

Foliar application of Karrikin₁ enhanced heat tolerance of creeping bentgrass by activating antioxidant defense, signaling transduction, and stress responsive pathways

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Abstract

Karrikin (KAR), a recently identified plant growth regulator, functions in seed germination, seedling development, and abiotic stress adaptation. Despite its recognized biological functions, the regulatory role and underlying mechanisms of KAR in mediating heat tolerance of turfgrass remain largely unknown. In the present study, creeping bentgrass plants were treated with or without KAR₁ (100 nM) and subjected to two temperature treatments (25/20 and 35/30 °C, day/night) for 20 d. Physiological parameters, antioxidant enzyme activities, as well as expression levels of genes involved in stress signaling and heat stress responses were analyzed. The results showed that heat stress caused negative effects on creeping bentgrass, including leaf water loss and membrane lipid peroxidation. Exogenous application of KAR₁ alleviated heat-induced damage on creeping bentgrass, resulting in improved leaf relative water content, decreased leaf electrolyte leakage, and increased antioxidant enzyme activities (including ascorbate peroxidase, catalase, and superoxide dismutase). In addition, KAR₁ treatment up-regulated the expression of KAR/KAI2-Ligand signal transduction genes (*AsKAI2*, *AsDLK2*), phytohormone biosynthesis and signaling genes (*AsSnRK2.6*, *AsICS1*), transcription factors (*AsNAC053*, *AsbZIP58*, *AsHSFB-2b*, *AsHSFB-4d*), antioxidant defense genes (*AsAPX1*, *AsAPX2*, *AsCATA*, *AsCATC*, *AsFe-SOD*, *AsCuZn-SOD*, *AsMn-SOD*), and heat shock proteins (*AsHSP12*, *AsHSP16*, *AsHSP70*, *AsHSP90.5*) under heat stress. These results suggested that KAR₁ might act as an effective agent to enhance the heat tolerance of creeping bentgrass, which provides a new perspective on cool-season lawn maintenance.

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Introduction

As temperatures increase, heat stress has become a primary abiotic stress limiting plant growth and development^[1]. Heat stress induces a cascade of detrimental physiological effects, including protein denaturation, enzyme inactivation, and membrane lipid peroxidation, disrupting critical metabolic pathways, such as photosynthesis and respiration, and affecting cell membrane permeability, causing electrolyte leakage and water loss^[2–4]. In addition, heat stress results in the production of excessive reactive oxygen species (ROS), which can lead to oxidative stress and damage cellular components, including proteins, lipids, and nucleic acids, further impairing cell function^[5].

Plants have evolved a series of physiological and molecular mechanisms to mitigate heat damage and help them adapt to high temperatures^[6]. For instance, antioxidant defense pathways, heat stress response pathways, hormone biosynthesis or signaling pathways, as well as transcriptional regulation pathways, play significant roles in the regulation of heat stress responses^[7]. The accumulation of non-enzymatic antioxidants like carotenoids and enzymatic antioxidants, such as ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD), help to clear overproduced ROS and improve the thermotolerance^[8]. The enhanced heat tolerance of maize (*Zea mays*) seedlings was associated with increased expression levels of genes involved in salicylic acid (SA) biosynthesis (*PHENYLALANINE AMMONIA-LYASE 1*, *ZmPAL1*; and

