Transcriptional Regulation of Hydrogen Peroxide and Calcium for Signaling Transduction and Stress-defensive Genes Contributing to Improved Drought Tolerance in Creeping Bentgrass

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ABSTRACT. Small molecules, including H₂O₂ and Ca, mediate stress signaling and drought tolerance in plants. The objective of this study was to determine whether improvement in drought tolerance by H₂O₂ and Ca were associated with the regulation of transcription factors and stress-protective genes in perennial grass species. Plants of creeping bentgrass (*Agrostis stolonifera*) were sprayed with water (control), H₂O₂ (9 mM), or CaCl₂ (10 mM) and exposed to drought stress for 20 days in controlled-environment growth chambers. Foliar application of H₂O₂ or Ca led to significant improvement in drought tolerance of creeping bentgrass, as demonstrated by greater turf quality, leaf relative water content, chlorophyll content, photochemical efficiency, and cell membrane stability, as compared with the untreated control. The application of H₂O₂ and Ca resulted in significant up-regulation of genes in Ca signaling transduction pathways [*Ca-dependent kinase 26 (CDPK26)*, *mitogen-activated protein kinase 1 (MAPK1)*, and *14-3-3*] and transcript factors (*WRKY75* and *MYB13*). For genes encoding antioxidant enzymes, H₂O₂ mainly enhanced *superoxide dismutase (SOD)*, *catalase (CAT)*, *glutathione reductase (GR)*, and *dehydroascorbate reductase (DHAR)* expression, while Ca primarily improved transcript levels of *SOD*, *monodehydroascorbate reductase (MDHAR)*, and *GR*. In addition, *heat shock protein 70 (HSP70)*, *metallothionein 1 (MT1)*, and *glutamine synthetase 2 (GS2)* were also markedly up-regulated by H₂O₂ and Ca under drought stress. However, the transcript level of *lipoxygenase 3 (LOX3)* was significantly down-regulated by H₂O₂ and Ca under well-watered and drought conditions. These results imply that H₂O₂ and Ca commonly or differentially regulate genes expression in association with drought tolerance through activating Ca signaling pathway and regulating transcription factors and stress-protective genes expression, leading to the alleviation of lipid peroxidation, maintenance of correct protein folding and translocation, and enhancement of nitrogen metabolism under a prolonged period of drought stress in creeping bentgrass.

Plant responses to drought stress involve multiple mechanisms at molecular, biochemical, physiological, and metabolic levels (Bhargava and Sawant, 2013; Shanker et al., 2014; Todaka et al., 2015). Many small molecules, such as Ca and H₂O₂, play important roles in stress signaling and responses in plants (Apel and Hirt, 2004; Ermak and Davies, 2002; Reddy et al., 2011). Calcium could serve as an inorganic osmolyte for maintaining cell osmotic potential or signaling molecule for stress signal transduction in plants under abiotic stress, such as drought, high temperature, or salt stress (Bush, 1995). The

study of Jiang and Huang (2001) found that exogenous Ca application increased Ca concentration in cell saps contributing to improved osmotic adjustment and enhanced antioxidant capacity in two cool-season grass species under heat stress. Stress-triggered change of cytosolic Ca²⁺ in guard cells was correlated with stomatal closure, indicating the importance of Ca²⁺ signaling in drought responses (Fu and Lu, 2007). It has been found that exogenously applied Ca altered some antioxidant enzyme activities and enhanced root activity as well as the accumulation of osmolytes, which could improve drought tolerance of white clover (*Trifolium repens*), wheat (*Triticum aestivum*), and maize (*Zea mays*) (Li et al., 2015; Nayyar, 2003; Wang, 2010). Foliar Ca spray also effectively alleviated drought-induced growth inhibition, photoinhibition, and decline in leaf water in maize and sugar beet [*Beta vulgaris* (Hosseini et al., 2019; Naeem et al., 2018)]. These findings indicate positive function of Ca on improving drought tolerance involved in multiple physiological changes in plants.

