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Chitosan-enhanced heat tolerance associated with alterations in antioxidant defense system and gene expression in creeping bentgrass

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Abstract

As an effective plant growth regulator, chitosan plays a positive role in enhancing heat tolerance in perennial turfgrass. The objective of this study was to elucidate whether chitosan-promoted thermotolerance was associated with the antioxidant defense system under long-term heat stress in creeping bentgrass (*Agrostis stolonifera*). Plants were treated with or without 100 mg·L^{-1} chitosan under either heat stress (38/28 °C, day/night) or non-stressed condition (25/20 °C, day/night) for 42 d in growth chambers. Foliar application of chitosan significantly enhanced heat tolerance as reflected by the increased turf quality through inhibiting over-accumulation of reactive oxygen species, and increasing ascorbic acid content and antioxidant enzymes activities (peroxidase, POD; ascorbate peroxidase, APX; glutathione reductase, GR; dehydroascorbate reductase, DHAR). Chitosan-treated plants also had higher transcript levels of *AsCu/ZnSOD*, *AsCATB*, *AsPerox4*, *AsAPX2*, *AsAPX3*, *AsAPX4*, *AsAPX6*, *AsAPX8*, *AsGR2*, and *AsDHAR* genes in comparison to the untreated plants. The results suggested that chitosan-promotion in heat tolerance could be associated with non-enzymatic antioxidants, antioxidant enzymes, as well as relative gene expression.

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Introduction

Heat stress is one of the crucial environmental stresses limiting plant growth and development, and ultimately influencing productivity and quality of many plants including turfgrass species[1,2]. Under heat stress, the unfavorable disorders in fundamental metabolic processes often occur in turfgrass species, such as disturbance in homeostasis of reactive oxygen species (ROS)[3,4]. ROS are the by-products of enzymatic reactions, including superoxide anion (O_2^-), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2)[5]. Numerous evidence revealed that abiotic stress triggered overproduction of ROS, which would lead to oxidative damage on nucleic acids, proteins, lipids, and carbohydrates in various plants[6–8].

In order to resist oxidative injuries, plants have evolved a complex antioxidant defense system to scavenge and detoxify the excessive accumulation of ROS to maintain the relative steady balance between generation and elimination of ROS. The defense mechanism is composed of both non-enzymatic and enzymatic systems[9]. Non-enzymatic systems (ascorbic acid, glutathione, and nonprotein amino acids, etc.) are one of an important component of antioxidant defense systems[10]. Acting as electron donors, antioxidants directly or indirectly transform toxic ROS into less harmful oxidized products, and modulate redox homeostasis[11]. Enzymatic system involves several antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and ascorbate peroxidase (APX)[9]. The first line of defense in ROS-scavenging enzyme is SOD that catalyzes O_2^- to H_2O_2 and molecular oxygen (O_2)[11]. Based on different metal co-factors, SOD could be distinguished into three classes including Cu/ZnSOD, MnSOD, and FeSOD, which have various subcellular localization[12]. CAT, POD, and the ascorbate-glutathione cycle further convert H_2O_2 into H_2O and O_2[13]. Ascorbate-glutathione cycle including two antioxidants (ascorbic acid and glutathione), as well as four important enzymes (APX, GR, DHAR, and MDHAR), plays a key role in detoxifying the excess H_2O_2[14,15]. However, redox homeostasis is still destroyed when plants are exposed to prolonged heat stress[16,17]. Therefore, maintaining the stability of antioxidant defense system is essential for enhancing heat tolerance in plants, especially in cool-season grass species[18,19].

As a cool-season turfgrass, creeping bentgrass (*Agrostis stolonifera*) is widely used in golf greens and other high-quality turfs due to its fine texture and superior tolerance to low mowing height[20]. However, creeping bentgrass is sensitive to heat stress, and likely to be injured by heat stress during the summer season[21,22]. Thus, it is an urgent need for turf managers to promote heat tolerance in creeping bentgrass. Previous studies have demonstrated that foliar application of plant growth regulators was an effective and convenient strategy to alleviate stress-induced damages[23,24]. Chitosan is a natural, biocompatible, and biodegradable polysaccharide produced from chitin[25]. Chitosan has nucleophilic behavior, which allows it to form multifunction *via* structural chemical modifications[26]. Exogenous chitosan with the appropriate concentration and method could greatly mitigate harmful

effects of abiotic stresses on plants. Under salt stress, chitosan application led to a significant improvement in salt tolerance through promoting plant growth, and enhancing the expression and activation of alternative oxidase in maize (*Zea mays*) seedlings[27]. Under drought stress, chitosan effectively alleviated drought stress in association with the altered antioxidant metabolism and various metabolic processes in white clover (*Trifolium repens*)[28], bermudagrass (*Cynodon transvaalensis* × *C. dactylon*)[29], and creeping bentgrass[30]. Under heat stress, foliar application of 200 and 800 mg·L^{-1} chitosan had positive effect on mitigating heat damage in kimchi cabbage (*Brassica rapa* ssp. *Pekinensis*)[31] and cotton (*Gossypium hirsutum*)[32], respectively. Exogenous 100 mg·L^{-1} chitosan also improved heat tolerance in creeping bentgrass through regulating chlorophyll metabolism, antioxidant defense, and the heat shock pathway[33]. In our previous study, foliar application of chitosan significantly enhanced heat tolerance in creeping bentgrass by inhibiting the decline in photosynthesis and maintaining cell membrane stability[34]. Despite present knowledge, chitosan-induced regulating mechanism on antioxidant defense system has not been well understood in creeping bentgrass under heat stress. Therefore, the objective of this study was to elucidate whether chitosan-promoted thermotolerance associated with the antioxidant defense system under long-term heat stress, including the content of antioxidants, activities of antioxidant enzymes, as well as the expression level of genes encoding antioxidant enzymes. Our findings would be expected to provide a new understanding for chitosan-induced promotion on heat tolerance in perennial turfgrass species.

Materials and methods

Plant material and growth conditions

Creeping bentgrass seeds (*A. stolonifera* cv. 'Penn A-4') were sown in polyvinyl chloride tubes (25-cm height and 10-cm diameter) filled with sand. Plants were grown for 2 months in a greenhouse at 25/20 °C (day/night) and 14 h photoperiod[34]. During establishment, plants were irrigated daily, fertilized once a week with water-soluble fertilizer (Scotts Miracle-Gro Company, USA), and trimmed every 2 d in order to maintain a canopy height of 4–5 cm. Plants were moved to a growth chamber (XBQH-1, Jinan Xubang, Jinan, Shandong Province, China) with temperature of 25/20 °C (day/night), 14 h photoperiod, and 60% relative humidity. Plants were pre-acclimated to the environment of growth chambers for one week before treatments.

