



Research article

Comparative proteomic responses of two bermudagrass (*Cynodon dactylon* (L. Pers.) varieties contrasting in drought stress resistanceHaitao Shi ^{a,1}, Tiantian Ye ^{a,b,1}, Zhulong Chan ^{a,*}^a Key Laboratory of Plant Germplasm Enhancement and Specialty Agriculture, Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan 430074, China^b University of Chinese Academy of Sciences, Beijing 100039, China

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ABSTRACT

Drought (water-deficit) stress is a serious environmental problem in plant growth and cultivation. As one of widely cultivated warm-season turfgrass, bermudagrass (*Cynodon dactylon* (L. Pers.) exhibits drastic natural variation in the drought stress resistance in leaves and stems of different varieties. In this study, proteomic analysis was performed to identify drought-responsive proteins in both leaves and stems of two bermudagrass varieties contrasting in drought stress resistance, including drought sensitive variety (Yukon) and drought tolerant variety (Tifgreen). Through comparative proteomic analysis, 39 proteins with significantly changed abundance were identified, including 3 commonly increased and 2 decreased proteins by drought stress in leaves and stems of Yukon and Tifgreen varieties, 2 differentially regulated proteins in leaves and stems of two varieties after drought treatment, 23 proteins increased by drought stress in Yukon variety and constitutively expressed in Tifgreen variety, and other 3 differentially expressed proteins under control and drought stress conditions. Among them, proteins involved in photosynthesis (PS), glycolysis, N-metabolism, tricarboxylic acid (TCA) and redox pathways were largely enriched, which might be contributed to the natural variation of drought resistance between Yukon and Tifgreen varieties. These studies provide new insights to understand the molecular mechanism underlying bermudagrass response to drought stress.

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1. Introduction

Drought (water-deficit) stress is one of the major environmental stresses that seriously limits plant distribution, growth and yield

worldwide (Shi et al., 2012, 2013a, b; 2014; Zhao et al., 2011). With the changes of environment and global climate, drought stress not only seriously affects plant growth and development, but also breaks the ecological balance of the ecosystem (Shi et al., 2012, 2013a, b; 2014; Zhao et al., 2011).

Bermudagrass (*Cynodon dactylon* (L. Pers.) is one of widely cultivated warm-season turfgrass on lawns, parks and sport fields, with drastic natural variation in the drought stress resistance among different varieties (Shi et al., 2012; Zhao et al., 2011). Recently, we identified three groups of bermudagrass that differed in drought resistance, including drought sensitive variety (Yukon), moderately tolerant variety (SR9554) and drought tolerant variety (Tifgreen) (Shi et al., 2012). Comparative physiological analysis among these varieties suggested that changes of water status, osmolyte accumulation and antioxidant defense system during drought stress might contribute to the natural variation of bermudagrass drought resistance (Shi et al., 2012). However, the molecular mechanisms underlying bermudagrass response to drought stress remain largely unknown. Recent studies in plant proteome have made it powerful and effective approach to identify proteins

Abbreviations: 2-DE, two-dimensional electrophoresis; Asc, ascorbate; BSA, bovine serum albumin; CBB, coomassie brilliant blue; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; EL, electrolyte leakage; GAPC, cytosolic glyceraldehyde 3-phosphate dehydrogenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GS, glutamine synthetase; H₂O₂, hydrogen peroxide; HSP, heat shock protein; IEF, isoelectric focus; IPG, immobilized pH gradient; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; MDH, malate dehydrogenase; NF, normalized frequency; OEC, oxygen-evolving complex; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PLD, phospholipase D; PRK, Phosphoribulokinase; PS, photosynthesis; ROS, reactive oxygen species; RuBisCO, ribulose-1,5-bisphosphate carboxylase oxygenase; RuBP, ribulose-1,5-bisphosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SOD, superoxide dismutase; TCA, tricarboxylic acid; TFA, trifluoroacetic acid.

* Corresponding author. Tel.: +86 27 87510823; fax: +86 27 87510251.

E-mail address: zhulongch@wbcas.cn (Z. Chan).

¹ These authors contributed equally to this study.

that were essential of stress response (Komatsu et al., 2012; Mohammadi et al., 2012; Shi et al., 2013a; Xu and Huang, 2010; Zhao et al., 2011). Currently, the drastic natural variations among different bermudagrass varieties provide effective approach to investigate the molecular, genetic, proteomic, and metabolic mechanisms during drought stress response (Mohammadi et al., 2012; Shi et al., 2012; Xu and Huang, 2010; Zhao et al., 2011). Bermudagrass grows and reproduces rapidly through propagation by both seeds and cutting branches (Shi et al., 2012, 2013a, b). When grown under drought stress condition, the leaves of bermudagrass turned yellow and brown or died off, but the branch and rhizome were still alive (Shi et al., 2012, 2013a, b).

Bermudagrass adapts to different cultivation conditions all over the world, however, research about bermudagrass stress response is limited because of non-availability of genome information. Most research groups mainly paid attention to physiological level changes and focused on the leave samples (Hu et al., 2009; Shi et al., 2012, 2013a, b; Xu and Huang, 2010; Zhao et al., 2011). In this study, we identified 39 drought-responsive proteins in both leaves and stems of two bermudagrass varieties contrasting in drought stress resistance using two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). Among these proteins, 23 proteins were up-regulated by drought stress in leaves or stems of Yukon variety and were constitutive expressed in Tifgreen variety. Further cluster and pathway analyses provided some new insights to understand the molecular mechanism underlying bermudagrass response to drought stress.

2. Materials and methods

2.1. Plant materials and growth conditions

Two bermudagrass varieties of Yukon [provided by American Seed Research of Oregon Company (<http://www.sroseed.com/>)] and Tifgreen [provided by Wuhan Green Garden Turfgrass Company (<http://www.whcp66.com/>)] were used in this study. The healthy cutting shoot-tips from these two bermudagrass varieties were cultured in the soil in the growth room as previously described (Shi et al., 2012), which was controlled at an irradiance of about $150 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, $25 \pm 2^\circ\text{C}$, 65%–75% relative humidity, and 16 h light and 8 h dark cycles.

2.2. Drought stress treatment

The healthy cuttings in pots were re-cultivated for about 14 d to nearly the same crown size. Then the plants were subjected to well-watered condition (control) and drought stress condition (soil water deficit) by withholding water in the soil for indicated days. To minimize the environment effects, the pots with soil and plants were rotated daily with the same growth condition. For the following assays, the leaf and stem samples from each independent experiment were harvested from at least 30 seedlings per variety.