ISOCHORISMATE SYNTHASE 1, *ZmICS1*), resulting in elevated endogenous SA levels to scavenge ROS^[9]. In addition, transcriptional factors (TFs), such as tryptophan-arginine-lysine-tyrosine (WRKY), myeloblastosis (MYB), NAM, ATAF1/2, and CUC2 (NAC), basic leucine zipper (bZIP), basic helix-loop-helix (bHLH), and heat shock transcription factors (HSFs) have been proven to be closely related to heat tolerance in plants^[7,10]. A class HSF member, *HSFA3*, enhances thermotolerance including positively regulating expression of *HSFA2a* in perennial ryegrass (*Lolium perenne*)^[11]. Overexpression of *ZmHSF21* from maize increased POD, SOD, and CAT activities and decreased ROS accumulation in *Arabidopsis* under heat stress^[12]. *PIWRKY47* directly binds to the promoter of *cytosolic glyceraldehyde-3-phosphate dehydrogenase 2* (*PIGAPC2*), activates *PIGAPC2* expression, inhibits ROS generation, and enhances thermotolerance in herbaceous peony (*Paeonia lactiflora*)^[13]. Similarly, *NAC042* is an important NAC TF to regulate thermal memory, and overexpression of *NAC042* could induce the expression levels of several HSFs and heat shock proteins (HSPs), thus improving heat tolerance in *Arabidopsis*^[14].

Foliar application of exogenous substances can effectively improve heat tolerance in plants, by maintaining a favorable antioxidative level, membrane stability, and water status^[15,16]. Karrikin (KAR), which was initially isolated from smoke from burning plant material, is defined as a kind of novel hormone analog^[17]. It has been demonstrated that KAR have a butenolide moiety similar to strigolactone (SL) and have the capacity to promote seed germination, and the subsequent growth and development of seedlings of

many plant species under various stress conditions^[18–20]. For instance, exogenous application of KAR₁ enhanced the germination rate of wheat (*Triticum aestivum*) seeds under salt stress, and alleviated salt stress damage via maintaining redox and K⁺/Na⁺ homeostasis in wheat seedlings^[18]. KAR₁ can enhance drought tolerance in creeping bentgrass by activating KAR-responsive genes and TFs, promoting proline accumulation and antioxidant capacity, as well as suppressing leaf senescence^[20]. KAR₁ treatment significantly increased antioxidant enzyme activities, as well as the contents of organic acids and amino acids, and altered the expression of abscisic acid (ABA) signaling genes in oil plant *Sapium sebiferum* under both drought and salt stress conditions^[21]. In addition, KAR₁ has been shown to interact with plant hormones, including ABA, SA, cytokinin (CK), gibberellin (GA), auxin (IAA), and ethylene (ETH), to stimulate seed germination and seedling growth under abiotic stresses^[21–25]. The KAR receptor KARRIKIN INSENSITIVE2 (KAI2)-mediated signaling positively regulates heat tolerance in *Arabidopsis thaliana* via the activation of HSF-related and HSP-related pathways^[26].

Although previous research indicates that KAR₁ plays a crucial role in regulating seed germination and abiotic stress tolerance in plants, how KAR₁ affects signal transduction, antioxidant defense and stress-responsive gene expression in regulating plant thermotolerance remains largely uncharacterized. Creeping bentgrass (*Agrostis stolonifera*) is an important perennial turfgrass species that is widely used on golf courses, tennis courts, and in landscaping worldwide^[27]. However, as a cool-season grass, creeping bentgrass is sensitive to high temperatures, and heat waves during summer months may cause detrimental effects on creeping bentgrass^[28]. The purpose of this study is to investigate the effects of the exogenous application of KAR₁ on water balance, membrane integrity, antioxidant defense, and expression of stress response and signaling genes under heat stress. This study would be useful in revealing potential KAR₁-mediated heat tolerance pathways and provide a solution for mitigating heat damage in creeping bentgrass.

Materials and methods

Plant materials and growth conditions

Seeds of creeping bentgrass (*A. stolonifera* cv. 'Penn-A4') were sown in PVC tubes (11 cm in diameter and 25 cm in height) filled with a mixture of potting soil and vermiculite (V:V, 3:1). Plants were established in a growth chamber (XBQH-1, Jinan Xubang, Jinan, Shandong Province, China) for 2 months with 25/20 °C (day/night), 14 h photoperiod, 70% relative humidity, and 650 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetically active radiation. Plants were trimmed weekly to maintain the canopy height at about 3 cm and fertilized once a week with half-strength Hoagland's nutrient solution^[29].