Experimental design and treatments

After acclimation, the two-month-old plants were divided into four groups as four treatments: (1) control + H_2O, foliar application with 10 mL deionized water under non-stressed condition (25/20 °C, day/night); (2) control + chitosan, foliar application with 10 mL chitosan under non-stressed condition (25/20 °C, day/night); (3) heat + H_2O, foliar application with 10 mL deionized water under heat stress (38/28 °C, day/night); (4) heat + chitosan, foliar application with 10 mL chitosan under heat stress (38/28 °C, day/night). During 42 d of treatments, plants were sprayed with either deionized water or chitosan every 7 d. For chitosan treatment, chitosan concentration was set as 100 mg·L^{-1} based on our previous study in creeping bentgrass[34]. This experiment was arranged in a completely randomized design with four biological replicates (four tubes) for each treatment. Each tube was rearranged daily to reduce impacts of the environment.

Turf quality and staining of ROS

Turf quality is a common parameter of overall turf performance rated on a scale of 1 (lowest) to 9 (best) based on texture, color, uniformity, and density[35]. A rating of 1 represented plants that were completely dead with brown leaves, and a rating of 9 indicated plants that were healthy with green and dense turf canopy. Turf quality at the minimal acceptable level was rated a 6.

The presence of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) at 0, 21, and 42 d of treatments were detected based on the method documented by Xu et al.[18] with some modifications. Histochemical staining for H_2O_2 was used 1% (w/v) 3,3-diaminobenzidine (DAB) in 50 mM Tris-HCl buffer (pH 7.5). Staining of O_2^- was used 0.5% (w/v) nitro blue tetrazolium (NBT) in 25 mM HEPES buffer (pH 7.6). All leaves were stained for 16 h at room temperature in darkness. Then, the staining solution was discarded, 85% ethanol was added, and incubated at 85 °C until leaf chlorophyll was dissolved completely. The DAB- or NBT-stained leaves were rinsed with deionized water.

Non-enzymatic antioxidant content and antioxidant enzyme activity measurements

Glutathione content and ascorbic acid content in leaves of creeping bentgrass at 0, 21, and 42 d of treatments were determined using kits (Nanjing Yurun Biotechnology Co., Ltd., Jiangsu, China; Suzhou Comin Biotechnology Co., Ltd., Jiangsu, China). Antioxidant enzyme activity in leaves was measured using the previously described method[36,37] with some modifications. Fresh leaf tissues (0.35 g) at 0, 21, and 42 d of treatments were sampled, then stored at −80 °C to conduct further analysis. Leaves were ground with liquid nitrogen to a fine powder, then extracted with 4 mL of cold extraction buffer (50 mM potassium phosphate, 1 mM ethylenediaminetetraacetic acid, 1% polyvinylpyrrolidone, pH 7.8). The extraction solution was centrifuged at 15,000 g for 30 min at 4 °C and the supernatant was collected for determination of antioxidant enzyme activity. SOD activity was determined based on the rate of NBT reduction in absorbance at 560 nm. CAT, POD, APX, DHAR, MDHAR, and GR activities were assayed following the increase or decrease in absorbance at 240, 470, 290, 265, 340, and 340 nm, respectively. All enzyme activities were determined using a spectrophotometer (Ultrospec 2100 pro, Biochrom Ltd., Cambridge, UK) and expressed on the basis of protein content, which was measured according to Bradford's method[38].

Gene expression analysis of antioxidant enzyme in leaves

The transcription levels of selected gene encoding antioxidant enzyme in leave tissue at 0, 21, and 42 d of treatments were detected by real-time quantitative polymerase chain reaction (qRT-PCR). Total RNA was isolated from leaves with using a FlaPure Plant Total RNA Extraction Kit (Genesand, Beijing, China) according to the manufacturer's instructions. Determination in RNA concentration was conducted by Tecan Infinite 200 pro (Grödig, Austria). The RNA was reverse-transcribed to cDNA by using MonScript™ RTIII Super Mix with dsDNase (Two-Step) (Monad, Wuhan, China).

For detecting transcript levels of genes in heat-treated and chitosan-treated conditions, Roche LightCycler480 II machine (Roche Diagnostic, Rotkreuz, Switzerland) and MonAmp™ ChemoHS qPCR Mix (Monad, Wuhan, China) were used for performing qRT-PCR. Primer sequences of SOD (*AsFeSOD*, *AsCu/ZnSOD*, *AsMnSOD*), CAT (*AsCATA*, *AsCATB*, *AsCATC*), POD (*AsPerox4*), APX (*AsAPX1*, *AsAPX2*, *AsAPX3*, *AsAPX4*, *AsAPX5*, *AsAPX6*, *AsAPX8*), GR (*AsGR1*, *AsGR2*), DHAR (*AsDHAR*), MDHAR (*AsMDHAR*) genes, and reference gene (*ACT2*) are provided in Table 1[39]. The specificity of each primer pair was confirmed by analyzing melting curve in qRT-PCR. PCR condition for all genes was set on the basis of the following parameters: 95 °C at 10 min, 15 s at 95 °C (40 cycles of denaturation), annealing for 15 s at 60 °C, and extending for 20 s at 72 °C. The relative expression level between interest genes and reference gene was to calculate according to $2^{-\Delta\Delta C_T}$ method[40].

Statistical analysis

Data were analyzed using the SPSS statistics software (SPSS 21.0; SPSS Inc., Chicago, IL, USA). The means ± standard error (SE) was calculated for all measured parameters in column charts. ANOVA analysis and Duncan multiple comparison were applied to determine significant differences between mean values for each parameter at the probability level of 0.05.

Results

Turf performance and ROS

Heat stress led to a significant decrease in turf quality at 21 and 42 d of treatments, and increased accumulation of both H_2O_2 and O_2^- as reflected by the darker color in leaves of creeping bentgrass at 42 d of treatments compared with non-stressed control regardless of chitosan application (Figs 1 & 2). Chitosan-treated plants kept better shoot phenotype and showed 1.2-fold increase in turf quality than untreated plants at 42 d of heat stress (Fig. 1a, b). Exogenous chitosan played a positive effect on decreasing the accumulation of both H_2O_2 and O_2^- under 42 d of heat stress (Fig. 2a, b).

Non-enzymatic antioxidant content

At 21 and 42 d under heat stress, chitosan-treated plants had 54% and 32% higher ascorbic acid content than untreated plants, respectively (Fig. 3a). Plants with or without chitosan under heat stress exhibited an obviously decrease in glutathione content at 21 and 42 d compared with non-stressed control (Fig. 3b). Chitosan-treated plants had higher glutathione content than untreated plants at 21 d of heat stress, but were not observed the significant difference in glutathione content at 42 d of heat treatment.