2.3. Determination of electrolyte leakage (EL)

The EL was assayed as previously described (Shi et al., 2012, 2013a, b). Briefly, about 0.2 g plant leaves and stems were incubated in 20 ml of deionized water, shaken on a gyratory shaker at room temperature for 8 h. In the beginning, the initial conductivity (C_i) was measured using a conductivity meter (Leici-DDS-307A, Shanghai, China). The samples were then boiled at 121°C for 20 min to completely induce all electrolytes of the solution, and the conductivity of the killed tissues (C_{max}) was assayed after the

solution cooling to room temperature. The relative EL (%) was calculated as $(C_i/C_{\text{max}}) \times 100$. Student's *t*-test was used to analysis the significant difference, and Asterisk symbols (*) indicates the significant difference of $P < 0.05$.

2.4. Protein extraction and 2-DE

Total protein extraction was determined according to previous described method (Shi et al., 2013a) with some modifications. Briefly, 2 g plant leave and stem samples were ground in liquid nitrogen, and then transferred in 5 ml of pre-cooled homogenization buffer [20 mM Tris–HCl (pH 7.5), 1.05 M sucrose, 10 mM EGTA, 1 mM DTT, 1 mM PMSF and 1% (v/v) Triton X-100] to homogenized extensively on ice, and the homogenate was centrifuged at 10,000 g for 30 min at 4°C . The supernatant was shifted to a new centrifugal tube and mix with one volume of Tris–HCl (pH 7.8) buffered phenol. After centrifugation at 10,000 g for 30 min at 4°C , the above phenol phase was mixed with five volumes of ice-cold saturated ammonium acetate in methanol overnight at -20°C . The total proteins were collected through centrifugation (at 10,000 g for 30 min at 4°C) and washed three times with ice-cold saturated ammonium acetate in methanol and acetone, respectively. Then vacuum-dried protein precipitate was stored at -80°C or dissolved in the lysis buffer [7 M urea, 2 M mithiurea, 4% (w/v) of 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 65 mM DTT and 0.2% (w/v) of carrier ampholyte (pH3.5–10)]. After dissolving extensively and centrifugation, the protein supernatant was quantified through the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as the standard.

The 2-DE was performed as previously described (Shi et al., 2013a) with minor modification. Briefly, 800 μg of total proteins was applied onto an immobilized pH gradient (IPG) strip (17 cm, pH 4–7, Bio-Rad, USA), and rehydrated extensively for 16 h at room temperature. Then the strips were transferred to isoelectric focus (IEF) in the Protein IEF system (Bio-Rad, USA), and IEF was carried out at 200 V for 1 h, 500 V for 1 h, 10,000 V for 5 h, 10,000 V for 60000 Vh and 500 V for 2 h. After IEF, the strips were initially incubated in the first equilibration buffer [6 M urea, 0.375 M Tris–HCl pH8.8, 2% (w/v) of SDS, 20% (v/v) of glycerol and 2% (w/v) of DTT] with shaken for 15 min, and then incubated in the second equilibration buffer [6 M urea, 0.375 M Tris–HCl pH8.8, 2% (w/v) of SDS, 20% (v/v) of glycerol and 2.5% (w/v) of iodoacetamide] for the next 15 min. The equilibrated strips were performed with 12% of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as previously described (Shi et al., 2013a).

2.5. Gel image analysis

After SDS–PAGE, 2-D gels were stained in coomassie brilliant blue (CBB)–R250 staining buffer [containing 50% (v/v) of methanol, 15% (v/v) of acetic acid and 0.1% (w/v) of CBB–R250] for 4 h and destained in the buffer containing 30% (v/v) of ethanol and 8% (v/v) of acetic acid overnight. Then the protein gels were scanned using an EPSON PERFECTION™ V700 PHOTO scanner (Epson) at 600 dpi resolution and 48-bit full color image. All the images of three independent experiments were analyzed with PDQuest™ 2-DE Analysis Software (BIO-RAD, USA) according to the instruction.

2.6. Protein spot identification by MALDI-TOF-MS

The protein spots with differential intensities [$\log_2$ (fold change) > 1 and $\log_2$ (fold change) < -1] were excised from the 2-DE gel manually, washed with double distilled water twice, and destained twice with 50 mM $\text{NH}_4\text{—HCO}_3$ in 50% (v/v) acetonitrile

for 1 h at room temperature until the gel was mostly colorless. After carefully remove the liquid, 50 μ l acetonitrile was added to dehydrate the gel, and the gel spots were then incubated in 25 mM NH_4HCO_3 containing 10 pmol trypsin at 37 °C overnight. After trypsin digestion, the gel slices were washed with 0.1% trifluoroacetic acid (TFA) in 50% (v/v) acetonitrile three times to collect the peptides. The extracted peptides were used for MALDI-TOF–MS analysis using AXIMA-CFR plus (Shimadzu Biotech, Kyoto, Japan) according to the manufacturer's instruction.

After MALDI-TOF–MS analysis, the MS–MS data was matched against NCBI nr and Swiss-Port protein sequence databases using MASCOT software (Mascot Wizard 1.2.0, Matrix Science Ltd, <http://www.matrixscience.com>). In the searching process, peptide masses were assumed to be monoisotopic, and 100 ppm was used as mass accuracy, and one missing cleavage site was the maximum, and modifications were also considered. Additionally, the minimum score of 24 and the minimum sequence coverage of 2% in MOWSE analysis were used to keep the confidence of the identification results. Only the best matched protein with high confidence score was selected as the final result.

2.7. Cluster analysis and metabolic pathway analyses

Hierarchical cluster analysis was performed using CLUSTER program (<http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/>) (de Hoon et al., 2004), and resulting tree figures were displayed using the software package and Java Treeview (<http://jtreeview.sourceforge.net/>). The differentially expressed proteins were classified using the Classification SuperViewer Tool (http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi) (Provart and Zhu, 2003), and functional categories of every protein were assigned using MapMan (<http://mapman.mpimp-golm.mpg.de/general/ora/ora.html>) (Thimm et al., 2004). The pathway graph of carbon metabolism was obtained from KEGG (http://www.genome.jp/kegg-bin/search_pathway). Normalized frequency (NF) of each functional category was assayed as sample frequency of each category in this experiment/background frequency of each category in genome.

3. Results

3.1. Comparison of drought-triggered cell damage in leaves and stems of Yukon and Tifgreen varieties

Under control condition, no significant differences were obtained in plant growth and cell damage (leaves and stems) between Yukon and Tifgreen varieties (Fig. 1A–C). When subjected to drought stress condition, plant growth was inhibited and EL in both of leaves and stems gradually increased in both bermudagrass varieties (Fig. 1A–C). However, Tifgreen variety showed better growth than Yukon variety under drought stress condition, with lower EL in both of leaves and stems (Fig. 1A–C). Additionally, plant leaves exhibited higher EL than plant stems in both Yukon and Tifgreen varieties (Fig. 1A–C), indicating the natural variation in different organs of bermudagrass.