KAR₁ and stress treatments

KAR₁ (3-methyl 2H-furo [2,3-c] pyran-2-one) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). The KAR₁ stock and working solution were prepared according to Shah et al.^[21]. For KAR₁ treatment, 20 mL of 100 nM KAR₁ solution was foliar-sprayed onto the canopy before heat stress and every 5 d during heat stress. The control plants were sprayed with water. The concentration for KAR₁ was selected according to the study by Tan et al.^[20]. Plants were treated with the following treatments for 20 d: (a) KAR₁-treated plants exposed to heat stress (35/30 °C, day/night) (KH), (b) KAR₁-treated plants exposed to optimal temperature (25/20 °C, day/night) (KC), (c) water-treated plants exposed to heat stress (CH), and (d) water-treated plants exposed to optimal temperature (CC). Each treatment had four biological replicates.

Determination of relative water content and electrolyte leakage

Leaf relative water content (RWC) and electrolyte leakage (EL) were measured at 20 d of heat stress. Leaf RWC was determined using fully expanded leaves, as described by Barrs & Weatherley^[30]. Approximately 0.1 g leaf samples were cut and immediately weighed as fresh weight (FW), then immersed in deionized water for 24 h at 4 °C in the dark and immediately weighed as turgid weight (TW). Leaf samples were then dried in an oven at 80 °C for at least 72 h to estimate dry weight (DW). The RWC was calculated following the formula: $\text{RWC} = (\text{FW} - \text{DW})/(\text{TW} - \text{DW}) \times 100\%$. Leaf EL was determined to indicate cell membrane stability. In brief, about 0.1 g of leaves were placed into centrifuge tubes filled with 35 mL deionized water, and shaken on a shaker at room temperature for 24 h. The initial conductance (C_i) was measured using a conductance meter (Thermo Scientific, Beverly, USA). Leaves were killed by autoclaving at 121 °C for 20 min and then shaken for another 24 h before measuring maximum conductivity (C_{max}). EL was calculated as the ratio of C_i to C_{max} and expressed as a percentage^[31].

Determination of antioxidant enzyme activities

About 0.3 g of fresh leaf samples were taken at 20 d of heat stress, frozen immediately with liquid nitrogen, and stored at –80 °C for antioxidant enzyme activity analysis. Leaf samples were extracted using an extraction solution (50 mM phosphate buffer (pH 7.8), 1% polyvinylpyrrolidone, 1 mM EDTA) with a pre-chilled mortar and pestle. The extractions were centrifuged for 20 min at 12,000 rpm at 4 °C and the supernatants were used to determine antioxidant enzyme activities. APX activity was determined by measuring the decrease in absorbance at 290 nm for 1 min following the protocol described by Nakano & Assada^[32]. The 3 mL reaction mixture contained enzyme extract, 100 mM HAC-NaAc (pH 5.8), 0.003 mM EDTA, 10 mM ascorbic acid, and 5 mM H₂O₂, which initiated the reaction. The activity of SOD was measured according to the method of Beyer & Fridovich^[33] with modifications. The reaction medium was composed of 50 mM phosphate buffer (pH 7.8), 195 mM L-methionine, 0.003 mM EDTA, 0.06 mM riboflavin, 1.125 mM nitroblue tetrazolium, and an appropriate amount of leaf extract in a final volume of 3 mL. The reaction mixture was illuminated under light of 13,000 lux for 15 min and the absorbance was determined at 560 nm. CAT activity was determined by measuring the decline of absorbance at 240 nm for 90 s following the procedure of Maehly & Chance^[34]. The absorbances were measured using a spectrophotometer (Ultrospec 2100 pro, Biochrom Ltd., Cambridge, UK). Antioxidant enzyme activities were expressed on the basis of per unit protein, which was quantified using the Bradford method and bovine serum albumin as the standard.