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The higher concentration of H_2O_2 can cause lipid peroxidation, proteins degradation, accelerated senescence, and even programmed cell death, whereas the lower level and rapidly alteration of H_2O_2 acts as critical regulatory roles in intermediate signaling transduction for activation of defense mechanisms in plants during early phases of stress responses (Ray et al., 2012; Suzuki et al., 2012; Yu, 1994). Previous studies demonstrated that exogenous H_2O_2 enhanced heat tolerance through improving cell membrane stability, leaf photosynthesis, and antioxidant enzyme activities in creeping bentgrass [*Agrostis stolonifera* (Larkindale and Huang, 2004, 2005)]. The appropriate low concentration of foliar H_2O_2 application alleviated drought damage in soybean (*Glycine max*) and marigold (*Tagetes erecta*) associated with carbohydrate accumulation and roots development (Ishibashi et al., 2011; Liao et al., 2012). Absciscic acid (ABA) triggered H_2O_2 signaling to induce antioxidant defense, thereby alleviating drought-caused oxidative damage in bermudagrass [*Cynodon dactylon* (Lu et al., 2009)]. It has been widely reported that both of H_2O_2 and Ca^{2+} could regulate stress-related downstream genes expression associated with improved drought tolerance in different plant species (Li et al., 2015; Neill et al., 2002; Xu et al., 2015; You et al., 2013). However, H_2O_2 - and Ca-regulated signaling transduction, and key genes controlling drought tolerance were not well documented in plants exposed to severe or a prolonged period of drought stress.

Objectives of this study were to 1) assess effects of H_2O_2 and Ca signaling molecules on improving drought tolerance through physiological analysis; and 2) examine genes transcript level encoding signaling transduction, transcription factors, antioxidant enzymes, and stress-related proteins that may be commonly or differentially regulated by H_2O_2 and Ca associated with drought tolerance in creeping bentgrass under a prolonged drought stress.

Materials and Methods

PLANT MATERIAL AND TREATMENT. Creeping bentgrass ('Pennncross') sod plugs (5 cm diameter) were collected from Rutgers University Horticultural Farm II (North Brunswick, NJ) and planted in plastic containers (40 cm length, 30 cm width, and 35 cm height). A total of eight containers were used, and each container includes three sod plugs. Fritted clay was used as soil matrix, and all containers were placed in a greenhouse [average 23/18 °C of day/night, 790 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetically active radiation (*PAR*)]. Plants were trimmed to maintain a canopy height of 3 cm and irrigated twice per week with Hoagland's solution (Hoagland and Arnon, 1950) for 2 months in the greenhouse during September–October. Plants were then moved to controlled growth chambers (Environmental Growth Chamber, Chagrin Falls, OH) that provided 21/19 °C (day/night), 70% relative humidity, and 12-h photoperiod at 660 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ *PAR*. After acclimation in the growth chamber for 1 week, each sod plug was sprayed with 10 mL of 9 mM H_2O_2 or 10 mM CaCl_2 solution or distilled water (untreated control) once each day for 3 d. After pretreatments, plants were cultivated under the well-watered condition (plants were irrigated every 2 d and soil water content was maintained at the pot capacity) or exposed to drought stress (stopping irrigating) for 20 d during the month of November. The concentration of H_2O_2 and CaCl_2 were selected based on a preliminary experiment. Each treatment has four replications in

four different containers that were placed in four growth chambers. Leaf samples were collected from plants at 20 d of drought stress treatment.

