced serine may lead to low abundance of GABA and pyroglutamic acid. In TE-treated plants, the glycolate level increased, which could lead to higher levels of glycine and H_2O_2. These metabolomic data are consistent with the above-mentioned proteomic results of increased catalase abundance. However, in TE-treated plants serine abundance decreased even with high glycine level, likely as a result of regulation of GDC enzyme. Proteomic data showed that GDC was downregulated in both TE-treated and nontreated plants exposed to drought stress. The abundance responses of glycine and serine were observed earlier during drought treatment than that of GDC, which suggests that GDC responds to stress at both activity and abundance levels. An increased glycine level in TE-treated plants might result from a high glycolate level instead of reduced conversion of glycine to serine because serine content largely decreased in both TE-treated and nontreated plants.

The content of metabolites in group II decreased under drought stress regardless of TE treatment; these included shikimic acid, citric acid, oxalic acid, serine, ethanolamine, pyroglutamic acid, GABA, and floridoside. As discussed above, the disturbed photorespiration might result in reduced levels of pyroglutamic acid, serine, and GABA. GABA is a four-carbon nonprotein amino acid that has been associated with various physiological responses, including the regulation of cytosolic pH, carbon fluxes in the TCA cycle, nitrogen metabolism, protection against oxidative stress, osmoregulation, and signaling (Bouché and Fromm 2004). Many experiments showed that GABA is rapidly produced in response to biotic and abiotic stresses (Kinnersley and Turano 2000; Lugan and others 2009). Another metabolite with the same response pattern is ethanolamine. Ethanolamine is a substrate for the synthesis of phosphatidylethanolamine, which is the second most abundant phospholipid in most eukaryotic membranes and plays an important role in determining the chemical and physical properties of these membranes and their proteins (Sparace and others 1981; Dawidowicz 1987). The reduction of GABA and ethanolamine in the present study may be associated with growth inhibition because they decreased not only under drought stress but also in TE-treated plants even under well-watered conditions.

Group III metabolites included threonic acid, itaconic acid, 2,4-dihydroxybutanoic acid, and sucrose. Sucrose as an osmoprotectant may protect cell membranes exposed to stress conditions (Kaplan and others 2004). Threonic acid is a product of ascorbic acid catabolism and increased in *Arabidopsis* under heat stress (Loewus 1999; Kaplan and others 2004). However, how the organic acids threonic acid, itaconic acid, and 2,4-dihydroxybutanoic acid are related to drought tolerance is unknown.

In summary, TE foliar application improved drought tolerance in Kentucky bluegrass as manifested by physiological changes, which could be associated with alterations at both protein and metabolite levels. The improved tolerance could result from reduced AOS production and enhanced antioxidant defense, because TE foliar application increased the abundance of ferritin, catalase, and GST. The improved tolerance to severe water deficit could also result from the enhanced ability to prevent and reverse incorrectly folded proteins given that the abundance of HSP 70 and chaperonin 81 increased only in TE-treated plants. TE application also resulted in an increased content of palmitic acid, α-linolenic acid, linoleic acid, and octadecanoic acid, which may contribute to improved drought tolerance because these fatty acids can maintain membrane stability and also regulate energy-dissipating systems in mitochondria to act against oxidative stress. The alteration of metabolism favoring photorespiration in TE-treated plants could also be associated with improved defense against drought stress. Our work highlights the complexity of the cellular responses to TE application under water deficit and indicates that many levels of regulation are at play. The relationship of proteins and physiological parameters altered by TE deserves further investigation.

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