Real-time quantitative polymerase chain reaction (qRT-PCR) analysis

To investigate the effects of KAR₁ on regulating gene expression, transcript levels of genes were evaluated using qRT-PCR. Leaf samples were collected at 24 h of heat stress and optimal temperature. Total RNA was extracted using the E.Z.N.A.® Plant RNA Kit (Omega Bio-Tek, Norcross, Georgia 30071, USA), following the manufacturer's instructions. The RNA was then reverse-transcribed into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Bio Inc., Noshigashi 7-4-38, Kusatsu, Shiga 525-0058, Japan). cDNA was amplified using Tag SYBR® Green qPCR Premix (Yugong Biotech, Nanjing, Jiangsu, China) on a Roche Light Cycler480 II thermocycler (Roche Diagnostic, Rotkreuz, Switzerland). The primer sequences used in the present study are shown in [Supplementary Table S1](#)^[20,35]. The relative expression levels of target genes under

different treatments were calculated according to the formula $2^{-\Delta\Delta CT}$ using *AsACTIN* as the reference gene^[36,37].

Statistical analysis

The experiment was arranged as split-plot design with temperature treatments as main plots and KAR₁ treatments as sub-plots. All data were subjected to analysis of variance (ANOVA) using SPSS 27 (IBM, Armonk, NY, USA). Significant differences among treatments were tested based on Duncan's multiple range test at $p < 0.05$.

Results

Effects of KAR₁ on physiological parameters of creeping bentgrass under heat stress

Under optimal temperature, exogenous application of KAR₁ had no significant effect on leaf RWC (Fig. 1a). However, KAR₁-treated plants had significantly higher (by 3.16%) leaf RWC and 11.44% lower EL than water-treated plants under heat stress (Fig. 1a–b). Heat stress reduced the activities of APX and SOD, while KAR₁ treatment significantly increased the activities of APX, CAT, and SOD by 65.63%, 29.69%, and 27.31%, respectively (Fig. 2a–c). Under optimal temperature, no significant differences in the activities of CAT and SOD were found between KAR₁-treated and water-treated plants, however, KAR₁-treated plants exhibited a 34.69% significantly higher activity of APX compared to water-treated plants (Fig. 2a–c).

Effects of KAR₁ on expression levels of antioxidant defense genes under heat stress

Under optimal temperature, the expression levels of *AsAPX1* and *AsAPX2* in KAR₁-treated plants exhibited a 2.23-fold and 1.66-fold

increase, respectively, compared to those treated with water (Fig. 3a). Exogenous application of KAR₁ significantly elevated the expression levels of *AsAPX1* and *AsAPX2* (by 2.70-fold and 1.46-fold) under heat stress (Fig. 3a). Under optimal temperature, KAR₁ treatment significantly enhanced the expression levels of *AsCATA* and *AsCATC* (by 6.00-fold and 4.16-fold) but had no significant effect on *AsCATB* (Fig. 3b). Under heat stress, compared with water-treated plants, the expression levels of *AsCATA* and *AsCATC* were significantly induced by KAR₁ treatment (1.79- and 2.82-fold, respectively) (Fig. 3b). Heat stress significantly increased the expression levels of three SOD-encoding genes (*AsFe-SOD*, *AsCuZn-SOD*, and *AsMn-SOD*), and the expression levels of *AsFe-SOD*, *AsCuZn-SOD*, and *AsMn-SOD* in KAR₁-treated plants were significantly higher than those in water-treated plants (Fig. 3c). Under optimal temperature, the expression level of *AsMn-SOD* was significantly higher in KAR₁-treated

