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Research article

iTRAQ-based proteomics reveals key role of γ-aminobutyric acid (GABA) in regulating drought tolerance in perennial creeping bentgrass (*Agrostis stolonifera*)

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ABSTRACT

γ-Aminobutyric acid (GABA), a non-proteinaceous amino acid, modulates plant growth and stress tolerance. However, the potential role of GABA in regulating key metabolic pathways and stress-defensive proteins against drought in plants has never been explored. Creeping bentgrass (*Agrostis stolonifera*) plants were pretreated with or without GABA and then subjected to water stress for 8 days in controlled growth chambers (23/19 °C, day/night). Physiological analysis showed that elevated endogenous GABA level via exogenous GABA application significantly mitigated water stress damage to creeping bentgrass, as manifested by increased leaf relative water content, water use efficiency, osmotic adjustment (OA), photochemical efficiency (Fv/Fm), net photosynthetic rate, and reduced oxidative damage. iTRAQ-based proteomics found that enhanced chaperones accumulation, carbohydrates, amino acids, and energy metabolism played important roles in protein protection, OA, energy maintenance, and metabolic balance, which is important adaptive response to drought stress in creeping bentgrass. The GABA further promoted energy production and conversion, antioxidant defense, and DHN3 accumulation that were essential for energy requirement, ROS-scavenging, and the prevention of cell dehydration in leaf during drought stress. In addition, GABA-treated plants maintained significantly higher abundance of dicarboxylate transporter 2.1, ATP-dependent zinc metalloprotease, receptor-like protein kinase HERK1, o-acyltransferase WSD1, omega-6 fatty acid desaturase, and two-component response regulator ORR21 than untreated plants under drought stress. The result provides new evidences that GABA-induced drought tolerance is possibly involved in the improvement of nitrogen recycling, protection of photosystem II, mitigation of drought-depressed cell elongation, wax biosynthesis, fatty acid desaturase, and delaying leaf senescence in creeping bentgrass.

1. Introduction

Owing to global climate change and decline in available irrigation water, drought in conjunction with coincident heat stress poses serious constraints to crop yield and plant survival worldwide. As one of natural hazards, drought-induced loss in cash crops will further expand in many countries and regions (Chaves et al., 2003). Better understanding of drought tolerance in different plants is essential for crop production and management. Creeping bentgrass, an important C3 cool-season perennial turfgrass, is widely used on urban greening, landscaping, and golf course putting green due to its fine leaf texture, rapid extension of lateral shoots, and excellent tolerance to low mowing (Fry and Huang, 2004). However, limited water supply reduces turfgrass quality. More importantly, the frequently occurring drought causes a severe economic loss during turfgrass production and management. Various strategies have been proposed to improve drought tolerance of creeping bentgrass including management practice, transgenic technique, and breeding effort. For examples, an increase in endogenous cytokinin (CTK) content in transgenic creeping bentgrass with an *ipt* could significantly promote drought tolerance (Merewitz et al., 2012). In response to drought stress, creeping bentgrass maintained better tiller and stolon growth and leaf hydration in elevated carbon dioxide (CO_2) environment (Burgess et al., 2019). The study of Zhang et al. (2018) found that the cultivar 'Independence' and 'Crystal Bluelinks' had stronger drought tolerance among 23 commercially available creeping bentgrass cultivars. These two cultivars may be more adapted to arid and semi-arid regions.

The application of exogenous plant growth regulators (PGRs) is an

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important management strategy to overcome drought damage in plants. Foliar application of spermidine (Spd), glycinebetaine, abscisic acid (ABA), salicylic acid (SA), and CTK could effectively improve drought tolerance of creeping bentgrass (Gan et al., 2018; Chang et al., 2016; Li et al., 2015b, 2017). γ-Aminobutyric acid (GABA), a four carbon non-proteinaceous amino acid, acts as a new PGR for regulating cell pH, growth, carbon and nitrogen balance, signal transduction, and stress response in plant species (Michaeli and Fromm, 2015; Kinnersley and Turano, 2000; Bouche and Fromm, 2004). Plants normally maintain GABA at a relative low level, but abiotic stresses such as drought, heat, salinity, and anoxic stress induce significant increase in endogenous GABA level (Xing et al., 2007; Li et al., 2016b; Serraj et al., 1998; Mei et al., 2016). Recent studies found that elevated endogenous GABA content by exogenous GABA application could obviously enhance plant tolerance to various abiotic stresses. For examples, endogenous GABA level was improved by root feeding with GABA, which helped to alleviate heat damage through inducing physiological, transcriptional, and post-transcriptional changes in creeping bentgrass (Li et al., 2019). Exogenous GABA effectively improve salt tolerance of maize (*Zea mays*) associated with increases in endogenous GABA, proline, and soluble sugar accumulation in leaves (Wang et al., 2017). In contrast, GABA-depleted *Arabidopsis gad1/2* mutant was oversensitive to salt stress than wild type (Mekonnen, 2017). These findings indicate that GABA plays a positive role in regulating tolerance to abiotic stress in plants.

Facing with scarcity of water resource, plants display a range of morphologic, physiological, and biochemical mechanisms to conquer drought stress. In past ten years, omics such as genomics, transcriptomics, proteomics, and metabolomics provide faster and comprehensive progress in stress biology research (Thatcher et al., 2016; Li et al., 2019; Kosová et al., 2019). Based on proteome, the study of Shi et al. (2013) found that exogenous polyamine increased the accumulation of many proteins involved in electron transport, energy pathways, and antioxidant defense contributing to improved drought tolerance in bermudagrass (*Cynodon dactylon*); SA induced systemic drought tolerance in soybean (*Glycine max*) through enhancing the abundance of proteins in relation to carbon metabolism, redox balance and protein synthesis, and amino acids metabolism (Sharma et al., 2018); exogenous ABA mitigated drought damage associated with increases in the abundance of proteins that had roles in protein transport, carbon metabolism, and stress defense such as heat shock protein (HSP) in tea (*Camellia sinensis*) leaves (Zhou et al., 2014). Proteomics provides an effective approach for analyze PGRs-induced acclimation or adaptation to water deficit in different plant species. However, the potential role of GABA in regulating key metabolic pathways and stress-defensive proteins against drought stress in plants has never been explored.

At present, a large number of proteins have been identified in plants in relation to acclimation or adaptation to drought stress. Our previous study has found that foliage applied GABA could significantly enhance drought tolerance of creeping bentgrass associated with elevated accumulation of amino acids and secondary metabolites based on metabolomics analysis (Li et al., 2016a). However, it is still far from better understanding of GABA-regulated mechanism when plants respond to water deficit. The objective of this study is to identify key proteins and associated metabolic pathways regulated by drought stress and GABA in leaves of creeping bentgrass. Current study attempts to reveal adaptive response to drought stress and regulatory effects of GABA in response to water deficit in perennial non-model creeping bentgrass.

2. Materials and methods

2.1. Plant material and treatments

Seeds (creeping bentgrass, cv. Penncross, and 4 g/m^2) were sown in pots (25 cm length, 15 cm width, and 10 cm height) filled with distilled water and sterilized quartz sand. Seeds germinated in growth chambers (23/19 °C (day/night), 65% relative humidity, and 750 μmol m^{-2}·s^{-1} PAR) for 10 days. Seedlings were then irrigated with Hoagland's solution (Hoagland and Arnon, 1950) for another 20 days. Thirty-day-old plants were pretreated with or without 0.5 mM GABA solution for 3 days. For drought treatment, normal Hoagland's solution and GABA solution were replaced by polyethylene glycol (PEG) 6000 (−0.3 MPa) solution. The GABA or PEG was dissolved in Hoagland's solution. Three different treatments were set: 1) C, control (plants well grew in Hoagland's solution for 41 days); 2) DS, drought stress (plants well grew in Hoagland's solution for 33 days and then were subjected to drought stress for 8 days); and 3) DSG, drought stress + GABA (plants well grew in Hoagland's solution for 30 days, and were pretreated with GABA for 3 days, and then were subjected to drought stress for 8 days). The effective dose of GABA was chosen based on preliminary experiments. Hoagland's, GABA, and PEG solutions were replaced every day to avoid change of concentration. All treatments were completely arranged in growth chambers. Four biologic replicates for each treatment were used for the analysis of physiological parameters and three biologic replicates for each treatment were used for iTRAQ-based proteomics.