Antioxidant enzyme activity

At 21 d of non-stressed treatment, exogenous chitosan caused 38% and 48% significant increase in activities of SOD and POD, respectively, compared with untreated plants (Fig. 4a, c). At 21 d of treatments, heat stress significantly decreased POD activity compared with plants under non-stressed condition (Fig. 4c). At 42 d, activities of SOD, CAT, and POD increased significantly in response to heat stress compared with plants under non-stressed condition, but no significant difference in activities of SOD and CAT was found between plants with or without chitosan (Fig. 4a–c). Foliar application of chitosan had 30% higher POD activity than untreated plants at 42 d of heat stress.

At 21 d of non-stressed condition, exogenous chitosan significantly increased APX activity compared with plants without chitosan. Chitosan-treated plants had 58% higher increase in APX activity than untreated plants at 42 d of heat stress (Fig. 5a). Heat stress significantly increased activities of both GR and DHAR compared with non-stressed control at 21 and 42 d. Plants applied with chitosan demonstrated significant enhancement in GR and DHAR activities compared with untreated plants at 42 d under heat stress (Fig. 5b, c). Exogenous chitosan significantly increased MDHAR activity by 94% compared with untreated plants at 21 d of heat stress (Fig. 5d), but there was no significant difference in MDHAR activity between chitosan-treated and untreated plants at 42 d of heat stress.

Table 1. Primer sequences used in the experiment.

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
AsFeSOD	TGCTCGTCTGTCATCCTTGT	GGTTGGGTTTGGCTTGTCTT
AsCu/ZnSOD	AATGTGACAGCTGGAGTGGA	CCCTTGCCAAGATCATCAGC
AsMnSOD	AGGAACCAGGTTTGCTCCTT	GATGAATGCAGAGGGTGCTG
AsCATA	TACTCCGACGACAAGATGCT	TTCTTGAATCCGCACTTGGG
AsCATB	AGTGGATTCCAGGGACAGTG	GACCATCGATGCAGATCACG
AsCATC	CCTGGCTGCTTGAAGTTGTT	ACTTCCCGTCCAGGTTTGAT
AsPerox4	GATGTTGCCCATCTTGACCA	ACTACAGCAACCTCCTGTCC
AsAPX1	CTCCTACGCCGATCTCTACC	TGCCGAAGACTTGCCTTAGA
AsAPX2	GGAGAGAGGACAAGCCTGAG	AAACCCATCTGAGCGGAGAA
AsAPX3	TACATCGCGGAGATCGAGAG	GATCTTGAGCCCTGCATTGG
AsAPX4	CTGCAACTACTCCAGCAAGC	CACAAGAACTGGTGGTGCAA
AsAPX5	GCGGCTTAGTCAAGGAGTTG	CGACGAGATGGTCTCTGACA
AsAPX6	CAAGTCTCTGCATGGAACGG	CCATACTTTGCTGCTGCCAT
AsAPX8	TCCTTGTCATCAAGGCCCAT	CACAGCTCCTGAGCAATGTC
AsDHAR	TGCGTGAACTCTATCGCTCT	GAGCGTGCAGCTCCATTATT
AsGR1	TCCTCCGCAGTCCACATATC	GTTAGGGTTTGGAGGGTGGT
AsGR2	CACACGGCGAAACACATACT	AGAATCACAGCACGTTTCGG
AsMDHAR	GCACGTACTGGGTCAAAGAC	TTCATATGTTGGCGGCGAAG
ACT2	CCTTTTCCAGCCATCTTTCA	GAGGTCCTTCCTGATATCCA

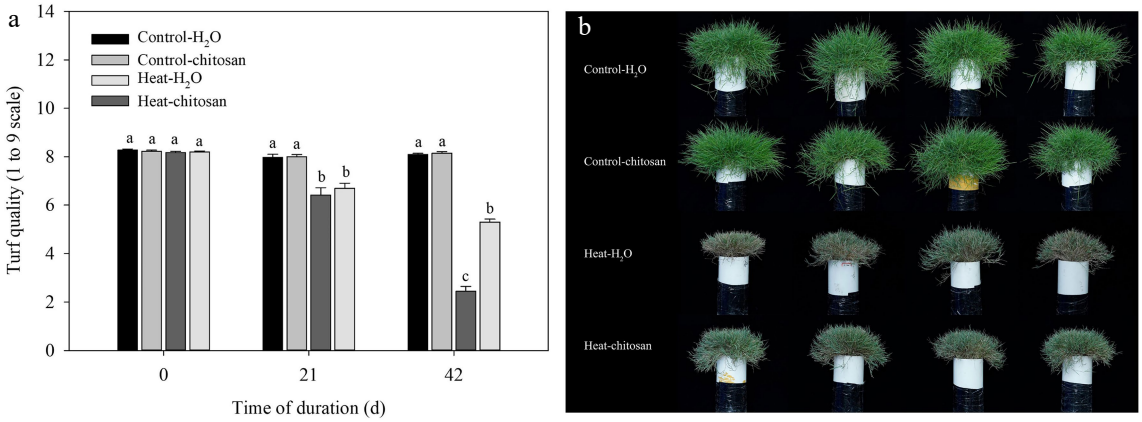

Fig. 1 Effects of heat stress and exogenous chitosan on (a) turf quality and (b) shoot phenotype in creeping bentgrass exposed to non-stressed control and heat stress. Control + H_2O, foliar application with deionized water under non-stressed condition (25/20 °C, day/night); Control + chitosan, foliar application with 100 mg·L^{-1} chitosan under non-stressed condition (25/20 °C, day/night); Heat + H_2O, foliar application with deionized water under heat stress (38/28 °C, day/night); Heat + chitosan, foliar application with 100 mg·L^{-1} chitosan under heat stress (38/28 °C, day/night). Shoot phenotype was taken at 42 d of treatments. Different lowercase letters represent significant difference between different treatments during the experimental period ($P \leq 0.05$). Error bars represent standard error (SE).