MEASUREMENTS OF TURF QUALITY AND PHYSIOLOGICAL PARAMETERS. Turf quality (TQ) was evaluated by using a scale of 1 to 9 according to color, density, and uniformity of turfgrass (Beard, 2001). Leaf relative water content (RWC) or electrolyte leakage (EL) was detected by using the method of Barrs and Weatherley (1962) or Blum and Ebercon (1981), respectively. Assay methods in details were recorded in our previous study (Li et al., 2016a). For chlorophyll (Chl) content, 0.1 g of fresh leaves were cut from plants and submerged in 10 mL of dimethyl sulphoxide. After being placed in the dark for 48 h, leaves extractions were measured at 663 and 645 nm using a spectrophotometer (Spectronic Instruments, Rochester, NY) (Arnon, 1949). For photochemical efficiency (Fv/Fm), leaves were pretreated into darkness through leaf clips for 20 min. Fv/Fm ratio was then recorded by using a fluorescence meter (Fim 1500; Dynamax, Houston, TX).

GENE EXPRESSION ANALYSIS. For genes expression analysis, real-time quantitative polymerase chain reaction (qRT-PCR) was used. RNeasy Mini Kit (Qiagen, Duesseldorf, Germany) was used for extracting total RNAs in fresh leaves according to manufacturer's instructions. The RNA was then reverse-transcribed into cDNA (A revert Aid First Stand cDNA Synthesis Kit; Fermentas, Burlington, ON, Canada). For the PCR protocol, the conditions were set: 5 min at 95 °C, and 40 repeats of denaturation at 95 °C for 15 s, annealing at 60 °C for 45 s, followed by heating the amplicon from 60 to 95 °C to obtain the melting curve. The Eq. The formula $2^{-\Delta\Delta\text{Ct}}$ was used for calculating the transcript level of all genes (Xia et al., 2009). Table 1 shows primer sequences of all genes, including reference gene *ACT2*.

STATISTICAL ANALYSIS. Experiment design was a split-plot design with water status as the main plot and small molecules (H_2O , Ca, and H_2O_2) treatments as the sub-plot. Data were analyzed by using the general linear model procedure for the analysis of variance (SAS version 9.1; SAS Institute, Cary, NC). The significance of differences was tested by using Fisher's protected least significance test with $P \leq 0.05$.

Results

PHYSIOLOGICAL RESPONSES TO H_2O_2 AND CA IN CREEPING BENTGRASS. H_2O_2 and Ca application had no significant effects on TQ, RWC, EL, Chl, and Fv/Fm in leaves under well-watered condition. Drought stress significantly decreased TQ, RWC, Chl content, and Fv/Fm ratio, but increased EL of leaves (Figs. 1 and 2). Under drought stress, H_2O_2 - and Ca-treated plants exhibited significantly higher TQ and RWC and lower EL than nontreated plants (Fig. 1). Both H_2O_2 - and Ca-treated plants maintained a 30% increase in Chl content compared with nontreated plants in response to drought stress, and Fv/Fm ratio was also significantly higher in H_2O_2 - and Ca-treated plants than that in nontreated plants under drought stress (Fig. 2).

EXPRESSION OF GENES INVOLVED IN SIGNALING TRANSDUCTION AND TRANSCRIPTION FACTORS BY H_2O_2 AND CA. In response to drought stress, most of genes expression levels were affected by H_2O_2 and Ca^{2+} (Fig. 3A). H_2O_2 and Ca induced significant changes in five genes, while four genes were only regulated by Ca under well-watered conditions (Fig. 3B). Twelve genes

Table 1. Primer sequences used for detecting transcript levels of genes in real-time quantitative polymerase chain reaction (qRT-PCR) and their corresponding GeneBank accession numbers of the analyzed genes.