2.2. Measurements of physiological parameters

For leaf relative water content (RWC), fresh leaves were collected from plants and immediately weighed to get fresh weigh (FW). Fresh leaves were then immerged in distilled water for 12 h to get turgid weight (TW). Turgid leaves dried at 105 °C for 30 min and 80 °C for 72 h to obtain dry weight (DW). The RWC was calculated based on RWC (%) = [(FW -DW)/(TW-DW)] × 100 (Barrs, 1962). For the determination of osmotic potential (OP), fresh leaf tissues were immersed in deionized water at 4 °C for 8 h to fully hydrate fresh leaves. Fully hydrate tissues were frozen in liquid nitrogen for further analysis. After thawing in an ice bath, a small hole was used to grind leaves to extract the leaf juice, and 10 ml of sap was inserted into the osmometer (Wescor, Logan, UT). The osmotic potential was converted based on MPa = $-c \times 2.58 \times 10^{-3}$. OA was calculated as the difference in OP between stressed leaves and well-watered control leaves (Blum, 1989). Leaf electrolyte leakage (EL) was calculated based on EL (%) = $C_{initial}/C_{max} \times 100$. Fresh leaves (0.1 g) were immediately immerged in 15 ml of distilled water for 12 h and solution conductance was detected as initial conductivity ($C_{initial}$). Leaves were autoclaved at 100 °C for 30 min and tissues conductivity was measured as maximum conductance (C_{max}) using a conductivity meter (model 32; Yellow Springs Instrument Co., Yellow Spring, OH) (Blum and Ebercon, 1981). The determination of malondialdehyde (MDA) content was performed according to the method of (Dhindsa et al., 1981). The method of euzymelinked immunosorbent assay was used for measuring endogenous GABA content using an Assay Kit (Shanghai euzymelinked Biotechnology Co., Ltd., China) according to manufacturer's instructions. For photochemical efficiency (Fv/Fm), leaves were placed in dark for 30 min and Fv/Fm was recorded by using a Chl fluorescence system (Pocket PEA, Hansatech, the United Kingdom). Net photosynthetic rate (Pn) and instantaneous water use efficiency (WUE) were estimated using a portable photosynthetic system (CIRAS-3, PP Systems, USA). For Pn, WUE, and Chl fluorescence, 15 individual leaves per replicate per treatment were used.

2.3. Protein extraction, iTRAQ labeling, and identification

Leaf samples were ground in liquid nitrogen to fine power. The powder of each sample (150 mg) was mixed with 1 ml of lysis buffer (Tris-base (pH 8), 8 M urea, 1% sodium dodecyl sulfate (SDS), complete protease inhibitor cocktail (Sigma-Aldrich, P9599). After being shaken and incubated on ice for 20 min, the homogenate centrifuged at 12000 g for 15 min at 4 °C. The 4 vol cold acetone containing 10 mM DL-Dithiothreitol (DTT) was added in the extract and placed at −20 °C for 2 h. After centrifuged at 4 °C, pellets were washed twice with cold acetone and then dissolved in 600 μL of buffer containing Tris-base (pH

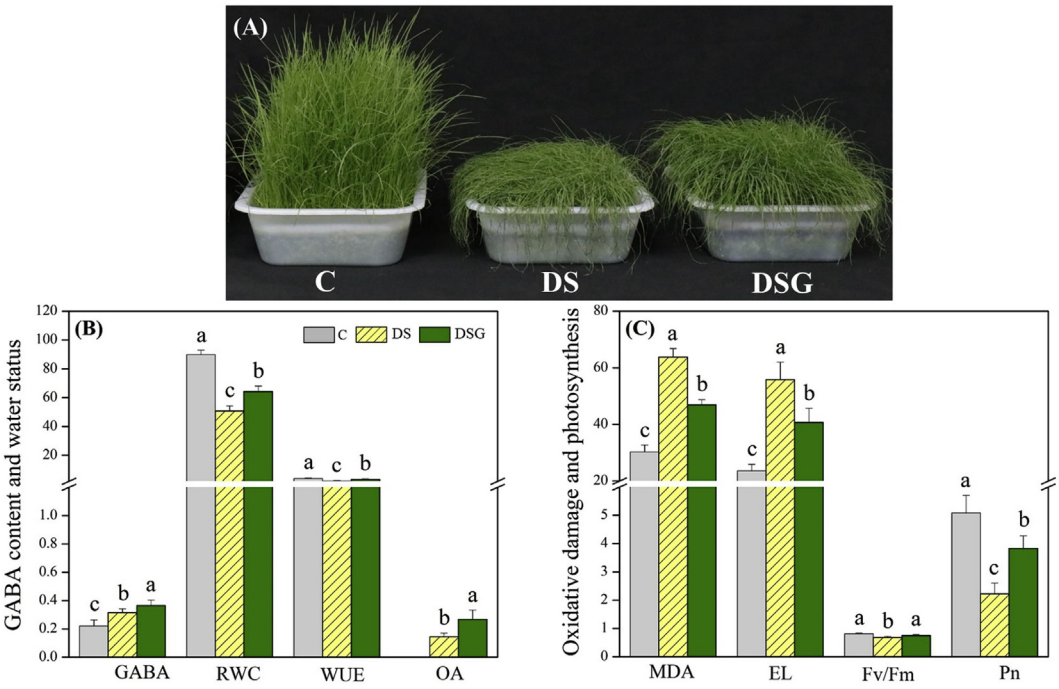

Fig. 1. Effects of drought and exongenous γ-aminobutyric acid (GABA) on endogenous GABA content and water status (GABA content (μmol g^{-1} DW); relative water content, RWC (%); water use efficiency, WUE (μmol CO_2 mmol H_2O^{-1}); osmotic adjustment, OA (MPa)) and (B) oxidative damage and photosynthesis (malondialdehyde, MDA (nmol g-1 DW); electrolyte leakage, EL (%); photochemical efficiency, Fv/Fm; net photosynthesis rate, Pn (μmol CO_2 m^{-2} s^{-1})) in leaf of creeping bentgrass. Vertical bars indicate ± SE of mean (n = 4) and different letters above columns indicate significant differences ($P \leq 0.05$). C, control (normal condition); DS, drought stress; DSG, drought-stressed plants pretreated with GABA.