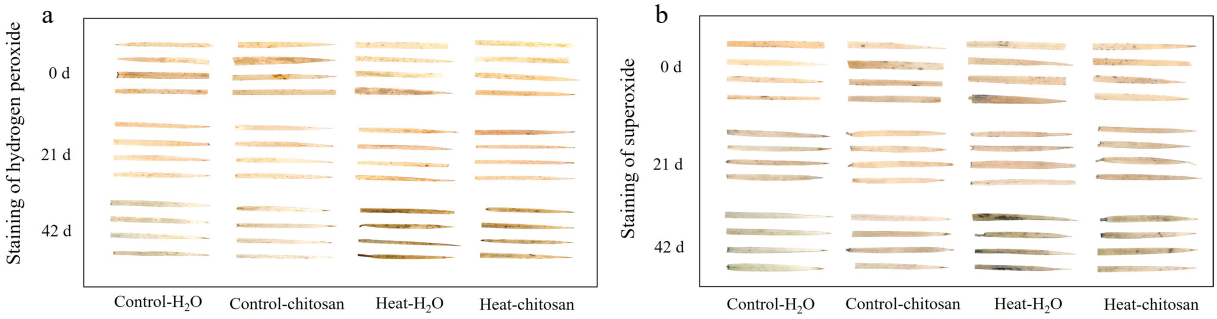

Fig. 2 Effects of heat stress and exogenous chitosan on staining of (a) hydrogen peroxide and (b) superoxide in creeping bentgrass. Control + H_2O, foliar application with deionized water under non-stressed condition (25/20 °C, day/night); Control + chitosan, foliar application with 100 mg·L^{-1} chitosan under non-stressed condition (25/20 °C, day/night); Heat + H_2O, foliar application with deionized water under heat stress (38/28 °C, day/night); Heat + chitosan, foliar application with 100 mg·L^{-1} chitosan under heat stress (38/28 °C, day/night).

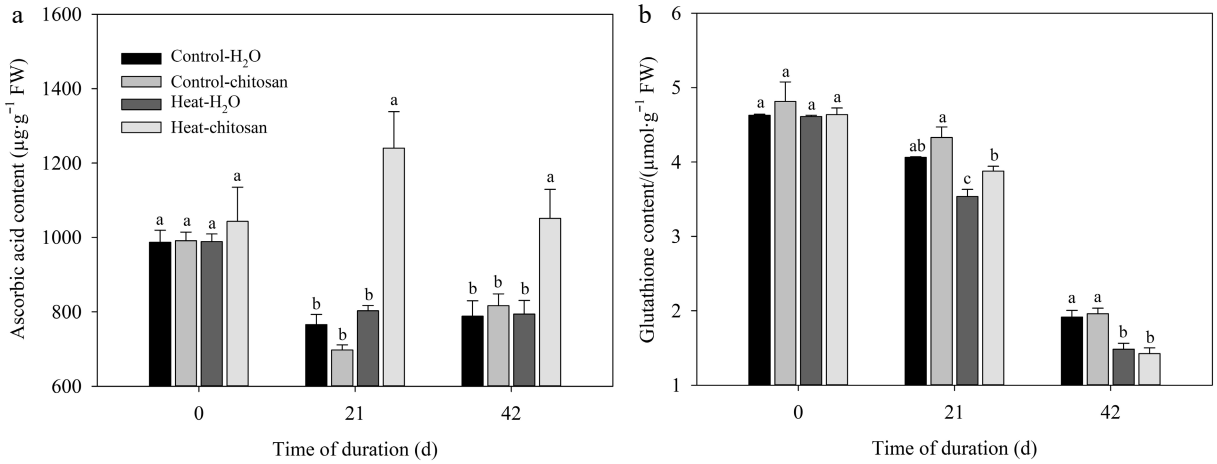

Fig. 3 Effects of heat stress and exogenous chitosan on content of (a) ascorbic acid and (b) glutathione in creeping bentgrass. Control + H_2O, foliar application with deionized water under non-stressed condition (25/20 °C, day/night); Control + chitosan, foliar application with 100 mg·L^{-1} chitosan under non-stressed condition (25/20 °C, day/night); Heat + H_2O, foliar application with deionized water under heat stress (38/28 °C, day/night); Heat + chitosan, foliar application with 100 mg·L^{-1} chitosan under heat stress (38/28 °C, day/night). Different lowercase letters represent significant difference between different treatments during the experimental period ($P \leq 0.05$). Error bars represent standard error (SE).

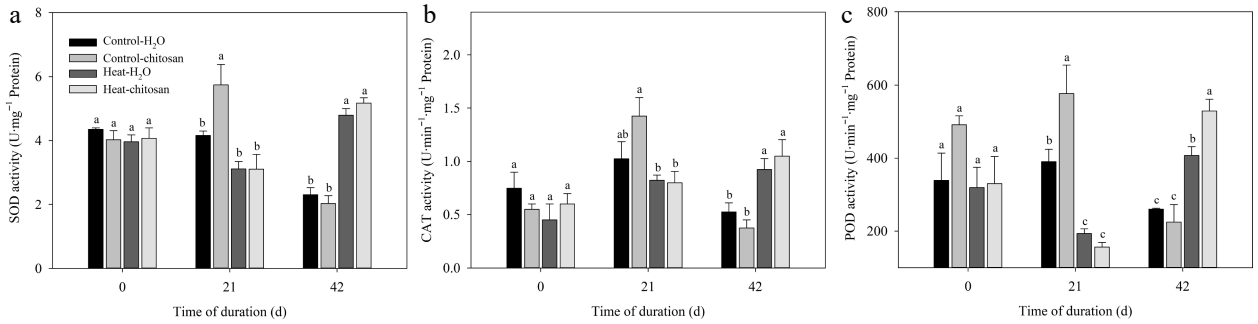

Fig. 4 Effects of heat stress and exogenous chitosan on activities of (a) SOD, (b) CAT, and (c) POD in creeping bentgrass. Control + H_2O, foliar application with deionized water under non-stressed condition (25/20 °C, day/night); Control + chitosan, foliar application with 100 mg·L^{-1} chitosan under non-stressed condition (25/20 °C, day/night); Heat + H_2O, foliar application with deionized water under heat stress (38/28 °C, day/night); Heat + chitosan, foliar application with 100 mg·L^{-1} chitosan under heat stress (38/28 °C, day/night). Different lowercase letters represent significant difference between different treatments during the experimental period ($P \leq 0.05$). Error bars represent standard error (SE).