3.2. Global protein expression profiles in leaves and stems of Yukon and Tifgreen varieties

To reveal the differences in plant leaves and stems of two bermudagrass varieties contrasting in drought stress resistance in response to drought stress, a proteomic approach was performed to identify drought-responsive proteins in leaves and stems of Yukon and Tifgreen varieties (Fig. 2). Generally, more than 400 proteins with different protein abundances were reproducibly examined for each variety and organ combination (Fig. 3).

3.3. Identification of differentially expressed proteins in leaves and stems of Yukon and Tifgreen varieties

After comparative proteomic analysis, 52 proteins were differentially expressed in two bermudagrass varieties under drought stress condition. Among them, we successfully identified 39 proteins via MALDI-TOF–MS, including 5 commonly regulated proteins (3 up-regulated proteins and 2 down-regulated proteins) by drought stress in leaves and stems of Yukon and Tifgreen varieties (group 1), 2 differentially regulated proteins in leaves and stems of

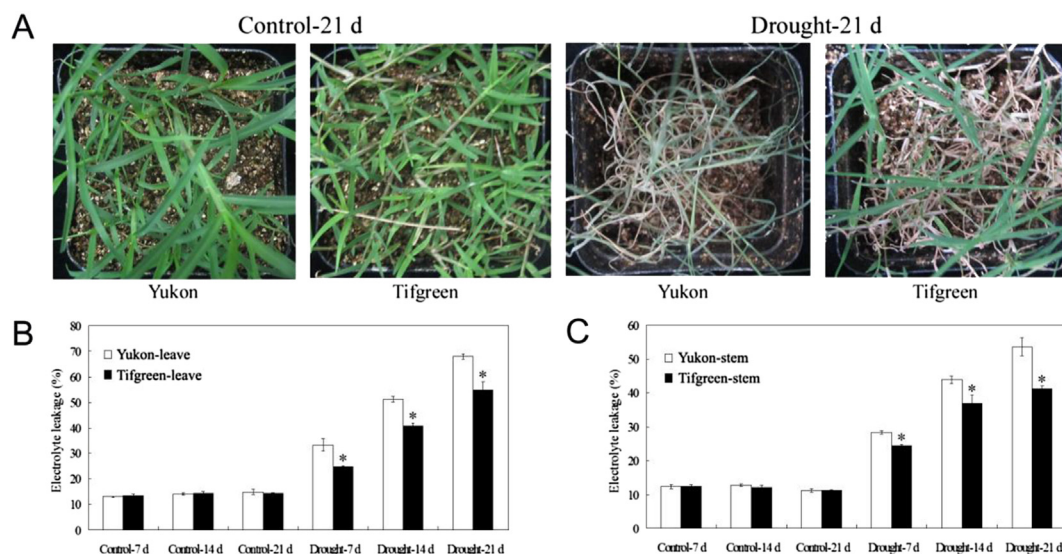


Fig. 1. Comparison of drought-triggered cell damage in leaves and stems of Yukon and Tifgreen varieties. (A) The photograph showing 3-week-old plants that subjected to control (well-watered) condition and drought condition for 21 d. EL of leaves (B) and stems (C) of Yukon and Tifgreen varieties under control and drought stress conditions at indicated days. The data represent the means of three independent experiment $\pm$ SDs, and * indicate the significant differences $p < 0.05$ (student's *t*-test).

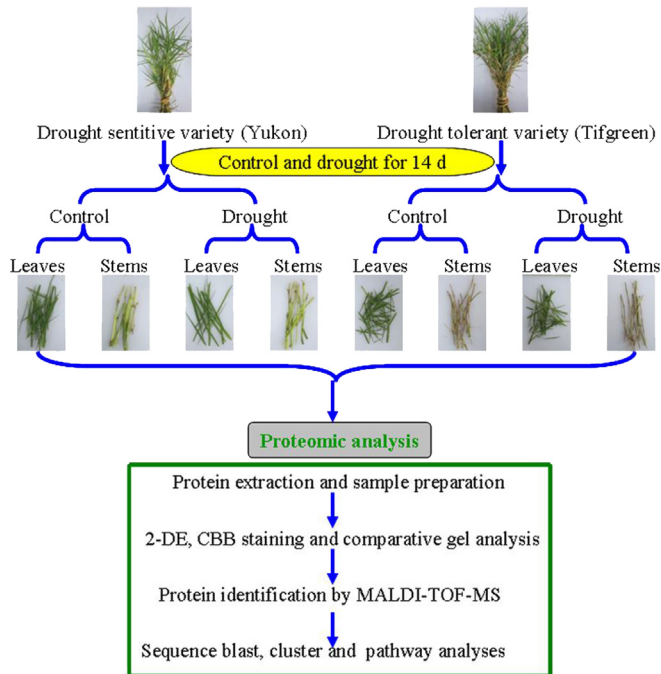


Fig. 2. Flowchart of the comparative proteomic and metabolomic analyses in leaves and stems of Yukon and Tifgreen varieties under control and drought stress conditions. The bermudagrass plants of Yukon and Tifgreen varieties with nearly the same crown size were subjected to watering condition (control) and drought condition (soil water deficit) for 14 d, then plant leaves and stems were harvest for proteomic and metabolomic analyses.

Yukon and Tifgreen varieties after drought stress (group 2), 23 proteins up-regulated by drought stress in leaves or stems of Yukon variety and constitutive expressed in Tifgreen variety (group 3), and 9 differentially expressed proteins in Yukon and Tifgreen varieties [3 proteins with $\log_2$ (Tifgreen vs. Yukon) > 1 and 6 proteins with $\log_2$ (Tifgreen vs. Yukon) < -1] under control and drought conditions (group 4) (Fig. 3 and Table 1).

Through MALDI-TOF-MS analysis, the MS data were matched against NCBI nr and Swiss-Port protein sequence databases using MASCOT software, and the best matched protein with high confidence score was selected as the final result of each protein spot (Table 1 and Table S1). Since bermudagrass is an un-sequenced species, the homologous proteins were blasted against sequenced plant species and functional categories of every protein were also assigned using MapMan (Table 1 and Table S1). Proteins including Cytochrome, RuBisCO, superoxide dismutase, and phosphoenolpyruvate carboxylase were identified in both leaf and stem samples in response to drought treatment (Table 1).