Target gene ^z	Accession no.	Forward primer (5'-3')	Reverse primer (5'-3')
<i>CDPK26</i>	GR281936.1	ATCCAGGCTGCTCACTCCGTA	AACCAACGCAGGGTAGGATTC
<i>MAPK1</i>	DV866362.1	AGCTGGCCCTGCATGGATAA	CAGGACAATGTTTCAGATGGAGGC
<i>14-3-3</i>	DV866921.1	TCATGGACAAGATCAAGGAGAAG	CAAACACCCAAGTGAGCTAAAC
<i>ABF3</i>	DV862003.1	ATCTGCCTGCGGAGGACACT	TGAAGCATCGGAACAGTGGC
<i>WRKY75</i>	DV867719.1	TGGTGGTGACGACATACGAGG	GGTTGGTAAAGGTTGAGGAGGTG
<i>MYB13</i>	GR279830.1	CATTCAAGTTTACCCGAGTGCG	CATAAAACATGACCCATCACAGCT
<i>SOD</i>	DV867103	CACTGGACCTCACTTCAAC	GTAGCAACACCATCCACTC
<i>CAT</i>	DY543619	TTGCCAATAAGAGGGAGAATG	CGAAGCCGAGCATGTAAG
<i>APX</i>	GR281667	AGGACATTGTTGCCCTTTC	GCTCCGTGAAGTAAGAGTTG
<i>MDHAR</i>	DV865007	CCATGAAGCTCTACAACGAG	GTAGAAGTAGGGCAGGTAGT
<i>DHAR</i>	DV853556	GAAAGGTGCCTGTGTTTAATG	GTGATGGAGTTGGGTACTTC
<i>GR</i>	AB277097	GATGGAGGCTACTTGCTTTG	GCTAAGACCCACGACAGATA
<i>HSP70</i>	DV860338.1	CCTGCCCAATTTGCATTACC	CAGACGGAGAAGCAACTGAA
<i>HSP90</i>	GR280041.1	CCACCCATACTCACCTGTCACG	CAAGGAGAAGTTTGAAGGGCTATG
<i>DHN3</i>	FE527922.1	CATGGCGTCTACTGCTTGTA	CAGAGGACTTGAACCCAGATAC
<i>MT1</i>	DV865927.1	TCTCCAAGCTCATCTTCTTCTCATT	TTCGTCCAGGTCAGGGTACATC
<i>GS2</i>	DV862105.1	GCTGGCGATGAACGTATGAAG	ACAGACAGTGCCTGACGGA
<i>LOX3</i>	GR279465.1	AGGGCTGGTCCTTGATGTCTG	TCTACTACCCAGCGACAGCAT
<i>ACT2</i>	DY543529	CCTTTTCCAGCCATCTTTCA	GAGGTCCTTCCTGATATCCA

^z*ABF3* = ABRE binding factor 3, *ACT2* = actin 2, *APX* = ascorbate peroxidase, *CAT* = catalase, *CDPK26* = Ca-dependent kinase 26, *DHAR* = dehydroascorbate reductase, *DHN3* = dehydrin 3, *GR* = glutathione reductase, *GS2* = glutamine synthetase 2, *HSP70* = heat shock protein 70, *HSP90* = heat shock protein 90, *LOX3* = lipoxygenase 3, *MAPK1* = mitogen-activated protein kinase 1, *MDHAR* = monodehydroascorbate reductase, *MT1* = metallothionein 1, *MYB13* = MYB transcription factor 13, *SOD* = superoxide dismutase, *WRKY75* = WRKY transcription factor 75.

were affected by both H₂O₂ and Ca, and two genes were specifically regulated by H₂O₂ under drought stress. Only one gene was specifically induced by Ca under drought stress (Fig. 3C). Under well-watered conditions, foliar spraying with Ca upregulated the expression levels of *CDPK26*, *MAPK1*, and *MYB13*, and the application of H₂O₂ only upregulated *MAPK1* expression compared with the well-watered control (Figs. 4 and 5). Drought stress induced significant increases in *CDPK26*, *MAPK1*, *14-3-3*, *ABF3*, *WRKY75*, and *MYB13* transcript levels with or without H₂O₂ and Ca application (Figs. 4 and 5). Under drought stress, both H₂O₂- and Ca-treated plants showed 4-fold increases in *CDPK26* and *14-3-3* as well as 5-fold increase in *MAPK1* expression level compared with nontreated plants (Fig. 4). For transcription factors, there was six times as high *WRKY75* and *MYB13* transcript levels in H₂O₂-treated plants as in nontreated plants under drought conditions (Fig. 5B and C). Similarly, *WRKY75* and *MYB13* transcript levels of Ca-treated plants were three times significantly higher than that of nontreated plants under drought stress (Fig. 5B and C). However, H₂O₂ and Ca had no effect on the transcript level of *ABF3* under well-watered and drought conditions (Fig. 5A).