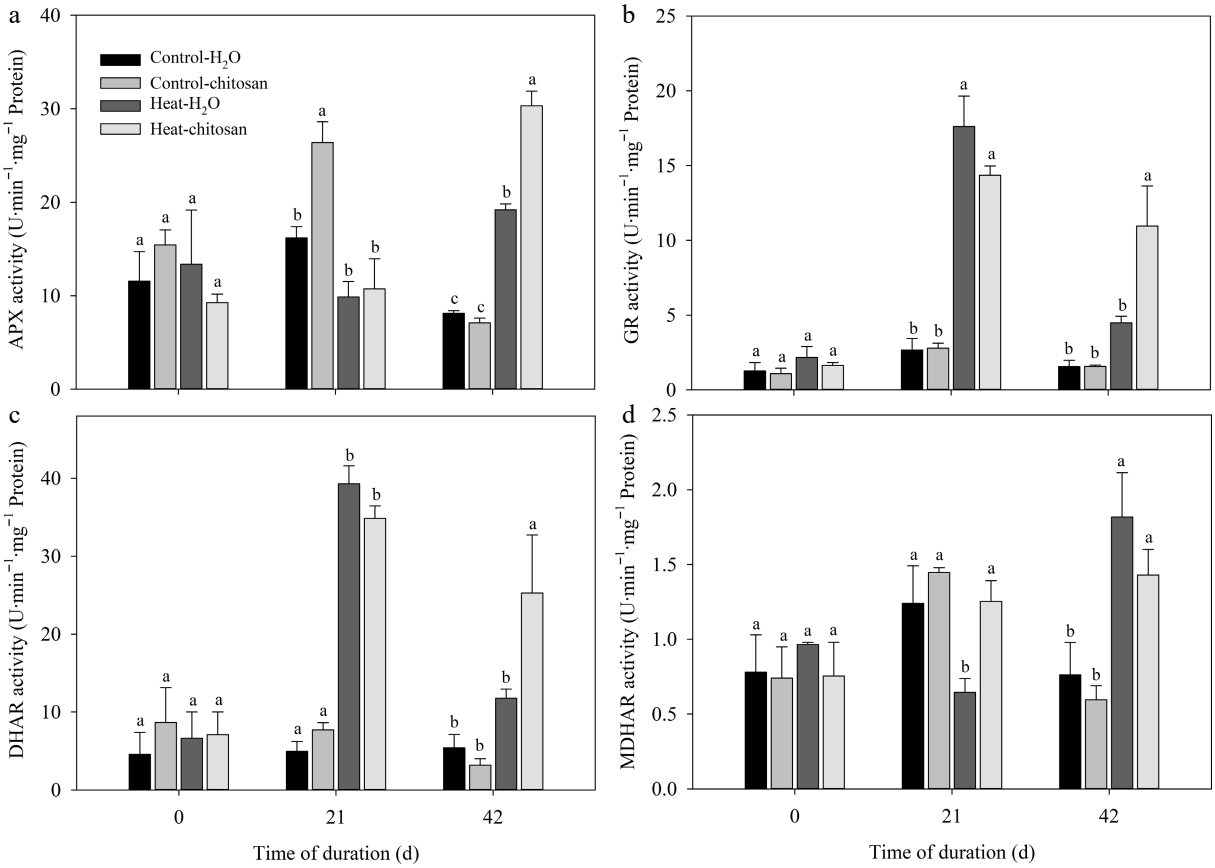

Fig. 5 Effects of heat stress and exogenous chitosan on activities of (a) APX, (b) GR, (c) DHAR, and (d) MDHAR in creeping bentgrass. Control + H_2O, foliar application with deionized water under non-stressed condition (25/2 °C, day/night); Control + chitosan, foliar application with 100 mg·L^{-1} chitosan under non-stressed condition (25/20 °C, day/night); Heat + H_2O, foliar application with deionized water under heat stress (38/28 °C, day/night); Heat + chitosan, foliar application with 100 mg·L^{-1} chitosan under heat stress (38/28 °C, day/night). Different lowercase letters represent significant difference between different treatments during the experimental period ($P \leq 0.05$). Error bars represent standard error (SE).

Gene expression of antioxidant enzyme

The expression levels of SOD (*AsFeSOD*, *AsCu/ZnSOD*, and *AsMnSOD*), CAT (*AsCATA*, *AsCATB*, and *AsCATC*), and POD (*AsPerox4*) genes in leaves were measured in this experiment (Figs 6 & 7). For SOD genes, expression levels of both *AsCu/ZnSOD* and *AsMnSOD* were down-regulated and up-regulated significantly compared with non-stressed control at 21 d and 42 d, respectively (Fig. 6b, c). Chitosan-treated plants showed 1.1-fold higher expression level in *AsCu/ZnSOD* than untreated plants at 42 d of heat stress (Fig. 6b). At 42 d of heat stress, exogenous chitosan significantly up-regulated the expression of *AsCATB* by 82% compared with untreated plants, but without significant effects on *AsCATA* and *AsCATC* genes (Fig. 7a–c). Chitosan-treated plants had 1.2-fold higher expression levels of *AsPerox4* than that without chitosan at 42 d of heat stress (Fig. 7d).

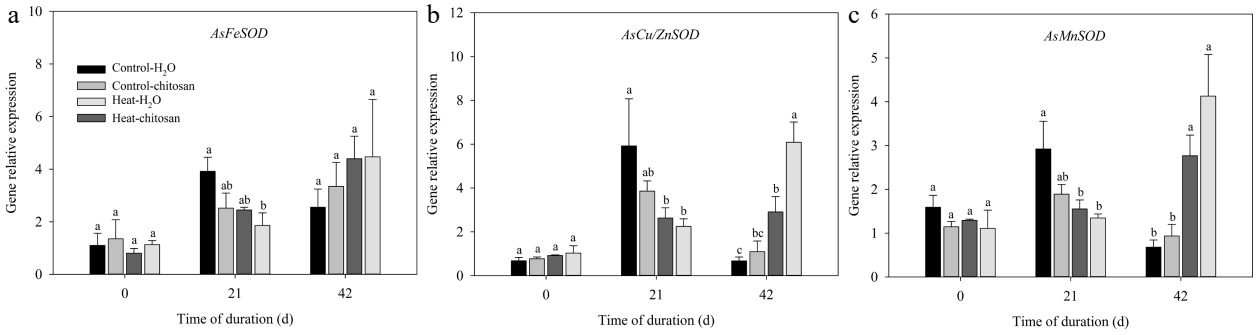

Fig. 6 Effects of heat stress and exogenous chitosan on expression levels of (a) *AsFeSOD*, (b) *AsCu/ZnSOD*, and (c) *AsMnSOD* in creeping bentgrass. Control + H_2O, foliar application with deionized water under non-stressed condition (25/20 °C, day/night); Control + chitosan, foliar application with 100 mg·L^{-1} chitosan under non-stressed condition (25/20 °C, day/night); Heat + H_2O, foliar application with deionized water under heat stress (38/28 °C, day/night); Heat + chitosan, foliar application with 100 mg·L^{-1} chitosan under heat stress (38/28 °C, day/night). Different lowercase letters represent significant difference between different treatments during the experimental period ($P \leq 0.05$). Error bars represent standard error (SE).