3.4. Cluster and pathway enrichment analysis of drought-responsive proteins in leaves and stems of Yukon and Tifgreen varieties

The expression patterns of 39 drought-responsive proteins in leaves and stems of Yukon and Tifgreen varieties were examined using cluster analysis (Fig. 4A–D and Table S2). Interestingly, expression levels of a group of proteins significantly increased in leaf tissue of drought sensitive Yukon variety, but slightly in drought tolerant Tifgreen variety after drought stress treatment. Those proteins were also constitutively highly expressed in Tifgreen indicating they might be involved in drought resistance of Tifgreen (Fig 4C). Pathway enrichment analysis revealed that

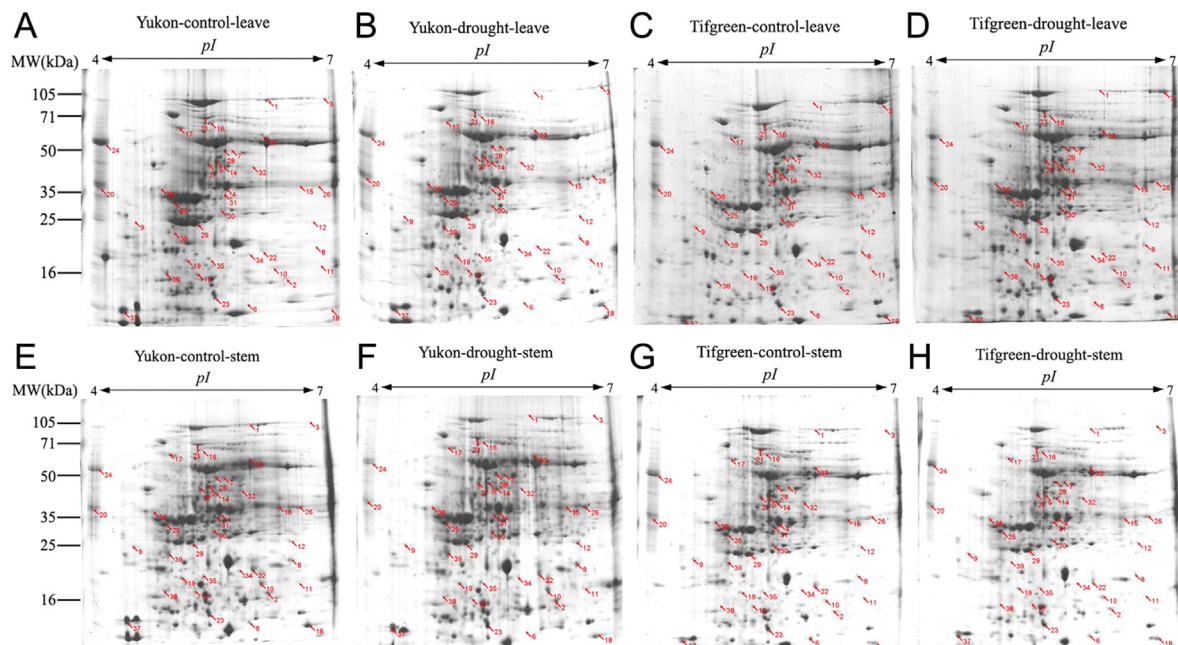


Fig. 3. Comparison of proteome patterns of bermudagrass leaves and stems in response to drought stress treatments. (A)–(H) Comparison of proteome patterns of Yukon leaves under control conditions (A) and under drought stress conditions (B), Tifgreen leaves under control conditions (C) and under drought stress conditions (D), Yukon stems under control conditions (E) and under drought stress conditions (F), Tifgreen stems under control conditions (G) and under drought stress conditions (H). Plant proteins from different organs under indicated conditions were separated in the first dimension on the IPG strip (pH 4–7), and in the second dimension on 12.5% SDS-PAGE. The differentially expressed proteins were marked with red arrows as shown. The data represent one gel from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
List of differentially expressed proteins in two bermudagrass varieties under control and drought stress conditions.