8) and 8 M urea. The protein concentration was detected by the method of Bradford (2015). For reduction and iodoacetamide alkylation, proteins (precisely 0.1 mg) reduction was done by using 5 mM DTT for 1 h at 60 °C and subsequently subjected to alkylation by using 10 mM iodoacetamide for 45 min at 25 °C in the dark. Proteins of each sample were digested with Trypsin Gold (Promega, Madison, WI) containing 1 μL of 0.3% trypsin and 500 μL of 100 mM triethylammonium bicarbonate (TEAB) at 37 °C for 16 h. After trypsin digestion, peptide was desalted with C18 cartridge to remove the high urea. Desalted peptides were labeled with iTRAQ reagents (iTRAQ® Reagent-8PLEX Multiplex Kit, Sigma), following the manufacturer's instructions (AB Sciex, Foster City, CA). Briefly, the 0.1 mg of peptides was mixed with 1 unit of labeling reagent. Peptides were dissolved in 20 μL of 0.5 M TEAB (pH 8.5), and the labeling reagent was added to 70 μL of isopropanol. The reaction was stopped with 50 mM Tris/HCl (pH 7.5) after incubation for 1 h. Differently labeled peptides were mixed equally and then desalted in 100 mg SCX columns (strata-x-c, Phenomenex: 8B–S029-EBJ). Samples were labeled with the iTRAQ tags as follows: for run 1, sample "C1" (115 tag), sample "C2" (116 tag), and sample "C3" (117 tag); for run 2, sample "DS1" (115 tag), sample"DS2" (116 tag), sample "DS3" (117 tag), sample "DSG1" (118 tag), sample"DSG2" (119 tag), and sample "DSG3" (121 tag). A 600 μg iTRAQ-labeled peptide mix was fractionated by using a C18 column (waters BEH C18 4.6 × 250 mm, 5 μm) on a Rigol L3000 HPLC operating at 1 ml/min. Eluent was collected every minute and then merged to 15 fractions. The samples were dried under vacuum and reconstituted in 20 μL of 0.1% (v/v) FA and 3% (v/v) acetonitrile (ACN) in water for subsequent analyses.

Fractions were dissolved in loading buffer (0.1% formic acid (FA) and 3% ACN) and separated by a C18 column (150 μm inner-diameter, 360 μm outer-diameter × 15 cm, 1.9 μm C18, Reprosil-AQ Pur, Dr. Maisch) for LC-MS/MS analysis. Mobile phase A consisted of 0.1% FA in water solution, and mobile phase B consisted of 0.1% FA in 80% ACN solution; a series of adjusted 60 min gradients (0 min: 95% mobile phase A and 5% B; 2 min: 90% A and 10% B; 51 min: 70% A and 30% B; 53 min: 50% A and 50% B; 55 min: 10% A and 90% B; 60 min: 10% A and 90% B) according to the hydrophobicity of fractions eluted in 1D LC with a flow rate of 300 nL/min was applied. Q-Exactive HF-X mass spectrometer was operated in positive polarity mode with capillary temperature of 320 °C. Full MS scan resolution was set to 60000 with AGC target value of 3e6 for a scan range of 350–1500 m/z. A data-dependent top 40 method was operated. HCD spectra was obtained at 15000 MS2 resolution with AGC target of 1e5 and maximum IT of 45 m s, 1.6 m/z isolation window, and NCE of 30, dynamically excluded of 60s. The resulting spectra from each fraction were searched separately by the search engines: Proteome Discoverer 2.2 (PD 2.2, Thermo). The searched parameters as follows: A mass tolerance of 10 ppm for precursor ion scans and a mass tolerance of 0.02 Da for the product ion scans were used. Carbamidomethyl was specified in PD 2.2 as fixed modifications. Oxidation of methionine, acetylation of the N-terminus and iTRAQ 8-plex of tyrosine, and lysine were specified in PD 2.2 as variable modifications. A maximum of 2 miscleavage sites was allowed.

For protein identification, protein with at least 1 unique peptide was identified at false discovery rate (FDR) less than 1.0% on peptide and protein level, respectively. Reporter Quantification (iTRAQ 8-plex) was used for iTRAQ quantification. Results of protein quantitation were statistically analyzed by Mann-Whitney Test. When the ratios were $|FC| \geq 1.2$ or ≤ 0.83 (fold change, FC) and $p < 0.05$, identified proteins were screened as the differentially expressed proteins (DEPs). Gene Ontology (GO) and InterPro (IPR) analysis were conducted using the interproscan-5 program against the non-redundant protein database (including Pfam, PRINTS, ProDom, SMART, ProSiteProfiles, PANTHER) (Jones et al., 2014), and the databases COG (Clusters of Orthologous Groups) and KEGG (Kyoto Encyclopedia of Genes and Genomes) were used to analyze the protein family and pathway. The probable interacting partners were predicted using the STRING-db server (http://string.embl.de/) based on the related species (Andrea et al., 2013). The enrichment pipeline was used to perform the enrichment analysis of

GO, IPR, COG and KEGG, respectively (Wei et al., 2009).

2.4. Statistical analysis

The General Linear Model procedure of SAS (version 9.1; SAS Institute, Cary, NC) was used to determine the significance for physiological parameters. The significance of differences among treatments were tested using the least significance test with $P \leq 0.05$.

3. Results

3.1. Effects of drought stress and exogenous GABA on physiological changes in leaf

Phenotypic changes showed GABA-treated creeping bentgrass growth better than untreated plants under drought stress (Fig. 1A). Physiological analyses found that drought stress significantly increased endogenous GABA content in leaves. Exogenous application of GABA further improved stress-induced GABA accumulation in leaves of creeping bentgrass (Fig. 1B). Leaf RWC and WUE obviously decreased after 8 days of drought stress. GABA-treated plants maintained 27%, 49%, and 87% increases in RWC, WUE, and OA as compared to untreated plants under drought stress (Fig. 1B). MDA content and EL remarkably increased when creeping bentgrass suffered from drought stress, but GABA-treated plants exhibited 26% and 27% lower MDA content and EL in leaves than untreated plants in response to drought stress, respectively (Fig. 1C). Drought stress only significantly inhibited Fv/Fm in leaves of untreated plants, and significantly decreased Pn of both GABA-treated and untreated plants. The 11% and 73% increases in Fv/Fm and Pn were observed in GABA-treated plants than that in untreated plants in response to drought stress (Fig. 1C).

3.2. Effects of drought stress and GABA on protein profile in leaf

More than 5000 proteins were identified. Total spectra, peptide, protein numbers, peptide length distribution, unique peptide distribution, and protein mass distribution were recorded in Fig. S1. For GO analysis of all identified proteins, the majority of proteins were located in nucleus, integral component of membrane, intracellular, cytoplasm, ribosome, and membrane (Fig. S2). Most of proteins had catalytic activity, protein kinase activity, oxidoreductase activity, nucleic acid binding, protein binding, and ATP binding function. More than half proteins were involved in intracellular protein transport, translation, carbohydrate metabolic process, proteolysis, protein phosphorylation, metabolic process, and oxidation-reduction process (Fig. S2). For COGs analysis, these identified proteins were mainly involved in signal transduction mechanism, posttranslational modification, protein turnover, chaperones, carbohydrate transport and metabolism, amino acid transport and metabolism, translation, ribosomal structure and biogenesis, and energy production and conversion (Fig. S3). The KEGG analysis found that all identified proteins were involved in different cellular processes, metabolism, and organismal systems (Fig. S4). For the analysis of DEPs, exogenous application of GABA changed the expression pattern of DEPs, as demonstrated by the difference in a clustering analysis of DEPs between DS vs. C and DSG vs. C (Fig. 2A). The 489, 375, and 31 increased DEPs were identified in WC vs. C, DSG vs. C., and DSG vs. DS, respectively. There were 237, 204, and 27 decreased DEPs in DS vs. C, DSG vs. C., and DSG vs. DS, respectively (Fig. 2B). Only 5 DEPs overlapped among WC vs. C, DSG vs. C. and DSG vs. DS; a total of 243, 98, or 43 DEPs were independently found in WC vs. C, DSG vs. C., or DSG vs. DS, respectively; DS vs. C and DSG vs. C had 472 common DEPs (Fig. 2C).