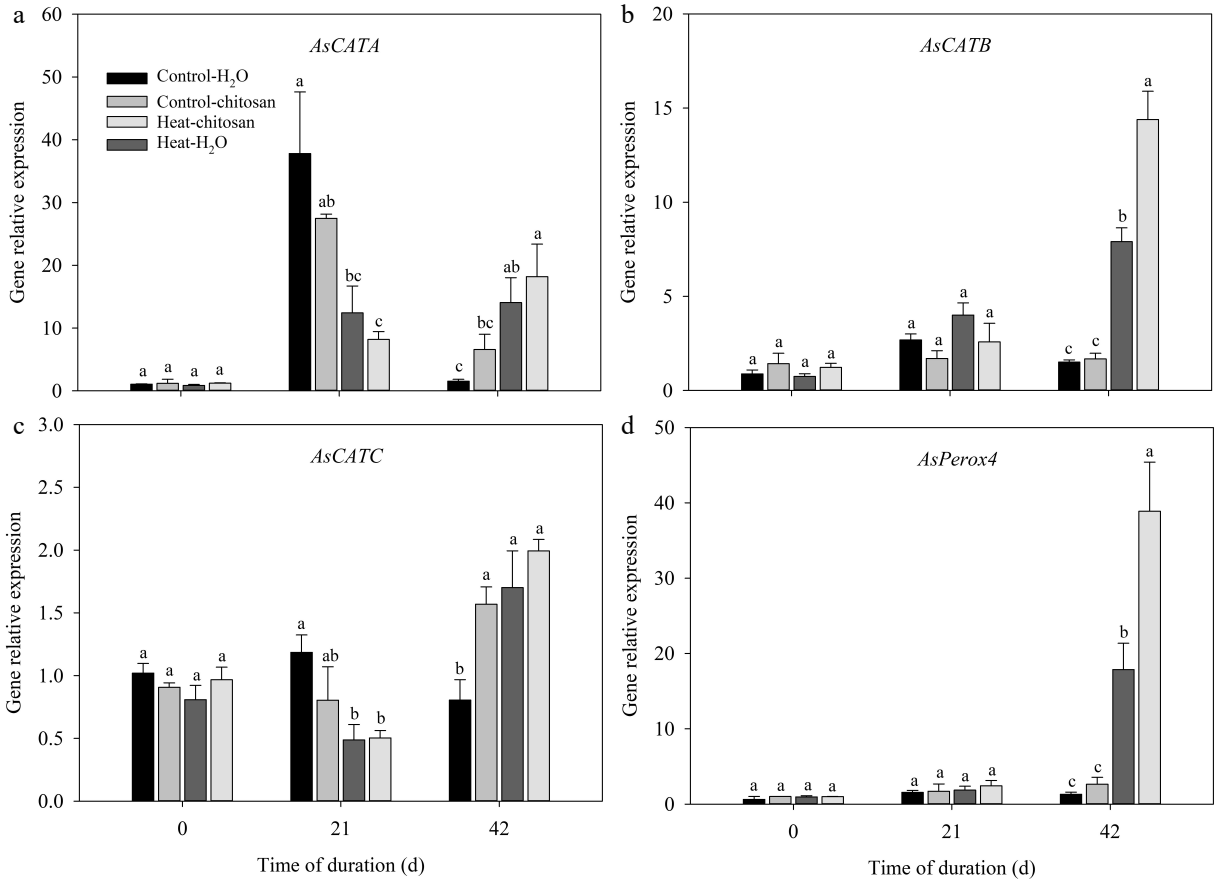

Fig. 7 Effects of heat stress and exogenous chitosan on expression levels of (a) *AsCATA*, (b) *AsCATB*, (c) *AsCATC*, and (d) *AsPerox4* in creeping bentgrass. Control + H_2O, foliar application with deionized water under non-stressed condition (25/20 °C, day/night); Control + chitosan, foliar application with 100 mg·L^{-1} chitosan under non-stressed condition (25/20 °C, day/night); Heat + H_2O, foliar application with deionized water under heat stress (38/28 °C, day/night); Heat + chitosan, foliar application with 100 mg·L^{-1} chitosan under heat stress (38/28 °C, day/night). Different lowercase letters represent significant difference between different treatments during the experimental period ($P \leq 0.05$). Error bars represent standard error (SE).

The transcript levels of APX, GR, DHAR, and MDHAR genes related to ascorbate-glutathione cycle were also quantified (Figs 8 & 9). Under normal condition, exogenous chitosan significantly up-regulated *AsAPX1* compared with untreated plants at 21 and 42 d. The expression level of *AsAPX1* in chitosan-treated plants also higher than untreated plants at 21 d of heat stress (Fig. 8a). At 42 d of heat stress, expressions of different APX genes were significantly up-regulated to certain extent due to foliar application of chitosan, including *AsAPX2*, *AsAPX3*, *AsAPX4*, *AsAPX6*, and *AsAPX8*, which demonstrated 1.4-fold, 1.2-fold, 1.0-fold, 0.8-fold, and 1.1-fold higher than untreated plants, respectively (Fig. 8b–d, f, g). At 42 d of