Expression pattern	No.	Accession	Homologous protein [species]	The. (Mr/pl)	Exp. (Mr/pl)	Score	Cov.	Class of bin code + subcategory
Group 1	1	gi 11991545	Phosphoenolpyruvate carboxylase [Chloris gayana]	109.0/5.76	100.9/6.07	138	19%	[4.1.16] glycolysis.cytosolic branch.phospho-enol-pyruvate carboxylase (PEPC)
	2	gi 301073308	Cu/Zn superoxide dismutase [Aeluropus lagopoides]	15.3/5.65	14.4/6.37	96	20%	[21.6] redox.dismutases and catalases
	3	gi 10241562	Putative C4 Phosphoenolpyruvate carboxylase [Cynodon dactylon]	45.2/5.76	102.9/6.9	118	27%	[4.1.16] glycolysis.cytosolic branch.phospho-enol-pyruvate carboxylase (PEPC)
	4	gi 148251625	Plastid high chlorophyll fluorescence 136 precursor [Zea mays]	43.1/8.71	36.4/5.70	160	34%	[29.8] protein.assembly and cofactor ligation
Group 2	5	gi 326516786	Predicted protein, beta-actin [Hordeum vulgare subsp. vulgare]	41.9/5.31	48.2/5.57	173	37%	[31.1] cell.organisation
	6	UCRIA_ORYSJ	Cytochrome b6-f complex iron-sulfur subunit, chloroplastic [Oryza sativa subsp. Japonica]	24.2/8.54	10.9/6.03	84	24%	[1.1.3] PS.lightreaction.cytochrome b6/f
	7	gi 28172915	Cytosolic 3-phosphoglycerate kinase [Zea mays]	31.7/7.71	56.9/5.76	120	13%	[4.1.12] glycolysis.cytosolic branch. 3-phosphoglycerate kinase (PGK)
Group 3	8	gi 413952229	Hypothetical protein ZEAMMB73_660929, flavodoxin-like quinone reductase 1 [Zea mays]	12.6/7.90	20.3/6.67	85	52%	[11.8] lipid metabolism."exotics" (steroids, squalene etc)
	9	gi 54303878	RuBisCO large subunit [Distichlis spicata]	44.3/6.29	26.2/4.52	37	2%	[1.3.1] PS.calvin cyle.rubisco large subunit
	10	gi 357111487	Oxygen-evolving enhancer protein 1, chloroplastic-like isoform 1 [Brachypodium distachyon]	34.8/5.17	15.7/6.27	153	46%	[1.1.1.2] PS.lightreaction.photosystem II.PSII polypeptide subunits
	11	gi 388564565	Cytochrome b6-f complex iron-sulfur subunit [Saccharum hybrid cultivar ROC22]	20.9/8.20	16.6/6.77	128	29%	[1.1.3] PS.lightreaction.cytochrome b6/f
	12	gi 222629986	Hypothetical protein, dehydroascorbate reductase [Oryza sativa Japonica Group]	11.1/4.72	26.5/6.67	75	32%	[21.2.1] redox.ascorbate and glutathione. ascorbate
	13	gi 297722003	Photosystem II oxygen evolving complex protein PsbP family protein [Oryza sativa Japonica Group]	25.8/7.63	14.4/5.36	86	22%	[1.1.1.2] PS.lightreaction.photosystem II.PSII polypeptide subunits
	14	gi 115448091	Phosphoribulokinase (PRK) [Oryza sativa Japonica Group]	45.2/5.68	43.8/5.64	132	20%	[1.3.12] PS.calvin cyle.PRK
	15	MDHM_CITLA	Malate dehydrogenase, mitochondrial [Citrus lanatus]	36.4/8.88	38.2/6.51	58	19%	[8.1.9] TCA/org. transformation.TCA.malate DH
	16	gi 187830110	Filamentation temperature-sensitive H ₂ B [Zea mays]	72.6/5.69	75.8/5.52	290	39%	[29.5.7] protein.degradation.metalloprotease
	17	gi 357167236	RuBisCO large subunit-binding protein subunit alpha [Brachypodium distachyon]	61.2/5.06	69.4/5.01	158	10%	[1.3.13] PS.calvin cyle.rubisco interacting
	18	gi 339759470	RuBisCO small subunit protein [Eleusine coracana]	5.2/5.06	10.0/6.95	107	72%	[1.3.2] PS.calvin cyle.rubisco small subunit
	19	gi 256708477	Chitinase [Leymus chinensis]	27.1/6.71	16.3/5.16	167	15%	[20.1.1001] stress.biotic
	20	gi 156617118	Glyceraldehyde 3-phosphate dehydrogenase [Mallotus paniculatus]	11.0/6.78	41.0/4.04	77	54%	[4.1.9] glycolysis.cytosolic branch. Glyceraldehyde 3-phosphate dehydrogenase
	21	gi 150035719	ATP synthase beta subunit [Bouteloua curtipendula]	35.2/5.52	78.8/5.44	302	62%	[1.1.4.2] PS.lightreaction.ATP synthase.beta subunit
	22	HSP7M_PEA	Heat shock 70 kDa protein, mitochondrial [Pisum sativum]	72.4/5.81	17.5/6.12	69	18%	[20.2.1] stress.abiotic.heat
	23	gi 2624326	Glycine-rich protein 1 [Oryza sativa Japonica Group]	15.9/5.52	11.2/5.63	97	30%	[27.4] RNA.RNA binding
	24	gi 1040909	RuBisCO large subunit, partial (chloroplast) [Monachather paradoxus]	50.1/6.23	63.7/4.05	186	28%	[35.1.1023] not assigned.no ontology
	25	gi 357148187	14-3-3-like protein GF14-A-like [Brachypodium distachyon]	29.0/4.80	31.5/4.91	75	26%	[30.7] signalling.14-3-3 proteins
	26	gi 225431124	Ferredoxin-NADP reductase, leaf isozyme, chloroplastic isoform 2 [Vitis vinifera]	38.8/8.92	39.4/6.76	86	24%	[1.1.5.3] PS.lightreaction.other electron carrier (ox/red).ferredoxin reductase
Group 4	27	gi 357147006	Actin-2-like [Brachypodium distachyon]	41.9/5.31	46.8/5.46	143	43%	[31.1] cell.organisation
	28	gi 297721387	Chloroplast translational elongation factor Tu - contains InterPro domain(s) [Oryza sativa Japonica Group]	50.6/5.68	53.4/5.64	234	33%	[29.2.4] protein.synthesis.elongation
	29	gi 255561530	Adenylate kinase, putative [Ricinus communis]	29.7/6.46	24.6/5.32	102	6%	[23.4.1] nucleotide metabolism.phosphotransfer and pyrophosphatases.adenylate kinase
	30	gi 295153532	NADH dehydrogenase subunit F [Erioneuron nealleyi]	27.7/9.13	28.8/5.66	48	18%	[1.1.6] PS.lightreaction.NADH
	31	gi 162463755	Glutamine synthetase root isozyme 3 [Zea mays]	39.6/5.34	35.2/5.65	129	32%	[12.2.2] N-metabolism.ammonia metabolism. glutamine synthase
	32	gi 20143564	ATPase alpha subunit, 3'-partial [Oryza sativa Japonica Group]	29.4/5.27	47.8/5.96	93	9%	[1.1.4.1] PS.lightreaction.ATP synthase.alpha subunit
	33	gi 338825700	ATP synthase CF1 alpha subunit [Eleusine coracana]	55.7/5.72	67.5/6.08	274	33%	[1.1.4.1] PS.lightreaction.ATP synthase.alpha subunit
	34	gi 290586402	RuBisCO large subunit [Acorus calamus]	26.4/7.78	18.4/5.99	170	32%	[35.2.1001] not assigned.unknown
	35	gi 356532101	Transducin beta-like protein 2-like [Glycine max]	47.6/8.61	16.9/5.50	79	24%	[33.99] development.unspecified
	36	gi 29367475	Fibrillin-like protein [Oryza sativa Japonica Group]	33.9/4.98	38.1/4.76	92	6%	[2.1.2.2] major CHO metabolism.synthesis. starch.starch synthase
	37	gi 2154728	Profilin 2 [Cynodon dactylon]	14.2/4.89	9.49/4.42	24	9%	[31.1] cell.organisation

38	gi418205218	RuBisCO, partial (chloroplast) [Allamanda schottii]	19.0/5.75	14.6/4.81	91	31%	[1.3.1] PS.calvin cycle.rubisco large subunit
39	gi357147242	Chitinase 8-like [Brachypodium distachyon]	27.1/6.71	22.8/4.94	126	23%	[20.1.1001] stress.biotic

No., protein spot number; The., Theoretical value; Exp., Experimental value; Mr, Mr (KD); Cov., sequence coverage. Group 1: Commonly regulated proteins by drought in leaves and stems of Yukon and Tifgreen varieties; Group 2: Differentially regulated proteins in leaves and stems of Yukon and Tifgreen varieties after drought stress; Group 3: Proteins up-regulated by drought stress in leaves or stems of Yukon variety and constitutive expressed in Tifgreen variety; Group 4: Differentially expressed proteins in Yukon and Tifgreen varieties under control and drought conditions.

proteins involved in photosynthesis (PS), glycolysis, N-metabolism, tricarboxylic acid (TCA) cycle and redox were largely enriched, while other proteins involved in cell, stress, lipid metabolism and development were also significantly changed in leaves and stems of Yukon and Tifgreen varieties (Table 2). Additionally, 7 proteins involved in carbon fixation were differentially expressed in Yukon and Tifgreen varieties (Fig. 5A–B), indicating the possible role of carbon metabolism in bermudagrass response to drought stress.

4. Discussion

In recent years, comparative physiological, transcriptomic, proteomic and metabolomic studies are the most important and effective strategies to reveal physiological and molecular mechanisms of plant genotypes with contrasting developmental phenotypes or stress resistance (Mohammadi et al., 2012; Shi et al., 2012, 2013a, 2014; Xu and Huang, 2010; Zhao et al., 2011). In the previous research, we found that the changes of water status, osmolyte accumulation and antioxidant defense system during drought stress might contribute to the natural variation of Tifgreen and Yukon varieties, which were differed in drought resistance (Shi et al., 2012). The physiological analyses of bermudagrass plants revealed that 14 d of drought stress treatment significantly reduced relative leaf water content but increased EL (Shi et al., 2012).