EXPRESSION OF GENES INVOLVED IN ANTIOXIDANT DEFENSE AFFECTED BY H₂O₂ AND Ca. Exogenous application of H₂O₂ and Ca significantly upregulated *CAT*, *MDHAR*, and *GR* transcript levels under well-watered conditions (Fig. 6B, D, and F). Under drought conditions, *SOD* was upregulated by exogenous H₂O₂ and Ca (Fig. 6A). Exogenous H₂O₂ application upregulated *CAT* and *DHAR*, but Ca had no significant effects on these two genes under drought stress (Fig. 6B and E). Neither H₂O₂ nor Ca application had significant effects on the expression of *ascorbate peroxidase* (*APX*) under well-watered or drought conditions (Fig. 6C). However, exogenous Ca upregulated *MDHAR* expression under drought stress, and the increased

percentage was 114% (Fig. 6D). *GR* transcript level in H₂O₂- and Ca-treated plants was significantly higher than that in nontreated plants by two times under drought stress (Fig. 6F).

EXPRESSION OF GENES INVOLVED IN STRESS-PROTECTIVE PROTEINS AND OTHER METABOLISM AFFECTED BY H₂O₂ AND Ca. Under well-watered condition, *heat shock protein 90* (*HSP90*) and *MT1* expression were not changed by foliar application of H₂O₂ and Ca, and exogenous H₂O₂ upregulated *HSP70* and *dehydrin 3* (*DHN3*); but Ca did not show significant effects on *HSP70* and *DHN3* expression level (Fig. 7). Under drought stress, *HSP70* expression level of H₂O₂- and Ca-treated plants increased by 60% compared with nontreated plants, and H₂O₂- and Ca-treated plants also had 3-fold increases in *MT1* transcript level compared with nontreated plants; but *HSP90* expression was unaffected by exogenous application of H₂O₂ and Ca (Fig. 7A and D). Drought stress induced more than 500 times increase in *DHN3* in all treatments relative to well-watered plants, but drought-stressed plants treated with H₂O₂ and Ca exhibited significantly lower *DHN3* expression than drought-stressed plants without chemical treatment (Fig. 7C). Foliar application of H₂O₂ and Ca had no significant effects on *GS2* expression, whereas *LOX3* was significantly downregulated by H₂O₂ under well-watered condition (Fig. 8). Under drought stress, the *GS2* transcript level in H₂O₂- and Ca-treated plants had three times increase compared with drought-stressed control plants (Fig. 8A). On the contrary, *LOX3* expression was significantly inhibited by H₂O₂ and Ca under well-watered and drought conditions (Fig. 8B).

Discussion

Plant tolerance to drought stress involves the activation of stress signaling transduction pathways, which include multiple

Fig. 1. Effects of H₂O₂ and CaCl₂ on (A) turf quality, (B) relative water content, and (C) electrolyte leakage in creeping bentgrass under well-watered and drought condition for 20 d. Vertical bars indicate $\pm$ SE (n = 4). Different letters above columns indicate significant differences among control, H₂O₂, and Ca treatment under a given condition (well-watered or drought); asterisk (*) indicates significant difference for one particular treatment (control, H₂O₂, or Ca) between well-watered and drought condition.

transcriptional factors or genes (Dortje et al., 2011). *CDPK* and *MAPK* are two critical kinases involved in Ca²⁺ signaling transduction pathways (Anil and Rao, 2001). *CDPK* and *MAPK* signaling transduction regulate transcription factors such as