3.3. Function annotation and enrichment analysis of DPGs in leaf

The GO analysis of DEPs found that most of DEPs were involved in oxidation-reduction process, response to cadmium ion, proteolysis, metabolic process, protein folding, and glycolytic process in DS vs. C and DSG vs. C (Fig. 3). The majority of DEPs in DSG vs. DS were related to oxidation-reduction process, proteolysis, response to stress, response to oxidative stress, translation, regulation of transcription, DNA-templated, and protein phosphorylation (Fig. 3). A large number of DEPs were located in plasma membrane, mitochondrion, chloroplast, and cytoplasmic vesicle in DS vs. C, DSG. Vs. C, and DSG vs. DS. Most DEPs had the function of ATP binding, metal ion binding, and zinc ion binding in DS vs. C, DSG. Vs. C, and DSG vs. DS (Fig. 3). In spite of GABA application, drought stress (DS vs. C and DSG vs. C) regulated many DEPs associated with biosynthesis of amino acids, carbon metabolism, phenylpropanoid biosynthesis, pyruvate metabolism, and ascorbate and aldarate metabolism in leaves (Fig. 4). Drought stress without exogenous GABA (DS vs. C) application induced changes of glycolysis/gluconeogenesis and pyruvate metabolism, and drought stress along with GABA application (DSG vs. C) affected ubiquinone and other terpenoid-quinone biosynthesis and linoleic acid metabolism (Fig. 4). According to KEGG analysis, drought stress with or without GABA application induced changes in most of DEPs related to post-translational modification, protein turnover, chaperones, carbohydrate transport and metabolism, amino acid transport and metabolism, energy production and conversion, and translation, ribosomal structure and biogenesis (Fig. 5). WGS vs. C exhibited more DEPs involved in secondary metabolites biosynthesis, transport and catabolism and cell wall/membrane/envelope biogenesis than DS vs. C (Fig. 5).

3.4. Effects of drought stress and GABA on stress-related key DEPs and interaction network of DEPs

Fig. 6 showed the interaction network of DEPs in DSG vs. C. This network displayed only known and direct interactions among identified DEPs. A total of 61 DEPs was involved in this interaction network containing 11 decreased DEPs (MIPS2, PSAF, LIL3:1, PHT2;1, G6PD4, PIP2-7, ARASP, AAO1, LIN2, RPS18C, and PL18a) and 50 increased DEPs (TPS9, SPS1F, UGP1, PPC1, SUS4, GBSS1b, PIG3, PFK2, GAPC1, OST1, PTI1, P5CS1, CAT2, ALDH12A1, GAD, IGPS, Hsc70-1, Hsp70-2, ALDH10A9, CAT1, ECA4, ZBD, HCAR, SRX, CDC5, P5CR, ATP synthase, TPR, ALDH10A8, NFS1, EFTu/EF1A, APX1, HSP70, ABCF3, ABCF1, LEA1, ACD2, Cu/ZnSOD, DHAR2, GSTU19, ETFBETA, HSP23.2, HSP17.9, HSP21, ECH2, GST3, GSTL2, HMA6, LEA14, and LEA31.). Most of DEPs in this interaction network were directly related to stress tolerance in plants and full names of these DEPs were recorded in Table S1. 14 key increased DEPs associated with stress tolerance were identified in DSG vs. DS in leaves (calvin cycle protein CP12-1, ATP-citrate synthase, dicarboxylate transporter 2.1, omega-6 fatty acid desaturase, salutaridine reductase, peroxidase 2, peroxidase 73, glutathione S-transferase 1, glutathione S-transferase 6, dehydrin 3, ATP-dependent zinc metalloprotease FTSH7, tow-component response regulator ORR21, Receptor-like protein kinase HERK1, and o-acyltransferase WSD1) (Fig. 7).

4. Discussion

The first and foremost effect of drought is the limitation of water supply from root to leaf resulting in plant wilting. It has been widely documented that the improvement of OA and the maintenance of higher WUE are important strategies for plants to overcome water deficit (Chaves et al., 2003). Previous studies found that foliage applied GABA improved resistance against terminal drought associated with significant increase in WUE in leaves of wheat (*Triticum aestivum*) (Farooq et al., 2017). The GABA priming could significantly mitigate drought-caused water loss through quickly reducing cell OP during drought stress (Vijayakumari and Puthur, 2016). In this study, drought stress significantly decreased leaf RWC and WUE, but creeping bentgrass could enhance OA to improve adaptability. More importantly,

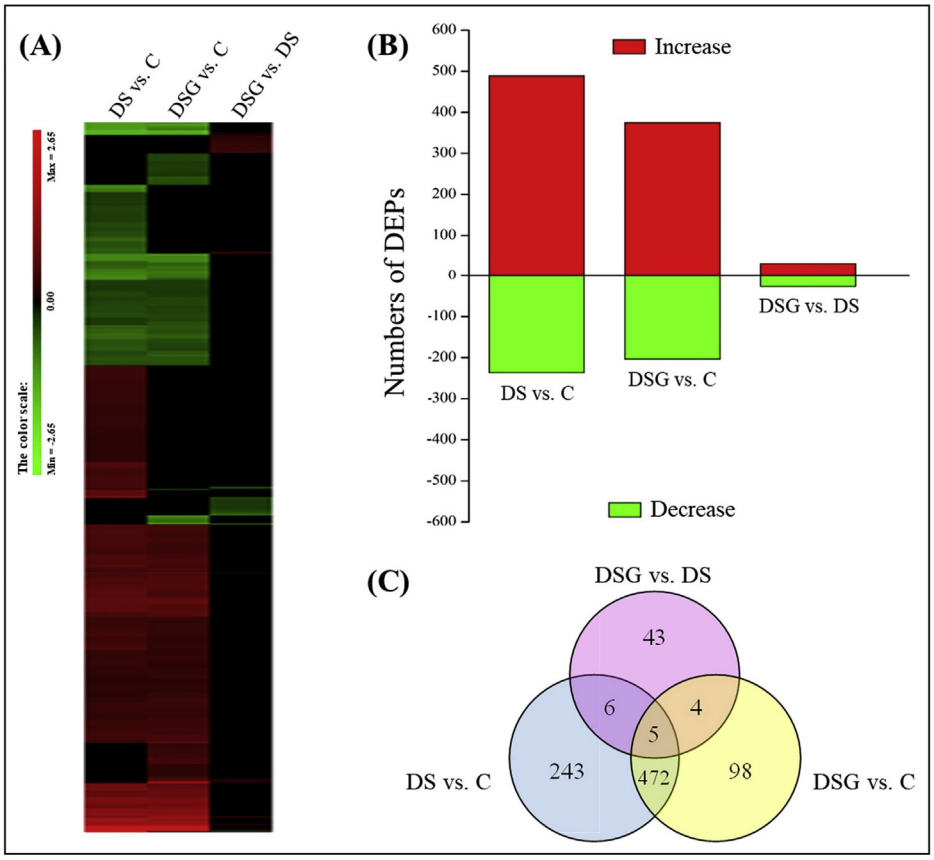

Fig. 2. (A) a clustering analysis of differentially expressed proteins (DEPs), (B) numbers of DEPs, and (C) venn diagram of DEPs. C, control (normal condition); DS, drought stress; DSG, drought-stressed plants pretreated with GABA.