In this study, we successfully identified 39 drought-responsive proteins in both leaves and stems of Tifgreen and Yukon varieties contrasting in drought stress resistance after 14 d of drought stress treatment (Table 1), providing new insights to the molecular mechanisms underlying bermudagrass response to drought stress. Compared with Zhao et al. (2011) that focused on the identification of differentially expressed proteins in bermudagrass leaves from hybrid varieties by water-deficit stress, this study paid special attention to the differentially expressed proteins in common by drought stress and variety variation effects. More importantly, organ-specific proteomic changes in the leaves and stems of bermudagrass under control and drought stress conditions were also compared (Figs. 2–4). Komatsu et al. (2012) have also clarified proteome changes in the root tips, roots, leaves, hypocotyls and young seedlings of flooded soybean plants, which shed new lights into characterization of soybean flooding resistance. Totally 39 drought-responsive proteins were divided into 4 different groups and several proteins functioned directly in carbon metabolism pathway (Table 1). Additionally, 42 identified metabolites including amino acids, organic acids, sugars and sugar alcohols exhibited higher levels in leaves and stems of drought tolerant Tifgreen variety, confirming the common modulation of bermudagrass variety in carbon metabolites.

4.1. Proteins involved in PS and glycolysis

Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) is an important enzyme involved in the first major step of carbon fixation, a process by which atmospheric carbon dioxide is converted by plants to energy-rich molecules such as glucose. In chemical terms, RuBisCO catalyzes the carboxylation of ribulose-1,5-bisphosphate (RuBP), the primary chemical reaction by which inorganic carbon enters the biosphere (Feller et al., 2008; Spreitzer and Salvucci, 2002). Phosphoenolpyruvate carboxylase (PEPC) is an enzyme in the family of carboxy-lyases that catalyzes the addition of bicarbonate to phosphoenolpyruvate (PEP) to form the four-carbon compound oxaloacetate, allowing for a separation of initial carbon fixation by contact with air and secondary carbon fixation into sugars by RuBisCO during the light-independent reactions of photosynthesis (Selwood and Jaffe, 2011). Phosphoribulokinase (PRK) is also an important enzyme in the Calvin cycle for CO₂

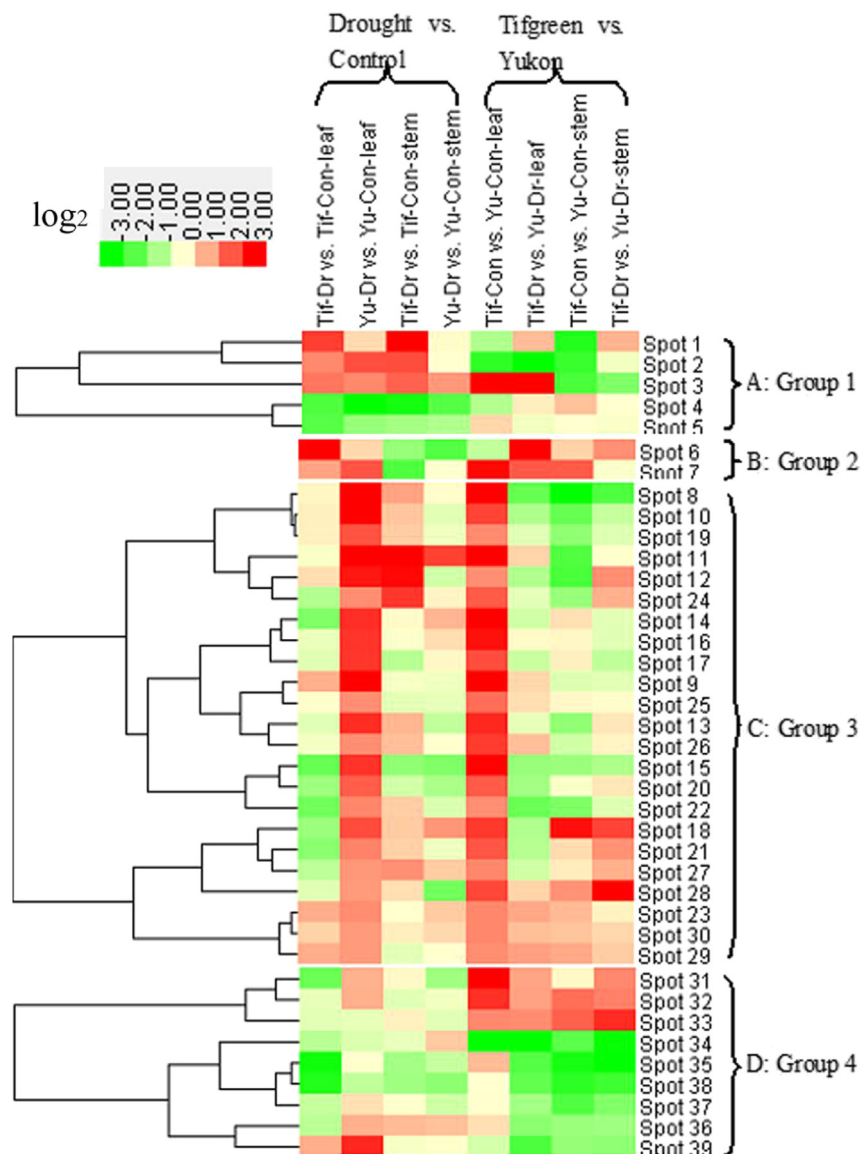


Fig. 4. Hierarchical cluster analysis of drought-responsive proteins in leaves and stems of Yukon and Tifgreen varieties. The resulting tree figure was displayed using the CLUSTER software package and Java Treeview. Yu, Yukon; Tif, Tifgreen; Con, control; Dr, drought.

assimilation, catalyzing phosphorylation of ribulose-5-phosphate to RuBP (Feller et al., 2008; Zhao et al., 2011). Both of RuBisCO and PEPC are essential of photosynthetic efficiency. In this study, six RuBisCO-related proteins (spots 9, 24 and 34, RuBisCO large subunit; spot 17, RuBisCO large subunit-binding protein subunit alpha; spot 18, RuBisCO small subunit protein; spot 38, RuBisCO), two PEPCs (spots 1 and 3) and PRK (spot 14) were commonly modulated by both drought stress effect and variety effect, indicating the involvement of carbon fixation in drought stress response.