WRKY and *MYB* families, which play critical roles in drought tolerance in plants via the activation of downstream signaling and stress-defensive genes (Boudsocq and Sheen, 2013; Chen et al., 2012; Danquah et al., 2014; Singh et al., 2002). Previous studies have proved that Ca²⁺ and reactive oxygen species (ROS) signaling are integrated in cells in most of cases. Ca²⁺ channels could be activated by H₂O₂, and ROS signaling production also could be directly regulated by Ca²⁺ signaling under normal and abiotic stress conditions (Camello-Almaraz et al., 2006; Gilroy et al., 2014; Pei et al., 2000). The research of Li et al. (2015) indicated that H₂O₂ interacting with Ca²⁺ signaling were involved in polyamine-regulated drought tolerance in white clover though activating *CDPK* signaling. The 14-3-3 proteins are important conserved signaling proteins involved in cellular signaling transduction and also have major regulatory function of carbohydrate and nitrogen metabolism in plants (Comparot et al., 2003; Roberts et al., 2002). In addition, 14-3-3 proteins also could regulate multiple signal transduction proteins such as *CDPK* in response to environmental stresses in higher plants (Roberts et al., 2002). Previous studies indicated that 14-3-3 protein or genes could be differentially mediated by salinity, drought, and cold, which is an important part of stress defense signaling in plants (Chen et al., 1994; Chen et al., 2006; Jarillo et al., 1994). It has been reported that overexpressing a tomato 14-3-3 gene enhanced salt tolerance in *Arabidopsis thaliana* (Xu and Shi, 2007). A “stay-green” phenotype and better drought tolerance were observed in transgenic cotton overexpressing a 14-3-3 gene (Yan et al., 2004). In this study, the application of H₂O₂ and Ca up-regulated 14-3-3, *CDPK26*, *MAPK1*, *WRKY75*, and *MYB13* in creeping bentgrass exposed to drought stress. Our results, together with reports in previous studies, suggest that H₂O₂ and Ca could regulate 14-3-3, *CDPK26*, *MAPK1*, *WRKY75*, and *MYB13*, which may contribute to improved drought tolerance due to the application of those two molecules in creeping bentgrass, as manifested by increased TQ, leaf RWC, Chl content, Fv/Fm, and cell membrane stability under drought stress.

In addition to transcriptional factors in signaling pathways, changes in downstream stress-protective genes also affect plant tolerance to drought stress (Shinozaki and Yamaguchi-Shinozaki, 2007). H₂O₂ and Ca up-regulated *HSP70* and *MTI* under drought stress in our study. HSPs are important molecular chaperones that help proteins to fold and assemble correctly related to plant adaption to abiotic stress (Sabeheh et al., 1998; Sørensen et al., 2003; Wang et al., 2004). HSPs not only play positive roles in regulating heat tolerance, but also are correlated with the improvement of drought tolerance in plants. For example, the transgenic tobacco (*Nicotiana tabacum*) constitutively expressing a *HSP70* obtained stress tolerance under progressive drought (Cho and Hong, 2006). *MTI* gene regulates synthesis of metallothionein, which plays roles in stabilization of cellular membranes, antioxidant, and metal ion homeostasis (Ruttkey-Nedecky et al., 2013). Transgenic rice (*Oryza sativa*) overexpressing an *OsMT1a* demonstrated the significant increase in drought tolerance associated with ROS scavenging and ions homeostasis (Yang et al., 2009). The improved accumulation of MT played an important role in spermidine-regulated drought tolerance in white clover (Li et al., 2016b). Current results suggest that H₂O₂ and Ca-regulated drought tolerance in creeping bentgrass could be associated with activation of *HSP70* and *MTI* expression. DHNs encoding dedrydrins are drought-inducible genes and their up-regulation may

Fig. 2. Effects of H₂O₂ and CaCl₂ on (A) chlorophyll content and (B) photochemical efficiency in creeping bentgrass under well-watered and drought condition for 20 d. Vertical bars indicate $\pm$ SE (n = 4). Different letters above columns indicate significant differences among control, H₂O₂, and Ca treatment under a given condition (well-watered or drought); asterisk (*) indicates significant difference for one particular treatment (control, H₂O₂, or Ca) between well-watered and drought condition.