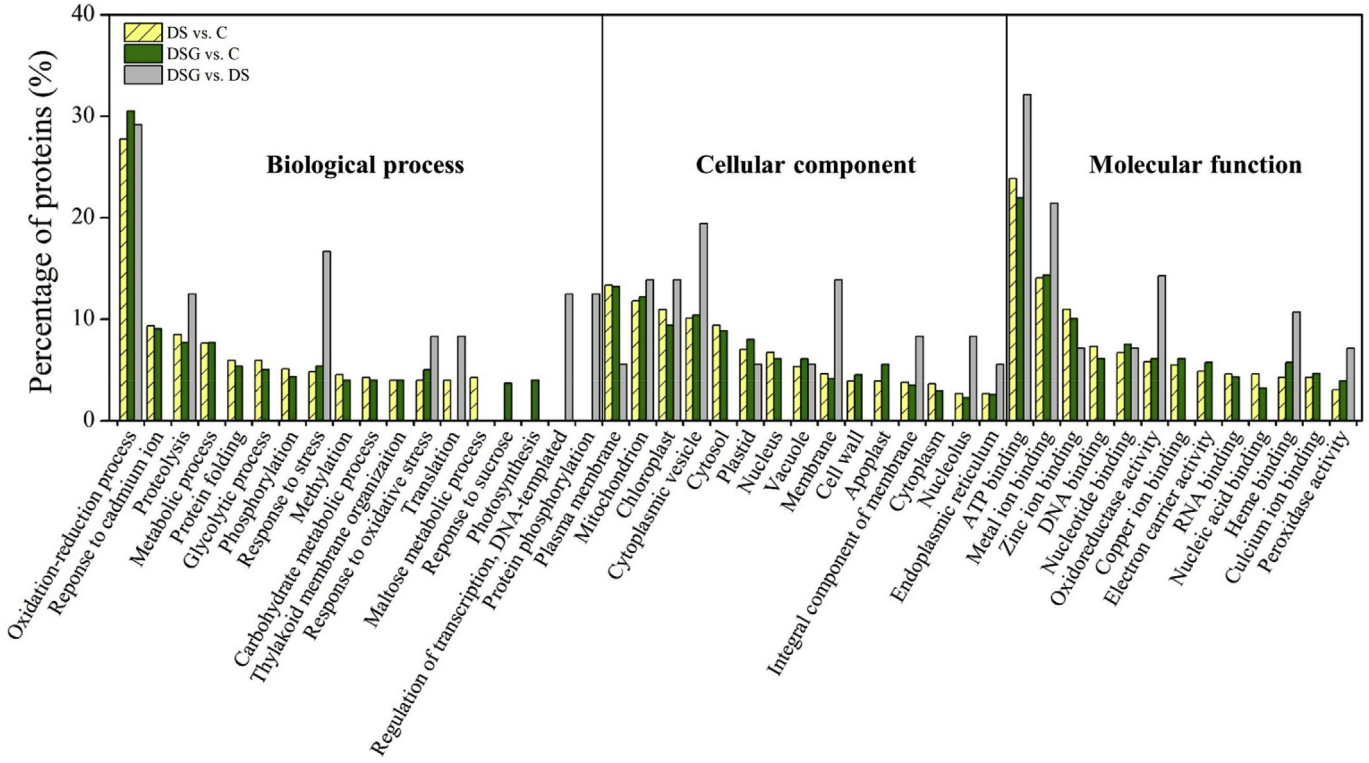

Fig. 3. Gene Ontology (GO) analysis of differentially expressed proteins (DEPs) in leaf of creeping bentgrass in response to drought stress. C, control (normal condition); DS, drought stress; DSG, drought-stressed plants pretreated with GABA.

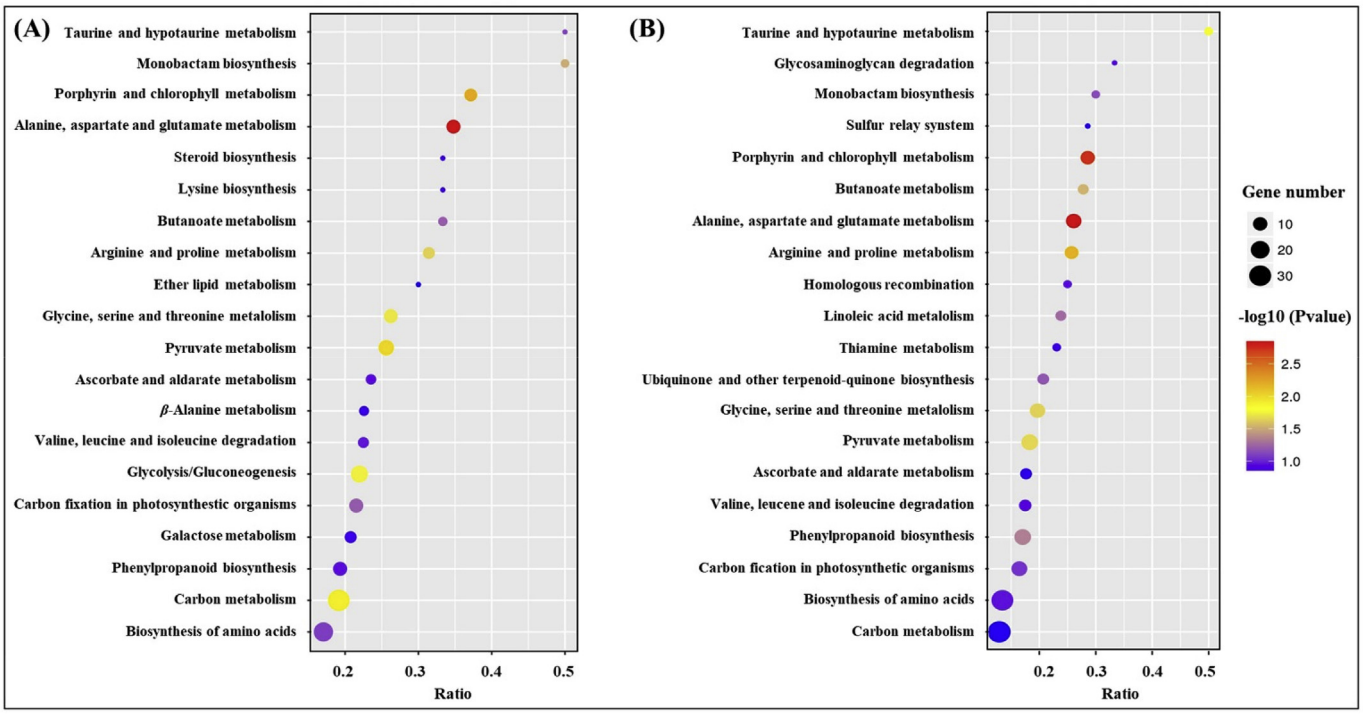

Fig. 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of identified differentially expressed proteins (DEPs) in leaf of creeping bentgrass in response to drought stress. (A) DS vs. C and (B) DSG vs. C. C, control (normal condition); DS, drought stress; DSG, drought-stressed plants pretreated with GABA. The ratio on X axis means the ratio of the number of identified DEPs in this study to total number of proteins in this pathway.

further increase in endogenous GABA content by exogenous GABA pretreatment obviously alleviated drought-induced declines in leaf RWC and WUE. The GABA application also further improved OA in leaf. These findings indicated that GABA played a positive role in regulating water balance under drought stress. In addition, drought stress caused cell membrane lipid peroxidation and decreased membrane stability, photochemical efficiency, and Pn in leaf of creeping bentgrass. The root feeding with GABA effectively alleviated these negative effects when creeping bentgrass were exposed to water limited environment. Earlier study also proved that the decrease in oxidative damage and increase in photosynthesis were closely related to GABA-induced tolerance to high temperature, chilling, and high salinity in plants (Li et al., 2016b; Aghdam et al., 2016; Wang et al., 2017). Key metabolic pathways and important stress-responsive proteins associated with adaptive responses to drought stress and GABA-induced drought tolerance in creeping bentgrass would be further discussed below based on proteome.