Glycolysis is an important metabolic pathway which is involved in carbohydrate metabolism almost present in all living organisms. The central role of glycolysis in plant metabolic pathway is to provide energy such as ATP and generates precursors for anabolism such as fatty acids and amino acids (Plaxton, 1996). In this study, four proteins [spot 1 and 3, PEPC; spot 7, cytosolic 3-phosphoglycerate kinase; spot 20, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] involved in glycolysis metabolism were identified as differentially expressed proteins by drought effect and by variety variation effect in bermudagrass. GAPDH is not only a

typical glycolytic enzyme, but also plays important roles in plant development and stress response (Guo et al., 2012; Zhang et al., 2011). Zhang et al. (2011) showed that *cytosolic GAPDH 3 (GAPC3)* overexpressing enhanced salt stress resistance in rice through the regulation of H_2O_2 levels, and Guo et al. (2012) found *AtGAPC1/2* modulate drought stress resistance through transducing H_2O_2 signal by the interaction between GAPC1/2 and phospholipase D (PLD δ) in Arabidopsis. Therefore, the modulation of these protein abundance (spots 1, 3, and 20) might be directly relative with drought resistance of bermudagrass.

Malate dehydrogenase (MDH) is a cytosolic NAD-dependent enzyme involved in malic acid synthesis. Yao et al. (2011a, b) examined the expression of the apple *cyMDH* gene (*MdcyMDH*) and found that *MdcyMDH* was involved in plant cell growth and resistance to cold and salt stresses. In this study, MDH (spot 15) was induced by drought stress and exhibited higher abundance of protein in the drought tolerant variety (Tifgreen), further indicating that plant MDH involved in not only cold and salt stress resistance but also drought stress response.

Table 2

Pathway enrichment analysis of proteins changed by variety variation effect and drought stress effect.

MapMan pathways	NF	p-value	Protein No.
PS	58.98	0.000	14
Glycolysis	45.83	0.000	4
N-metabolism	33.05	0.029	1
TCA / org. transformation	10.74	0.085	1
Major CHO metabolism	8.42	0.106	1
Redox	8.18	0.023	2
Nucleotide metabolism	4.77	0.171	1
Cell	3.12	0.055	3
Stress	2.10	0.116	3
Lipid metabolism	2.07	0.301	1
Development	1.08	0.372	1
Signalling	0.62	0.327	1
Protein	0.55	0.111	3
RNA	0.27	0.090	1
Not assigned	0.14	0.000	2

NF, normalized frequency of each functional category in genome. Protein No., protein number. Colour scales of NF are as following:

≥4.0	1.0–4.0	≤1.0
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Three proteins (spot 21, ATP synthase beta subunit; spot 32, ATPase alpha subunit; and spot 33, ATP synthase CF1 alpha subunit) are related to ATP synthesis, which is utilized in the light-reactions of PS for carbon assimilation. The higher expression of these ATP-related proteins in Tifgreen variety might improve energy supply such as ATP to protect Tifgreen plants from damage under drought stress condition. The oxygen-evolving complex (OEC) is a water-oxidizing enzyme involved in the photooxidation of water during the light reactions of photosynthesis. The protein abundances of spots 6 and 11 (cytochrome b6-f complex iron–sulfur subunit), spot 10 (oxygen-evolving enhancer protein 1, chloroplastic-like isoform 1) and spot 13 (photosystem II oxygen evolving complex protein PsbP family protein) were affected by both drought stress and variety variation effect.

The chloroplast ferredoxin is a redox protein that involved in both cyclic and non-cyclic photophosphorylation reactions of photosynthesis (Ciurli and Musiani, 2005). In non-cyclic photophosphorylation, ferredoxin is the last electron acceptor and reduces the enzyme NADP + reductase (Ciurli and Musiani, 2005). In this study, the expression of ferredoxin–NADP reductase (spot 26) and NADH dehydrogenase subunit F (spot 30) were induced by drought stress in Yukon variety and constitutive expressed in Tifgreen variety.

4.2. Proteins involved in various metabolic pathways

Superoxide dismutase (SOD) is the first step of enzymatic anti-oxidant defense response via converting superoxide to hydrogen

peroxide (H_2O_2), while dehydroascorbate reductase (DHAR) is a non-enzymatic antioxidant enzyme, reducing dehydroascorbate (DHA) to ascorbate (Asc) in the presence of glutathione (Miller et al., 2011; Suzuki et al., 2011). The differentially regulated proteins of spot 2 (Cu/Zn SOD) and spot 12 (DHAR) indicated the key roles of antioxidants during bermudagrass stress responses, which were consistent with our previous results (Shi et al., 2012).

Glutamine synthetase (GS) is widely known to be involved in assimilation of ammonium into amino acids and other nitrogen-related assimilation previously. Recently, Molina-Rueda et al. (2013) found that alteration in nitrogen metabolism in GS transgenic modulated ROS protection related genes' expression under drought conditions and resulted in drought resistance. Additionally, GS is also a good indicator of drought stress in drought tolerant and sensitive wheat cultivars (Nagy et al., 2013). Thus, the higher level of GS protein (spot 31, GS root isozyme 3) in Tifgreen variety than Yukon variety might be related to the natural variation of drought resistance in bermudagrass.

Additionally, three proteins (spot 8, flavodoxin-like quinone reductase 1; spot 29, adenylate kinase; and spot 36, fibrillin-like protein) that were involved in lipid metabolism (Laskowski et al., 2002), nucleotide metabolism and starch synthase of major CHO metabolism (Table 1), respectively, were affected by both drought stress and variety variation effects in bermudagrass. In summary, metabolic pathways including ROS, N, lipid, nucleotide and starch synthase of major CHO metabolisms might be contributed to drought stress response in bermudagrass.