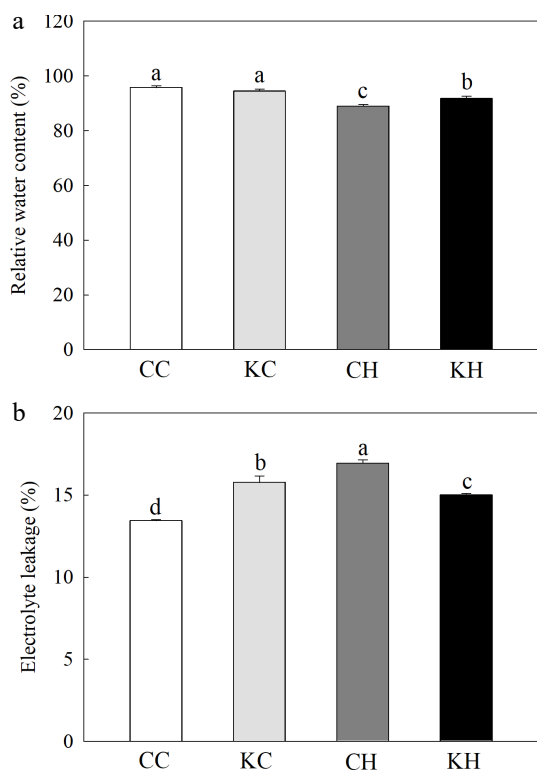


Fig. 1 Effects of KAR₁ on (a) RWC, and (b) EL in leaves of creeping bentgrass exposed to optimal temperature and heat stress. CC, foliar application of water + optimal temperature; KC, foliar application of 100 nM KAR₁ + optimal temperature; CH, foliar application of water + heat stress; KH, foliar application of 100 nM KAR₁ + heat stress. Data shown are the mean \pm SE of four biological replicates. Bars not sharing a common letter indicate significant differences at $p < 0.05$ level.

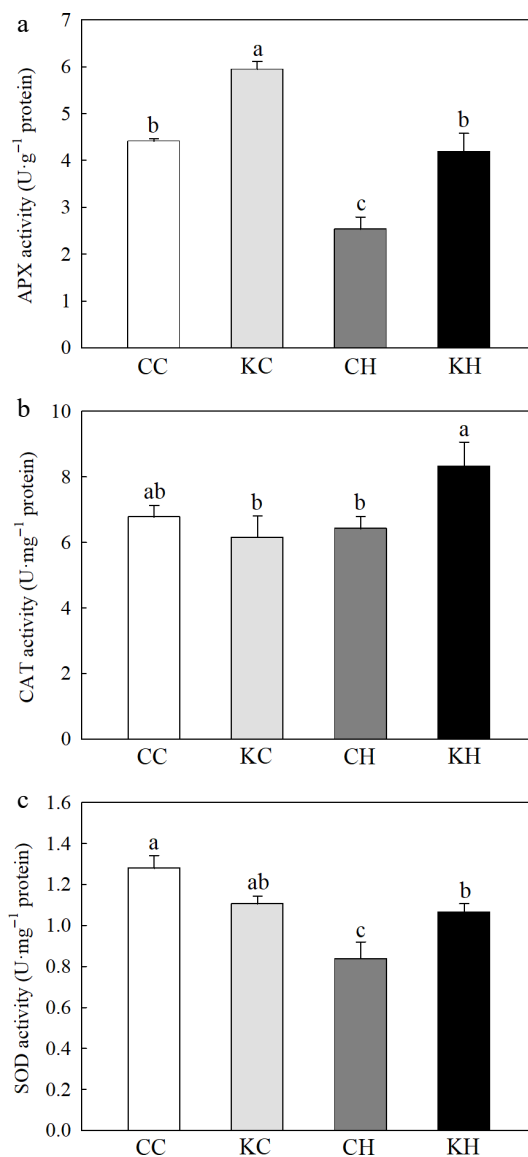


Fig. 2 Effects of KAR₁ on (a) APX activity, (b) CAT activity, and (c) SOD activity in leaves of creeping bentgrass exposed to optimal temperature and heat stress. CC, foliar application of water + optimal temperature; KC, foliar application of 100 nM KAR₁ + optimal temperature; CH, foliar application of water + heat stress; KH, foliar application of 100 nM KAR₁ + heat stress. Data shown are the mean \pm SE of four biological replicates. Bars not sharing a common letter indicate significant differences at $p < 0.05$ level.

(1.85-fold) plants after 24 h of heat stress compared to water-treated control (Fig. 3c).