Regulation and alteration of metabolic pathways are important adaptive responses in plants. In this study, carbon metabolism, amino acids transport and metabolism, energy production and conversion, and posttranslational medication, protein turnover, chaperones were affected most by drought stress in leaf of both GABA-treated and untreated creeping bengrass. Previous studies have proved that enhanced carbohydrates, amino acids, and energy metabolism improved acclimation to drought stress in grass species including creeping bentgrass (Li et al., 2016c; Merewitz et al., 2011; Shi et al., 2013). Meng et al. (2019) found that jasmonates (JAs) mediated many important metabolic processes such as photosynthesis, redox, and amino acid metabolism contributing to improved drought tolerance in *Arabidopsis*. The GABA-pretreated creeping bentgrass increased the abundance of alpha-trehalose-phosphate synthase 9 (TPS9), sucrose-phosphate synthase 3 (SPS3), sucrose synthase 4 (SUS4), glucose-6-phosphate 1-dehydrogenase 4 (G6PD4), delta-1-pyrroline-5-carboxylate synthase 1 (P5CS1), pyrroline-5-carboxylate reductase (P5CR), glutamate decarboxylase (GAD), and betaine aldehyde dehydrogenase (ALDH) involved in biosynthesis and metabolism of trehalose, sucrose, glucose, proline, γ-aminobutyric acid (GABA), and betaine under drought stress. These compatible osmolytes exhibited multiple positive function of OA, osmoprotection, antioxidant, and intermediates for metabolic balance when plants respond to drought stress (Ali and Ashraf, 2011; Ashraf and Foolad, 2007; Rosa et al., 2009; Ramesh et al., 2017). More importantly, GABA-treated creeping bentgrass accumulated significantly higher calvin cycle protein CP12 and ATP-citrate synthase than untreated plants under drought stress. Both of these two enzymes participate in energy production and metabolism in plants (Groben et al., 2010; Peng et al., 2015). In addition, many HSPs including HSP70-1, HSP70-2, HSP17.9, HSP21, and HSP23.2 significantly accumulated in GABA-treated creeping bentgrass in response to drought stress. As important molecular chaperones, HSPs perform the function of stabilizing proteins and preventing the aggregation of denatured proteins in plants under stressful conditions (Wang et al., 2004). It has been demonstrated that peanut wild relatives (*Arachis* spp.) increased the accumulation of HSP17.3 and HSP70 in response to drought stress (Carmo et al., 2019). The study of Katam et al. (2016) found that HSP70 was only highly abundant in leaf of drought-tolerant peanut (*Arachis hypogaea*) cultivar 'Vemana', but significantly decreased in sensitive cultivar 'Florunner' under drought stress (Katam et al., 2016). Significant increase in HSP70 abundance contributed to ABA-induced drought tolerance in tea (*Camellia sinensis*) plants (Zhou et al., 2014). Current study demonstrates that GABA pretreatment mitigates drought stress damage associated with the enhancement of carbon and amino acids metabolism, energy production and conversion, chaperones that are essential for OA, energy maintenance, protein protection, and metabolic balance in creeping bentgrass.

Antioxidant metabolism is a basic adaptive response to abiotic stress and of primary importance for plants to conquer adverse environments (Caverzan et al., 2016). In this study, drought stress induced the accumulation of many antioxidant enzymes such as superoxide dismutase [Cu–Zn] (Cu/ZnSOD), catalase 1 (CAT1), CAT2, ascorbate peroxidase 1 (APX1), glutathione S-transferase (GST), GST2, GST3, and GSTL2 in GABA-treated creeping bentgrass. Moreover, GABA-treated creeping

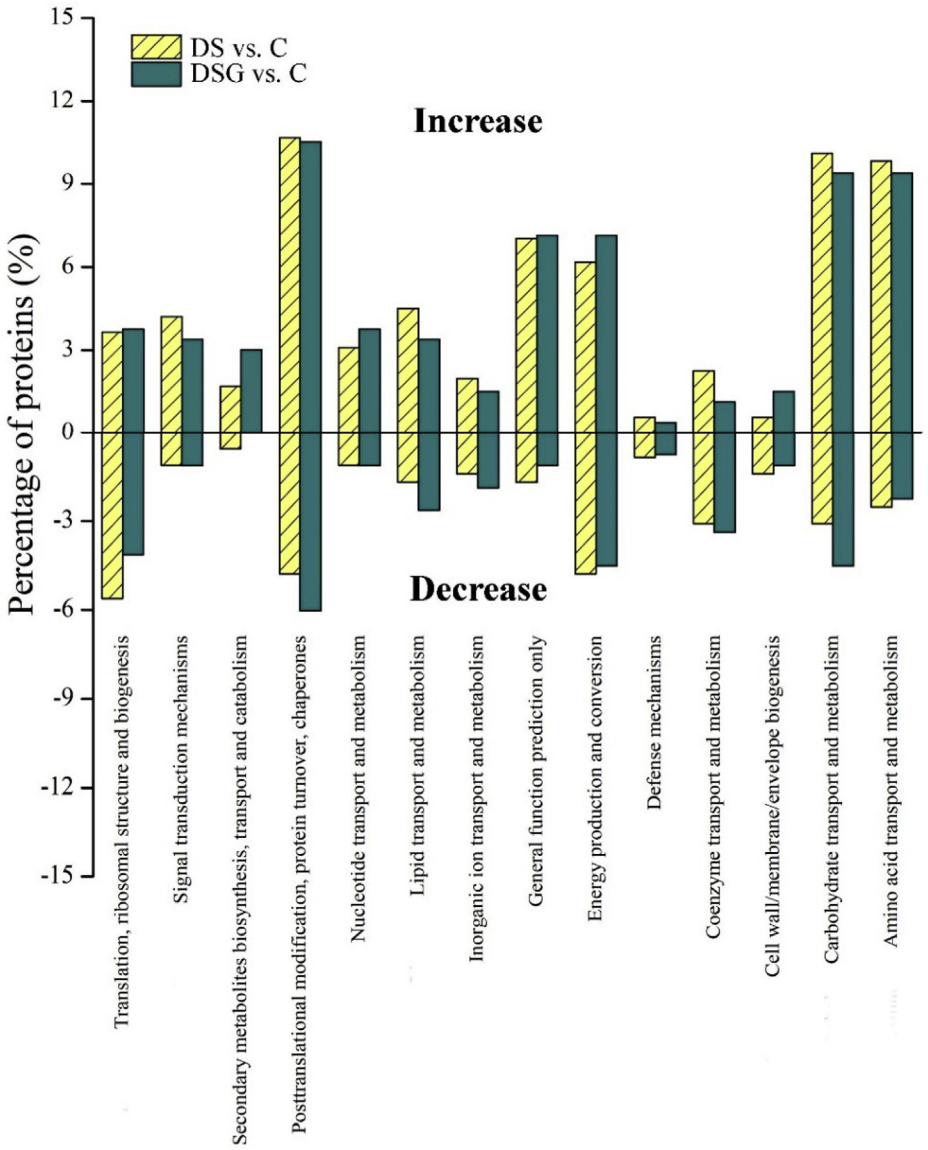

Fig. 5. The effect of drought stress without (DS vs. C) and with (DSG vs. C) GABA application on differentially expressed proteins (DEPs) within each functional category based on the clusters of orthologous groups (COGs) in leaf of creeping bentgrass. (A) DS vs. C and (B) DSG vs. C. C, control (normal condition); DS, drought stress; DSG, drought-stressed plants pretreated with GABA.

bentgrass maintained remarkably higher abundance of peroxidase 2 (POD2), POD73, GST1, and GST6 than untreated plants under drought stress. It is well known that SOD has the function of catalyzing the disproportionation of $O_2^{\cdot-}$ to H_2O_2, and CAT, POD and other enzymes involved in ascorbic acid-glutathione (ASA-GSH) cycle (APX and GST) mainly participate in H_2O_2 scavenging, thereby alleviating stress-caused reactive oxygen species (ROS) accumulation and oxidative damage in plants (Espinosadíez et al., 2015). Previous studies have widely reported that exogenous application of GABA improved antioxidant enzyme activities in relation to enhanced chilling tolerance in anthurium (*Anthurium andraeanum*) flowers (Aghdam et al., 2016), heat tolerance in creeping bentgrass (Li et al., 2016b), chromium stress tolerance in leaf mustard (*Brassic juncea*) (Mahmud et al., 2017), and drought tolerance in black cumin (*Nigella sativa*) (Rezaei-Chiyaneh et al., 2018). Proteomic analysis also proved that the drought-tolerant cotton (*Gossypium hirsutum*) cultivar 'KK1543' exhibited higher abundant APX and POD than the drought-sensitive 'Xinluzao26' in response to water deficit (Zhang et al., 2016). Less oxidative damage and better cell membrane stability were observed in GABA-treated creeping bentgrass as compared to that in untreated plants under water deficit. Our current findings together with previous studies indicate that GABA-improved ROS-scavenging capacity protects cells from oxidative damage under drought stress.