Fig. 3. (A) Heat map of changes in 18 genes expression levels in creeping bentgrass under well-watered and drought condition, (B) differential regulated genes induced by H₂O₂ and CaCl₂ under well-watered condition, and (C) differential regulated genes induced by H₂O₂ and CaCl₂ under drought condition in creeping bentgrass. The log₂ fold change ratios are shown in the results. Red indicates an upregulation, and green indicates a downregulation.

protect cells from drought damages and may reflect the level of drought stress that the plant has experienced, depending on stress duration and severity (Hara, 2010). In our study, the transcript level of *DHN3* increased by thousands of times in response to

drought stress, while H₂O₂ and Ca application suppressed the up-regulation of *DHN3* under drought stress, indicating H₂O₂- and Ca-treated plants might have suffered less stress damage.

Antioxidant defense is another stress-defense pathway including enzymatic and nonenzymatic components. Antioxidant enzymes function as scavengers of ROS in cells, thereby alleviating stress-induced oxidative damage (Hasanuzzaman et al., 2012). H₂O₂ and Ca act as mediators to participate in regulating antioxidant defense. For example, ABA triggered NADPH oxidase to release H₂O₂, resulting in the activation of *SOD*, *CAT*, and *APX* against oxidative damage in bermudagrass (Lu et al., 2009). Brassinosteroid-induced H₂O₂ accumulation was accompanied by increases in *SOD*, *CAT*, and key enzymes involved in an ascorbate-glutathione cycle leading to enhanced tolerance to oxidative stress in cucumber (*Cucumis sativus*) leaves (Xia et al., 2009). Polyamine could activate H₂O₂ and Ca signaling to regulate antioxidant enzyme activities and genes expression in white clover under water deficit condition (Li

et al., 2015). Foliar applied Ca significantly increased *CAT*, *GR*, and *APX* activities associated with significant declines in membrane lipid peroxidation in two cool-season grasses, tall fescue (*Festuca arundinacea*) and kentucky bluegrass

Fig. 4. Effects of H₂O₂ and CaCl₂ on genes expression of (A) Ca-dependent kinase 26 (*CDPK26*), (B) mitogen-activated protein kinase 1 (*MAPK1*), and (C) *14-3-3* in creeping bentgrass under well-watered and drought condition for 20 d. Vertical bars indicate $\pm$ SE (n = 4). Different letters above columns indicate significant differences among control, H₂O₂, and Ca treatment under a given condition (well-watered or drought); asterisk (*) indicates significant difference for one particular treatment (control, H₂O₂, or Ca) between well-watered and drought condition.

Fig. 5. Effects of H₂O₂ and CaCl₂ on genes expression of transcription factors (A) *ABRE binding factor 3* (*ABF3*), (B) *WRKY transcription factor 75* (*WRKY75*), and (C) *MYB transcription factor 13* (*MYB13*) in creeping bentgrass under well-watered and drought condition for 20 d. Vertical bars indicate $\pm$ SE (n = 4). Different letters above columns indicate significant differences among control, H₂O₂, and Ca treatment under a given condition (well-watered or drought); asterisk (*) indicates significant difference for one particular treatment (control, H₂O₂, or Ca) between well-watered and drought condition.

Fig. 6. Effects of H₂O₂ and CaCl₂ on genes encoding antioxidant enzyme (A) superoxide dismutase (*SOD*), (B) catalase (*CAT*), (C) ascorbate peroxidase (*APX*), (D) monodehydroascorbate reductase (*MDHAR*), (E) dehydroascorbate reductase (*DHAR*), and (F) glutathione reductase (*GR*) in creeping bentgrass under well-watered and drought condition for 20 d. Vertical bars indicate $\pm$ SE (n = 4). Different letters above columns indicate significant differences among control, H₂O₂, and Ca treatment under a given condition (well-watered or drought); asterisk (*) indicates significant difference for one particular treatment (control, H₂O₂, or Ca) between well-watered and drought condition.