Effects of KAR₁ on expression levels of genes involved in KAR/KL and SL metabolic and signaling pathways under heat stress

Heat stress significantly increased the transcript levels of KAR/KL signaling transduction genes, such as *MORE AXILLARY GROWTH2* (*AsMAX2*), and *DWARF 14-LIKE2* (*AsDLK2*) (Fig. 4b, d). Exogenous

application of KAR₁ significantly enhanced the expression levels of *KARRIKIN INSENSITIVE2* (*AsKAI2*) and *AsDLK2* (by 2.15-fold and 1.24-fold), and decreased the expression levels of *AsMAX2* and *KARRIKIN-UP-REGULATED F-BOX1* (*AsKUF1*) under heat stress (Fig. 4a–d). Heat stress significantly increased the transcript level of the SL biosynthetic gene, *AsMAX1*, while decreased the transcript levels of the SL biosynthetic gene, *AsD27* (Fig. 4e–f). Compared with the water-treated control, the exogenous application of KAR₁ enhanced the expression levels of *AsD27* and decreased the expression levels of *AsMAX1* under heat stress (Fig. 4e–f).

Effects of KAR₁ on expression levels of genes involved in ABA and SA metabolic and signaling pathways under heat stress

Plants under heat stress exhibited a significant increase in the transcript levels of the ABA catabolism-related gene, *CYTOCHROME P450 707A 1* (*AsCYP707A1*) (Fig. 5b). Exogenous application of KAR₁ significantly increased the expression level of the ABA signaling gene, *SNF1-RELATED PROTEIN KINASE2.6* (*AsSnRK2.6*, 1.61-fold) while decreased the expression level of *AsCYP707A1* under heat stress (Fig. 5a–b). Heat stress significantly induced the expression levels of two genes involved in SA biosynthesis, *AsICS1* and *AsPAL2*, while exogenous application of KAR₁ resulted in a significant increase in the expression level of *AsICS1* (1.93-fold) under heat stress (Fig. 5c).

Effects of KAR₁ on expression levels of TFs under heat stress

Heat stress significantly increased the expression levels of *AsMYB13*, *AsNAC053*, *AsbZIP58*, *AsbZIP60*, *AsWRKY75*, and *AsWRKY28* (Fig. 6a, c, e–h). Foliar application of KAR₁ significantly increased the expression levels of *AsNAC053* and *AsbZIP58* (by 1.33-fold and 1.64-fold, respectively), and decreased the expression level of *AsWRKY28* (0.61-fold) under heat stress (Fig. 6c, e, h). Under optimal temperature, KAR₁-treated plants had significantly higher expression level of *AsMYB13*, *AsbZIP58*, and *AsWRKY75* (by 3.87-fold, 2.40-fold, and 6.07-fold, respectively) (Fig. 6a, e, g).

Effects of KAR₁ on expression levels of HSFs and HSPs under heat stress

Heat stress significantly increased the expression levels of three HSFs (*AsHSFA-2c*, *AsHSFA-4b*, and *AsHSFB-2b*) and five HSPs (*AsHSP12*, *AsHSP16*, *AsHSP26.7*, *AsHSP70*, and *AsHSP90.5*). KAR₁-treated plants exhibited significant higher transcription levels of *AsHSFB-2b* (1.28-fold), *AsHSFB-4d* (1.87-fold), as well as four HSPs (*AsHSP12*, 2.73-fold; *AsHSP16*, 1.39-fold; *AsHSP70*, 2.07-fold, and *AsHSP90.5*, 2.06-fold) under heat stress (Fig. 7a, b). Under optimal temperature, application of KAR₁ induced the expression levels of *AsHSFB-4d* and *AsHSP70* by 2.32-fold and 7.55-fold, compared to water-treated control, respectively (Fig. 7a, b).