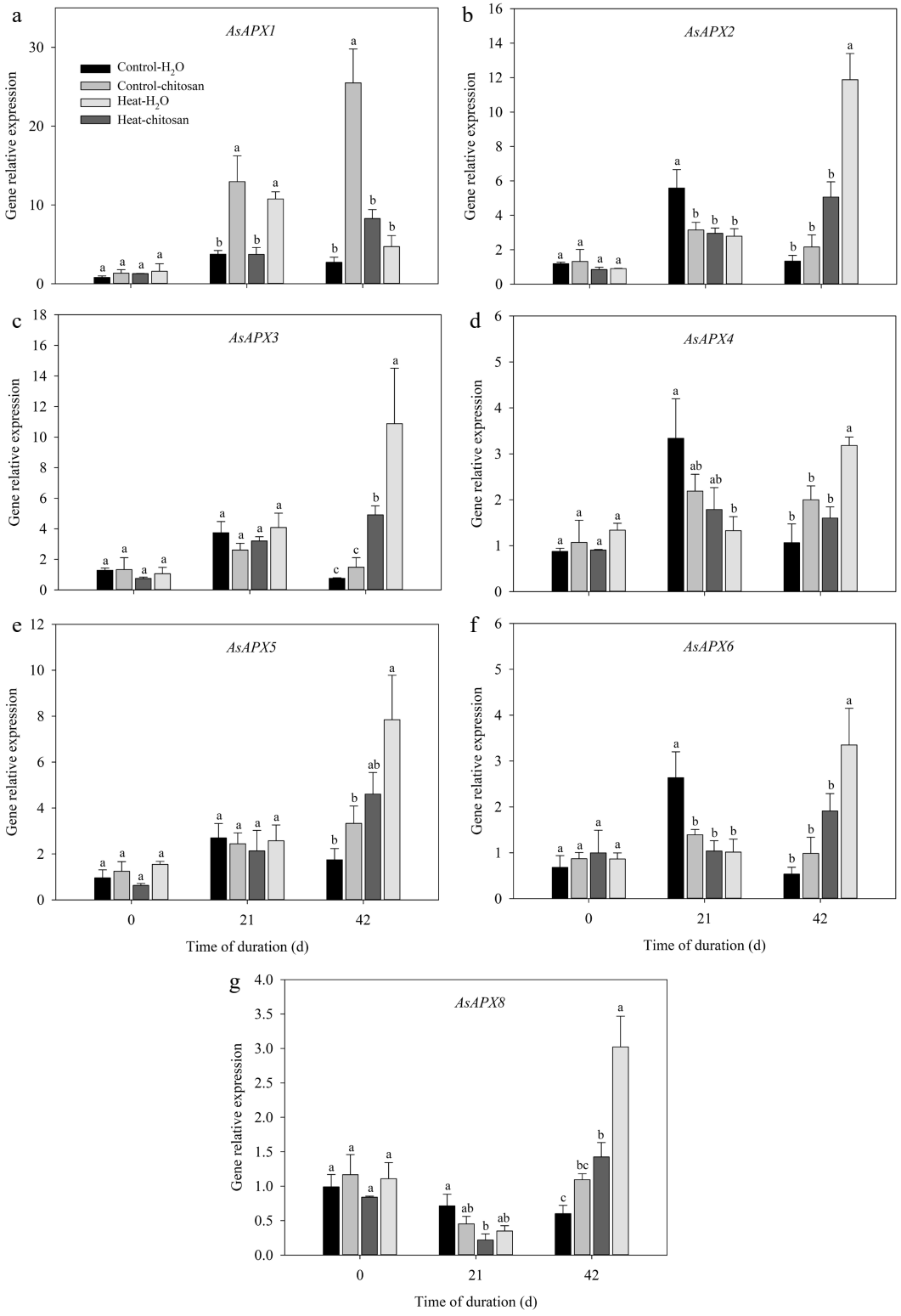

Fig. 8 Effects of heat stress and exogenous chitosan on expression levels of (a) *AsAPX1*, (b) *AsAPX2*, (c) *AsAPX3*, (d) *AsAPX4*, (e) *AsAPX5*, (f) *AsAPX6*, and (g) *AsAPX8* in creeping bentgrass. Control + H_2O, foliar application with deionized water under non-stressed condition (25/20 °C, day/night); Control + chitosan, foliar application with 100 mg·L^{-1} chitosan under non-stressed condition (25/20 °C, day/night); Heat + H_2O, foliar application with deionized water under heat stress (38/28 °C, day/night); Heat + chitosan, foliar application with 100 mg·L^{-1} chitosan under heat stress (38/28 °C, day/night). Different lowercase letters represent significant difference between different treatments during the experimental period ($P \leq 0.05$). Error bars represent standard error (SE).

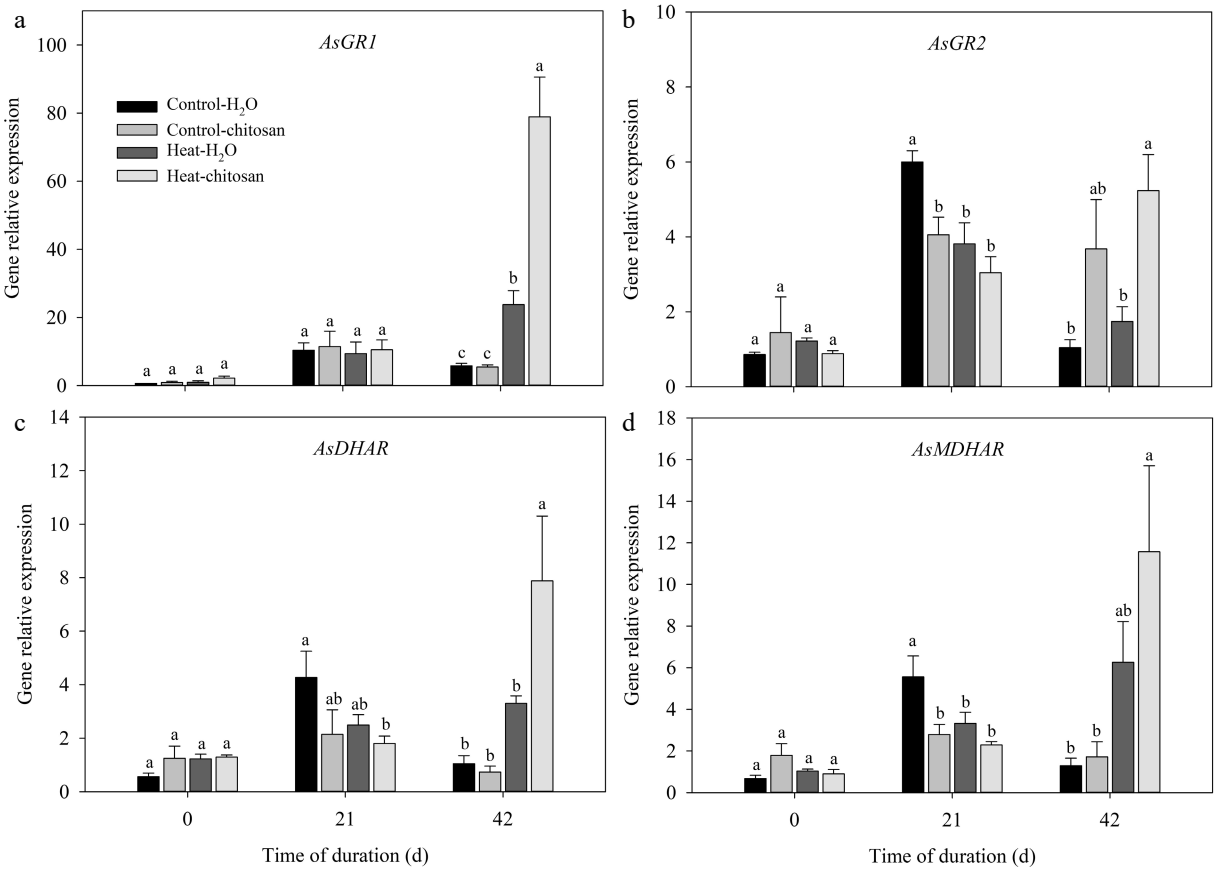

Fig. 9 Effects of heat stress and exogenous chitosan on expression levels of (a) *AsGR1*, (b) *AsGR2*, (c) *AsDHAR*, and (d) *AsMDHAR* in creeping bentgrass. Control + H_2O, foliar application with deionized water under non-stressed condition (25/20 °C, day/night); Control + chitosan, foliar application with 100 mg·L^{-1} chitosan under non-stressed condition (25/20 °C, day/night); Heat + H_2O, foliar application with deionized water under heat stress (38/28 °C, day/night); Heat + chitosan, foliar application with 100 mg·L^{-1} chitosan under heat stress (38/28 °C, day/night). Different lowercase letters represent significant difference between different treatments during the experimental period ($P \leq 0.05$). Error bars represent standard error (SE).

treatments, transcript levels of both *AsGR1* and *AsMDHAR* were significantly up-regulated compared with non-stressed control in response to heat stress, however, there was no significant difference in the expression of these genes between chitosan-treated and untreated plants under heat stress (Fig. 9a, d). Chitosan-treated plants showed 2.0-fold and 1.4-fold increase in expression level of both *AsGR2* and *AsDHAR*, respectively, compared with untreated plants at 42 d of heat stress (Fig. 9b, c).