Late embryogenesis abundant proteins (LEA), also known as dehydrins (DHNs), are produced in a wide variety of plants during environmental stresses including high and low temperature, drought, salinity, and heavy metal stress (Campbell and Close, 1997). Acting as critical intracellular stabilizer, DHNs protect proteins from aggregation and prevent cell dehydration due to desiccation or osmotic stress (Hanin et al., 2011). The increase in DHNs accumulation is key mechanism of adaptation to drought stress in plant species. The study of Shakirova et al. (2016) found that drought-resistant wheat cultivar 'Omskaya 35' accumulated significantly higher DHNs than drought-sensitive cultivar 'Salavat Yulaev' during seeds germination under drought stress. The 24-epibrassinolide-pretreatment could enhance drought tolerance through further improving stress-induced DHNs accumulation in both cultivars. Enhanced drought tolerance could be acquired in transgenic tobacco by overexpressing *Prunus nume* DHNs genes (Bao et al., 2017). Exogenous polyamines could significantly promote drought tolerance of white clover associated with DHNs

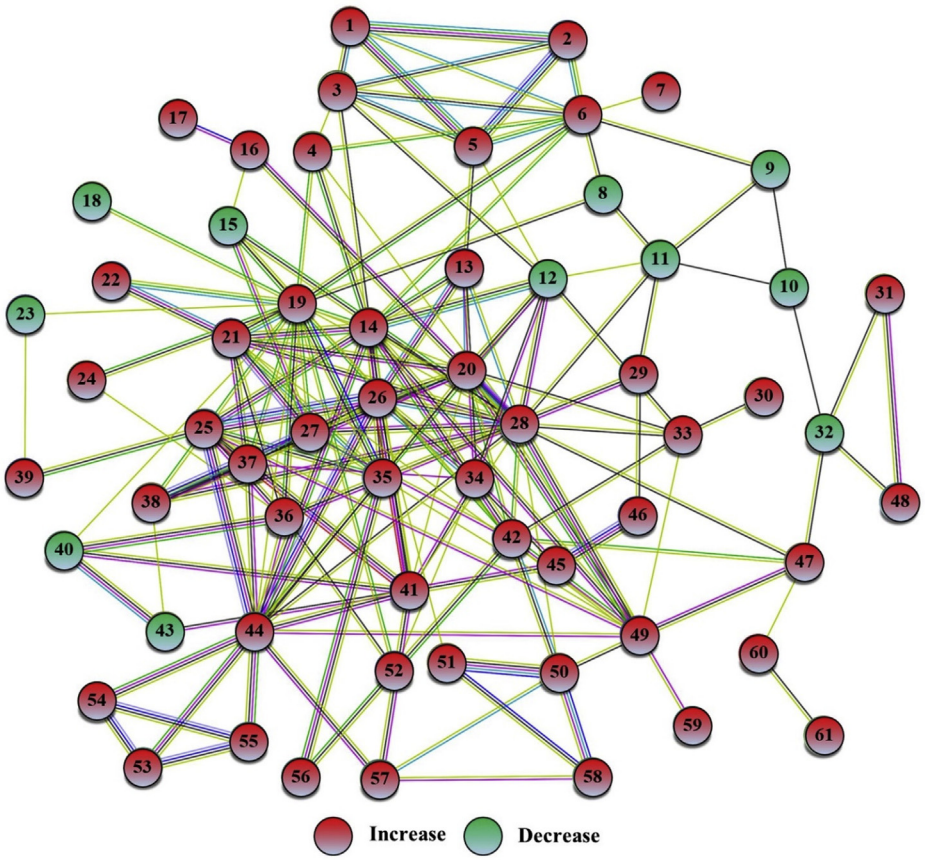

Fig. 6. Interaction network of the identified differentially expressed proteins (DEPs) when DSG compared to C (DSG vs. C). This network shows only known and direct interactions among identified DEPs. C, control (normal condition) and DSG, drought-stressed plants pretreated with GABA. 1, TPS9; 2, SPS3; 3, UGP1; 4, PPC1; 5, SUS4; 6, GBSS1b; 7, PIG3; 8, MIPS2; 9, PSAF; 10, LIL3:1; 11, PHT2;1; 12, G6PD4; 13, PFK2; 14, GAPC1; 15, PIP2-7; 16, OST1; 17, PTI1; 18, ARASP; 19, P5CS1; 20, CAT2; 21, ALDH12A1; 22, GAD; 23, AAO1; 24, IGPS; 25, Hsc70-1; 26, Hsp70-2; 27, ALDH10A9; 28, CAT1; 29, ECA4; 30, ZBD; 31, HCAR; 32, LIN2; 33, SRX; 34, CDC5; 35, P5CR; 36, ATP synthase; 37, TPR; 38, ALDH10A8; 39, NFS1; 40, RPS18C; 41, EFTu/EF1A; 42, APX1; 43, RPL18a; 44, HSP70; 45, ABCF3; 46, ABCF1; 47, LEA1; 48, ACD2; 49, Cu/ZnSOD; 50, GST2; 51, GSTU19; 52, ETFBETA; 53, HSP23.2; 54, HSP17.9; 55, HSP21; 56, ECH2; 57, GST3; 58, GSTL2; 59, HMA6; 60, LEA14; 61, LEA31. Known Interactions: from curated databases, and experimentally determined; Predicted Interactions: gene neighborhood, gene fusions, and gene co-occurrence; Others: textmining, co-expression, and protein homology.

accumulation (Li et al., 2015a, 2016c). Our earlier study also found that high abundance of DHN (65 KDa) contributed to GABA-priming alleviated salinity damage during seeds germination (Cheng et al., 2018). In response to drought stress, GABA-treated creeping bentgrass increased LEA1, LEA14, LEA31, and also had higher abundance of DHN3 than untreated plants. Higher expression of these proteins may explain GABA-induced drought tolerance in creeping bentgrass.