Discussion

Heat stress adversely affects plant physiological functions by disrupting normal metabolic processes, inducing excessive ROS accumulation, and causing severe damage to cell membranes, which can ultimately lead to plant death^[38]. The results of the present study demonstrated that exogenous application of KAR₁ could effectively alleviate heat stress-induced water loss and cell membrane damage in creeping bentgrass, suggesting a positive regulatory effect of KAR₁ on water homeostasis and membrane integrity under heat stress. Furthermore, qRT-PCR analysis showed that KAR₁-enhanced thermotolerance was associated with transcriptional regulation of multiple pathways, including those involved in antioxidant defense, phytohormone biosynthesis and signaling, key TFs, and heat stress responses.

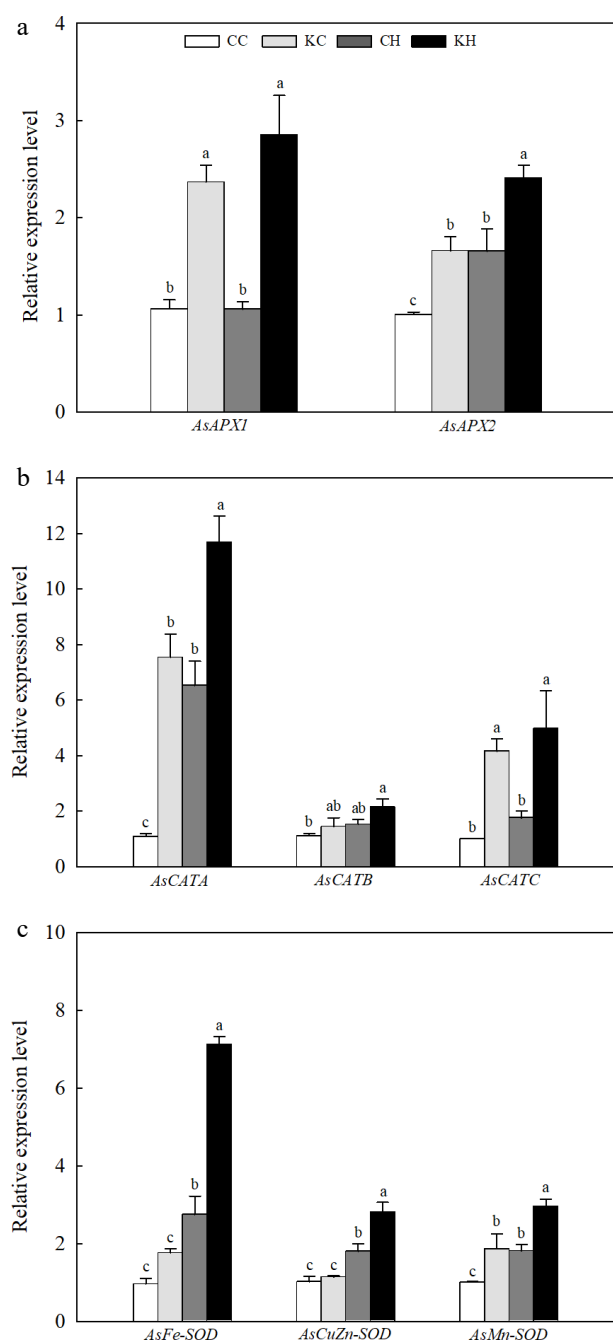


Fig. 3 Effects of KAR₁ on expression of (a) APXs, (b) CATs, and (c) SODs in leaves of creeping bentgrass exposed to optimal temperature and heat stress. CC, foliar application of water + optimal temperature; KC, foliar application of 100 nM KAR₁ + optimal temperature; CH, foliar application of water + heat stress; KH, foliar application of 100 nM KAR₁ + heat stress. Data shown are the mean ± SE of four biological replicates. Bars not sharing a common letter indicate significant differences at $p < 0.05$ level.