Discussion

It is generally known that the application of plant growth regulators is an effective method to enhance thermotolerance, which might be associated with changes in various physiological and biochemical processes under heat stress[41–43]. In this study, heat stress led to serious damages in creeping bentgrass, but chitosan application induced an improvement in heat tolerance as indicated by the reduction in ROS accumulation, better maintenance in antioxidant defense system, as well as the higher gene expression compared with the untreated plants under heat stress as discussed below.

The imbalance between the production and scavenging of ROS in heat-stressed plant resulted in oxidative stress, and influenced normal physiological activities in plants[44,45]. Jahan et al.[46] observed a significantly increased ROS (O_2^- and H_2O_2) content and decreased cellular membrane integrity in tomato (*Solanum lycopersicum*) seedings subjected to heat stress. Thus, maintaining ROS homeostasis is an important strategy to enhance heat tolerance, which is benefitial to promote plant growth and development[47,48]. Results in this study also showed that heat stress promoted ROS overaccumulation and decreased turf quality. Chitosan-treated plants had lower accumulation of both H_2O_2 and O_2^-, and superior turf quality in comparison to untreated plants under heat stress (Figs 1 & 2), which was consistent with previous report in creeping bentgrass[33]. Meanwhile, heat-induced adverse effects on cell membrane stability and photosynthesis were significantly alleviated in plants treated with exogenous chitosan according to our previous study[34]. Similar reports about better maintenance in ROS homeostasis in association with the enhancement in heat tolerance were also found in other plants, such as in wheat (*Triticum aestivum*)[49], tall fescue (*Festuca arundinacea*)[50], and perennial ryegrass (*Lolium perenne*)[51]. The result suggested that exogenous chitosan could contribute to the improved heat tolerance through maintaining the equilibrium between the ROS generation and elimination.

ROS scavenging system plays a key role in mitigating the adverse effects of oxidative stress and conferring heat tolerance in turfgrass species[52,53]. Various non-enzymatic

antioxidants and antioxidant enzymes including SOD, CAT, POD, and ascorbate-glutathione cycle components (ascorbic acid, glutathione, APX, GR, DHAR, and MDHAR) functions in detoxifying the overproduction of ROS[9]. Ascorbate-glutathione cycle is also referred to as the Asada–Halliwell pathway, which is a crucial antioxidant defense pathway to detoxify H_2O_2 and remain redox homeostasis in plant cells[54]. In lettuce (*Lactuca sativa*) seedlings, exogenous spermidine positively alleviated oxidative damage via enhancing ascorbate-glutathione cycle under heat stress[55]. Antioxidant enzymes involved in ascorbate-glutathione cycle showed a significant activation due to melatonin application, mitigating heat-induced damage in wheat seedlings[56]. In the present study, heat stress induced a significant increase in multiple antioxidant enzyme activities indicating that antioxidant defense system was triggered in response to heat stress to prevent plants from severe injury. Chitosan application improved heat tolerance mainly associated with positive changes in POD activities and ascorbate-glutathione cycle, as confirmed by significantly increased ascorbic acid content, POD, APX, GR, and DHAR activities compared with untreated plant under heat stress (Figs 3, 4 & 5). Huang et al.[33] previously found that chitosan-pretreated plants had higher heat tolerance through improving activities of four antioxidant enzymes (SOD, CAT, POD, and APX) at 15 d of heat stress. But in our study, no significant difference in activities of SOD and CAT was found between plants with or without chitosan at 42 d of long-term heat stress. Generally, activities of SOD and CAT increased and then declined under heat stress[57]. The different change in activities of SOD and CAT might indicate that both enzymes played an important role in the early duration of treatments. Moreover, heat stress also induced the up-regulation in transcriptional level of antioxidant enzyme genes in order to protect cellular components against oxidative stress[58]. Li et al.[19] revealed that exogenous application of γ-aminobutyric acid up-regulated the transcript of genes encoding antioxidant enzymes (SOD, CAT, POD, APX, MDHAR, DHAR, and GR) under water deficit stress. In this study, exogenous chitosan significantly up-regulated several relative gene expressions compared with untreated plants under heat stress, including SOD (*AsCu/ZnSOD*), CAT (*AsCATB*), POD (*AsPerox4*), APX (*AsAPX2*, *AsAPX3*, *AsAPX4*, *AsAPX6*, and *AsAPX8*), GR (*AsGR2*), and DHAR genes (*AsDHAR*) (Figs 6, 7, 8&9). Results showed that exogenous chitosan induced a significant increase in POD, APX, GR, and DHAR at both enzymatic and gene expression levels under heat stress. However, antioxidant enzyme activities were regulated by multiple factors, which might lead to variations in the transcript levels of antioxidant enzymes inconsistent with antioxidant enzyme activities[59]. Therefore, in the current study, foliar application of chitosan up-regulated transcript levels of *AsCu/ZnSOD* and *AsCATB*, but did not enhance activities of SOD and CAT. Our above results indicated that chitosan-induced promotion in heat tolerance resulted from alterations in antioxidant defense system including non-enzymatic antioxidants, antioxidant enzymes, as well as relative gene expression for ROS scavenging in creeping bentgrass.

In conclusion, exogenous chitosan significantly enhanced heat tolerance in creeping bentgrass. Chitosan-induced heat tolerance could be attributed to the less oxidative damage and better maintenance of antioxidant defense system detoxifying ROS. Foliar application of chitosan suppressed overproduction of ROS (H_2O_2 and O_2^-), improved ascorbic acid content and antioxidant enzymes activities (POD, APX, GR, and DHAR), as well as up-regulated transcript levels of *AsCu/ZnSOD*, *AsCATB*, *AsPerox4*, *AsAPX2, AsAPX4, AsAPX6, AsAPX8*, *AsGR2*, and *AsDHAR* compared with untreated control. In the future, further investigating into the molecular mechanisms of chitosan in improving heat tolerance is necessary.

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Conflict of interest

The authors declare that they have no conflict of interest.

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