Except for above these classical stress-defensive proteins, the GABA-enhanced accumulation of other important proteins may be related to drought tolerance in creeping bentgrass. Growth hindrance, photo-oxidative damage, and ammonium toxicity are some of the major detrimental effects of osmotic stress such as drought or salt stress in plants (Xu et al., 2018; Bittsánszky et al., 2015; Moran et al., 1994). Dicarboxylate transporter 2.1 catalyzes the assimilation of ammonia generated by photorespiratory pathway, which is essential for photorespiratory nitrogen recycling in plants (Taniguchi et al., 2002). ATP-dependent zinc metalloprotease is involved in thylakoid formation, photosystem II repair, and prevention of cell death caused by photo-oxidative damage (Lindahl et al., 2000). Recent study found that significant accumulation of ATP-dependent zinc metalloprotease could play protective role in maintaining proteins homoeostasis for SA-regulated drought tolerance and recovery in soybean (Sharma et al., 2018). The receptor-like protein kinase HERK 1 regulates the expression of multiple genes that are implicated in cell elongation during vegetative growth. The brassinosteroids (BRs) can induce *HERK1* expression to increase cell elongation in *Arabidopsis* (Guo et al., 2009). Differential protein profiling demonstrated that the abundance of dicarboxylate transporter 2.1, ATP-dependent zinc metalloprotease, and HERK 1 were significantly higher in water-stressed creeping bentgrass pretreated with GABA than that in water-stressed plants without GABA application. It may indicate that GABA-induced drought tolerance is possibly involved in the improvement of nitrogen recycling, mitigation of ammonia toxicity and drought-depressed cell elongation, and protection of photosystem II in creeping bentgrass. Ma et al. (2016) also reported that exogenous application of GABA effectively alleviated ammonium toxicity and restored the elongation of rice roots repressed by high NH_4^+.

The o-acyltransferase WSD1 catalyzes formation of cuticular wax biosynthesis by using long chain fatty alcohols in plants (Li et al., 2008). Cuticular wax, a protective barrier, plays an important role in protecting plant cells from abiotic and biotic stress damage. Increase in wax biosynthesis can effectively enhance drought tolerance in many plants. For examples, the overexpression of AP2 domain transcription factors *SHN* increased total cuticular waxes level, followed by the enhancement of drought tolerance and recovery in *Arabidopsis* (Aharoni et al., 2004). The *WXP1*-transgenic alfalfa plants with significant increase in cuticular waxes also displayed better drought tolerance and recovery (Zhang et al., 2005a). Fatty acid desaturases remove two hydrogen atoms from a fatty acid to produce unsaturated fatty acids such as 16: 3 and 18: 3 fatty acids (Schmidt et al., 1994). The maintenance of inherent level of fatty acid unsaturation and the ability to adjust fatty acid unsaturation are positively associated with salt and drought tolerance in plants (Upchurch, 2008). It has been found that the increase in fatty acid desaturation via overexpression of omega-3 desaturase genes *FAD3* and *FAD8* promoted drought tolerance in transgenic tobacco plants (Zhang et al., 2005b). Two-component response regulators such as ARR and ORR function as transcriptional activators involved in cytokinin signal transduction. Ectopic expression of a two-component response regulator *ARR2* in transgenic *Arabidopsis* promoted leaf differentiation and delayed leaf senescence (Hwang and Sheen, 2001). Enhanced endogenous cytokinin accumulation by overexpressing an *ipt* in creeping bentgrass alleviated leaf senescence contributing to improved drought tolerance (Merewitz et al., 2010). Under drought stress, the GABA-pretreated creeping bentgrass maintained significantly higher abundance of WSD1, omega-6 fatty acid desaturase, and ORR21. These results provide new evidences that GABA regulates drought

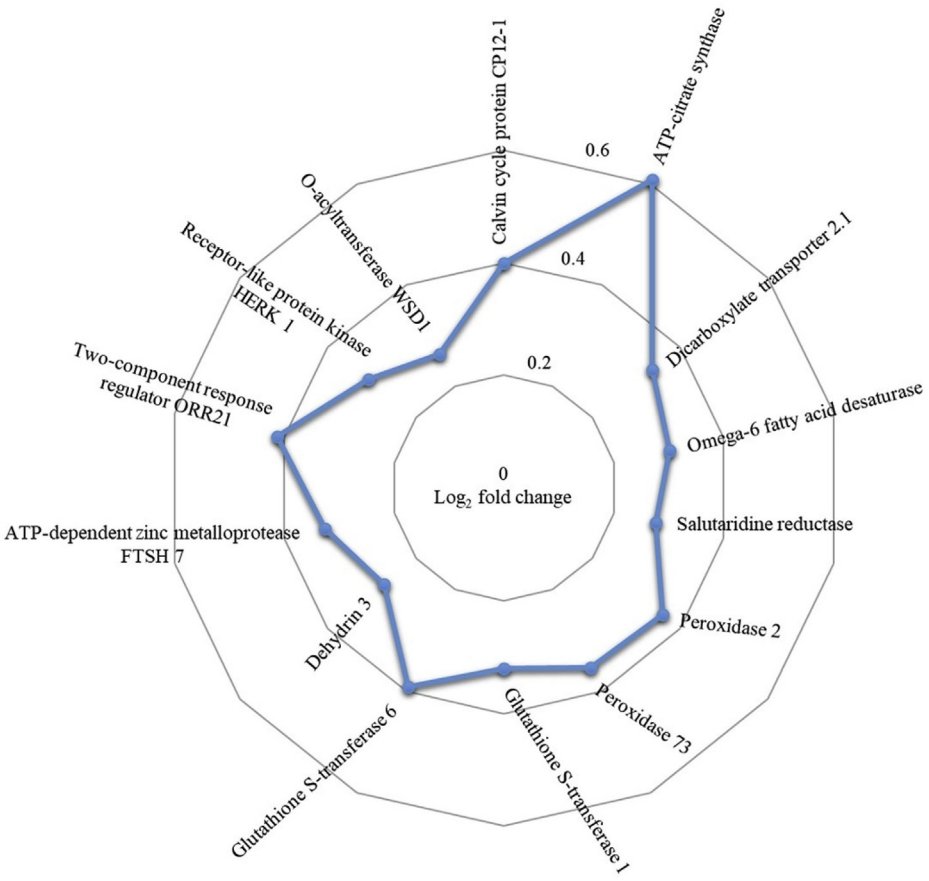

Fig. 7. Log_2 fold change of the identified differentially expressed proteins (DEPs) when DSG compared to DS (DSG vs. DS) in leaves of creeping bentgrass. C. DS, drought stress; DSG, drought-stressed plants pretreated with GABA.

tolerance in relation to wax biosynthesis, fatty acid desaturase, and delaying leaf senescence in creeping bentgrass.

In conclusion, elevated endogenous GABA level via exogenous application of GABA significantly mitigated drought stress damage in creeping bentgrass. Physiological responses demonstrated that GABA-treated creeping bentgrass maintained significantly higher leaf RWC, WUE, OA, Fv/Fm, and Pn and also exhibited lower oxidative damage than untreated plants under drought stress. iTRAQ-based proteomics found that enhanced chaperones accumulation, carbohydrates, amino acids, and energy metabolism are important adaptive responses to drought stress in creeping bentgrass. The GABA further promoted energy production and conversion, antioxidant defense, and DHN3 accumulation, which is essential for energy maintenance, ROS-scavenging, and the prevention of cell dehydration in leaf during drought stress. In addition, GABA significantly improved the abundance of dicarboxylate transporter 2.1, ATP-dependent zinc metalloprotease, HERK 1, WSD1, omega-6 fatty acid desaturase, and ORR21 in response to drought stress. These results provide new evidences that GABA-induced drought tolerance is possibly involved in the improvement of nitrogen recycling, mitigation of ammonia toxicity and drought-depressed cell elongation, and protection of photosystem II, wax biosynthesis, fatty acid desaturase, and delaying leaf senescence in creeping bentgrass. Future research will focus on GABA-regulated signal transduction pathways associated with drought tolerance and the function of specific proteins regulated by GABA on drought tolerance in creeping bentgrass based on transgenic approach.

Author contributions

ZL and XZ conceived and designed the experiments; ZL, TH, MT, and BC performed experiments; ZL, TH, and MT analyzed the data; ZL wrote the paper; XZ, TH, MT, and YP improved the paper. All authors have read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.10.018.

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