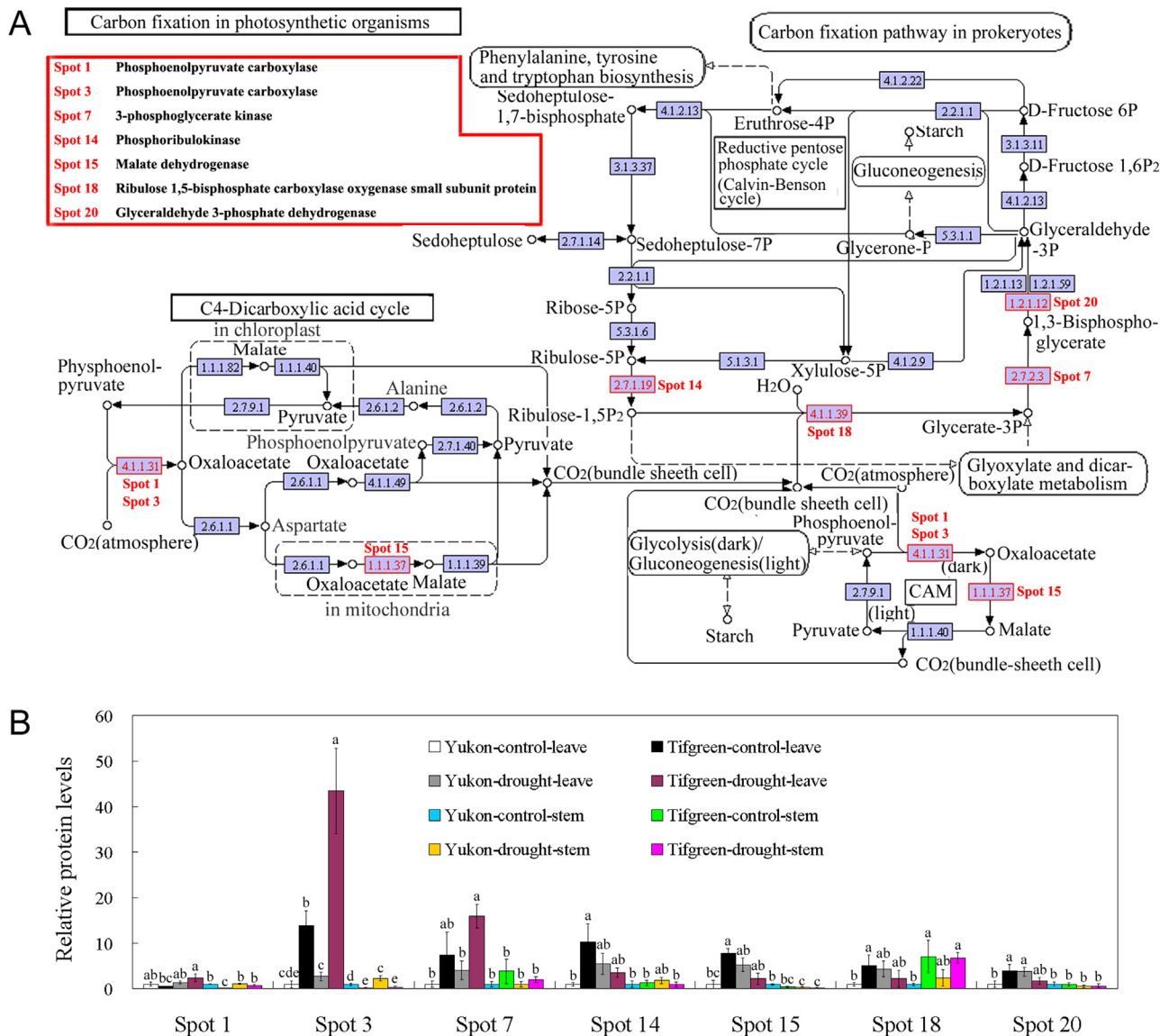


Fig. 5. Commonly regulated proteins that involved in carbon fixation in photosynthetic organisms by variety variation effect and drought stress effect. (A) Seven differentially expressed proteins by variety variation effect involved in carbon fixation in photosynthetic organisms. (B) Relative protein levels of seven differentially expressed proteins involved in the carbon fixation pathway. The abundance of each protein in leaves and stems of Yukon variety under control condition was set as 1.0. The data represent the average of three gels from three independent experiments $\pm$ SDs. Bars with different letters above the columns for protein level indicate significant differences at $p < 0.05$ (Duncan's range test).

4.3. Proteins involved in cell organization and elongation

As a principal component of microfilaments of cytoskeleton, actin is produced during cell elongation and organization, participating in cell division, elongation and cytokinesis, cell organization and signaling, cell shape and cell junctions. Elongation factor Tu is another protein involved in protein synthesis and cell elongation, via promoting aminoacyl-tRNA bind to the A-site of ribosomes during protein biosynthesis (Zhao et al., 2011). In this study, higher abundance of two proteins [spot 27, actin-2-like; spot 28, chloroplast translational elongation factor Tu ~ contains InterPro domain(s)] in Tifgreen variety in relative to Yukon variety might be contributed to the maintenance of cell organization and leaf water content under drought stress conditions. Additionally, abundance changes of spot 5 (beta-actin) by drought stress effect and spot 37 (profiling 2) by variety variation effect showed that these proteins might also play some roles in bermudagrass response to drought

stress. All these results indicated the relation between cell organization and drought stress response.

4.4. Modulation of stress-related proteins

As molecular chaperone, the heat shock protein 70 (HSP70) is responsive to multiple of environment stresses including thermal, oxidative, flooding and drought stresses (Jung et al., 2013; Komatsu et al., 2013). NtHSP70-1 could be induced by dehydration stress, and overexpressing NtHSP70-1 conferred activated thermoprotective activity and improved drought stress resistance in tobacco (Cho and Hong, 2006; Cho and Choi, 2009). In this study, HSP70 (spot 22) was up-regulated by drought stress in Yukon variety and constitutively expressed in Tifgreen variety, suggesting that increased expression of HSP70 might protect plants through quick degradation/reactivation of damaged proteins, thus obtaining enhanced drought resistance (Mohammadi et al., 2012). Cletus et al.

(2013) reviewed both constitutive expression and induced expression of plant chitinases, and described the largely involvement of plant chitinases in disease resistance. Although the *in vivo* role of chitinase in abiotic stress remain unclear, the expression of plant chitinases could be regulated by multiple of abiotic stresses (Cletus et al., 2013; Muoki et al., 2012), including drought stress in this study (spot 19, chitinase; spot 39, chitinase 8-like).

4.5. Changes of other proteins

The 14-3-3 proteins are highly conserved family in all eukaryotic organisms (Porcel et al., 2006; Sun et al., 2011; Takahashi et al., 2007). In plants, 14-3-3 proteins is involve in protein–protein interactions, mediating complex signal transduction pathways such as various metabolism processes, hormone cross-talks, gene transcription and protein modification, and stress response (Porcel et al., 2006; Sun et al., 2011; Takahashi et al., 2007). Recently, Expression of the maize *ZmGF14-6* gene and *OsGF14c* in rice conferred drought resistance (Campo et al., 2012; Ho et al., 2013), indicating the involvement of 14-3-3 protein in drought stress response in plant. In this study, higher protein abundance of spot 25 (14-3-3-like protein GF14-A-like) that was involved in signal transduction might play essential roles in drought stress response. Additionally, other proteins (spot 4, plastid high chlorophyll fluorescence 136 precursor; spot 16, filamentation temperature-sensitive H₂B; spot 23, glycine-rich protein 1; spot 35, transducin beta-like protein 2-like) with unknown assigned functions might also play some roles in bermudagrass response to drought stress.

5. Conclusion

Taken together, this study represents the first proteomic analysis in both leaves and stems of two bermudagrass varieties contrasting in drought stress resistance. Notably, 74% of 39 identified proteins exhibited higher protein abundance in the drought-tolerant variety under control and drought stress conditions, while 62% of 39 identified proteins were induced in the drought-sensitive variety under drought stress and were constitutively expressed in the drought-tolerant variety. These differentially expressed proteins were largely enriched in PS, glycolysis, N-metabolism and redox pathways, indicating the involvement of these proteins in the natural variation of bermudagrass in response to drought stress.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.plaphy.2014.06.006>.

Contributions

H Shi designed and performed the experiments, analyzed the data, and wrote the manuscript; T Ye analyzed the data; Z Chan designed the experiments, and revised the manuscript; and all authors approved the manuscript.

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