(*Poa pratensis*), under heat stress (Jiang and Huang, 2001). In this current study, *SOD*, *CAT*, *DHAR*, and *GR* were up-regulated by H₂O₂, whereas Ca mainly induced *SOD*, *MDHAR*, and *GR* expression under drought stress, which

may contribute to improved drought tolerance; however, H₂O₂ and Ca regulated antioxidant defense through different enzymatic components in creeping bentgrass. In addition to ROS-induced oxidative damages, dioxygenation of polyun-

Fig. 7. Effects of H_2O_2 and $CaCl_2$ on genes encoding stress-related proteins (A) heat shock protein 70 (*HSP70*), (B) heat shock protein 90 (*HSP90*), (C) dehydrin 3 (*DHN3*), and (D) metallothionein 1 (*MTI*) in creeping bentgrass under well-watered and drought condition for 20 d. Vertical bars indicate $\pm$ SE (n = 4). Different letters above columns indicate significant differences among control, H_2O_2 , and Ca treatment under a given condition (well-watered or drought); asterisk (*) indicates significant difference for one particular treatment (control, H_2O_2 , or Ca) between well-watered and drought condition.

Fig. 8. Effects of H_2O_2 and $CaCl_2$ on genes encoding stress-related proteins (A) glutamine synthetase 2 (*GS2*) and (B) lipoxygenase 3 (*LOX3*) in creeping bentgrass under well-watered and drought condition for 20 d. Vertical bars indicate $\pm$ SE (n = 4). Different letters above columns indicate significant differences among control, H_2O_2 , and Ca treatment under a given condition (well-watered or drought); asterisk (*) indicates significant difference for one particular treatment (control, H_2O_2 , or Ca) between well-watered and drought condition.

Fig. 9. Proposed pathways regulated by H_2O_2 and $CaCl_2$ contributing to drought tolerance in creeping bentgrass.

saturated fatty acids catalyzed by lipoxygenase (LOX) also causes membrane lipid peroxidation in plants (Ángel-Coronel et al., 2017; Fukuchi-Mizutani et al., 2000; Siedow, 1991). The suppression of LOX activity by silicon has been associated with mitigation of drought-induced oxidative damages in eight chickpea (*Cicer arietinum*) cultivars (Gunes et al., 2007). In this study, the transcript level of *LOX3* was significantly down-regulated by H_2O_2 and Ca in creeping bentgrass under drought stress, indicating that H_2O_2 and Ca could also alleviate oxidative damages involving LOX.

Abiotic stress causes excessive $NH_3-NH_4^+$ accumulation that is toxic to plants (Rare, 1990; Yu et al., 2005). Glutamine synthetase (GS) is responsible for amine assimilation and catalyzing the synthesis of glutamine under environmental stress. It helps plants to detoxify the excess free ammonia in cells in response to abiotic stress, leading to effective alleviation of stress-caused excessive $NH_3-NH_4^+$ accumulation (Guan et al., 2016; Yu et al., 2005). In this study, both H_2O_2 and Ca significantly up-regulated *GS2* expression under drought stress, which implies that H_2O_2 and Ca could regulate amine assimilation in plants suffering from drought stress.

Conclusions

In summary, the application of H_2O_2 and Ca significantly enhanced drought tolerance of creeping bentgrass, as demonstrated by improved turf quality, leaf RWC, Chl content, photochemical efficiency, and cell membrane stability under drought stress. The positive effects of H_2O_2 and Ca on drought tolerance could be associated with the activation of Ca signaling pathways and transcript factors (*14-3-3*, *CDPK26*, *MAPK1*, *WRKY75*, and *MYB13*), antioxidant defense (*SOD*, *CAT*, *GR*, *MDHAR*, and *DHAR*) alleviating oxidative damage, and stress protection genes (*HSP70*, *MT1*, and *GS2*) assisting protein folding and translocation, and maintaining nitrogen metabolism under a prolonged drought stress (Fig. 9).

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