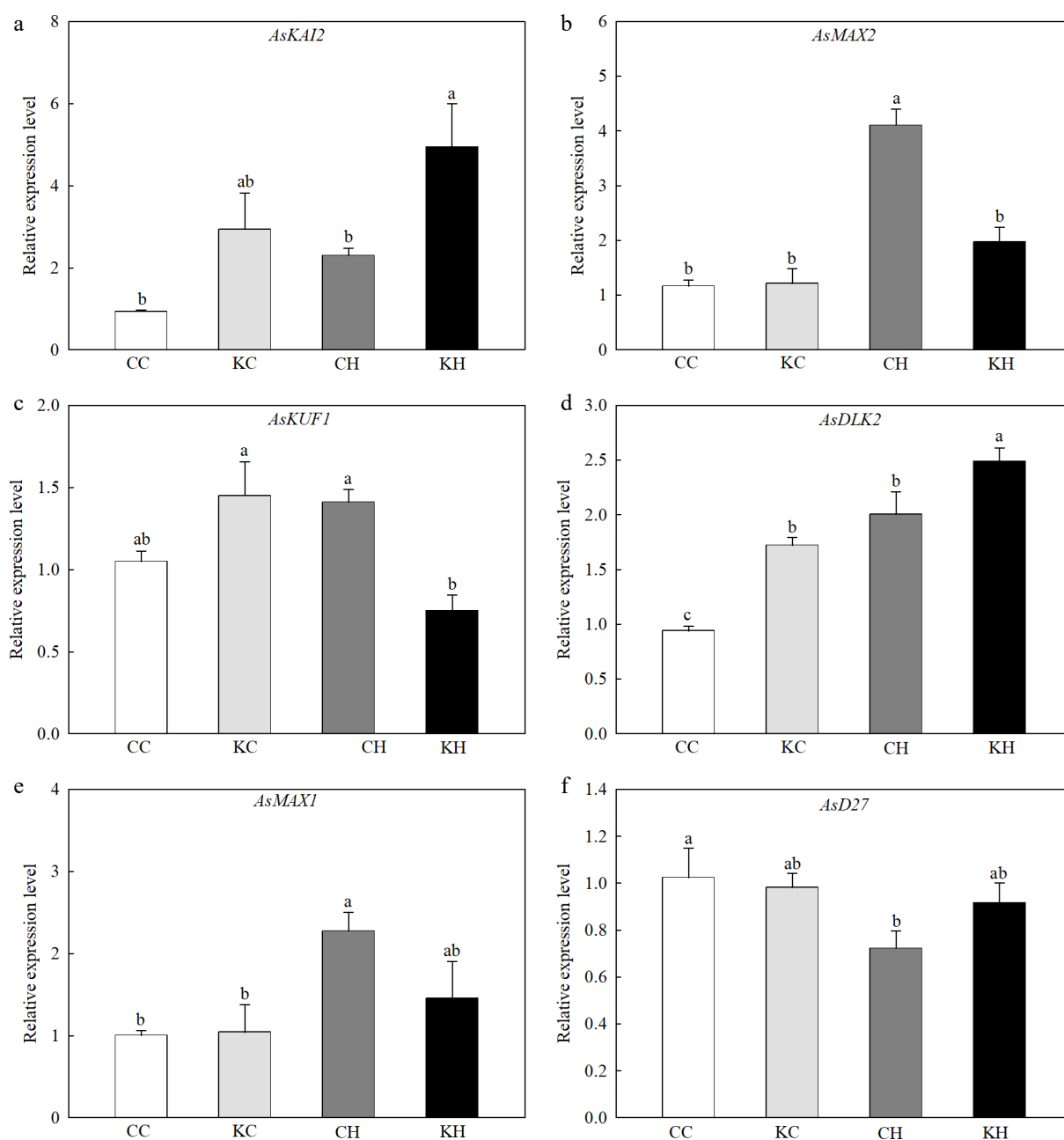


Fig. 4 Effects of KAR₁ on expression of (a) *AsKAI2*, (b) *AsMAX2*, (c) *AsKUF1*, (d) *AsDLK2*, (e) *AsMAX1*, and (f) *AsD27* in leaves of creeping bentgrass exposed to optimal temperature and heat stress. CC, foliar application of water + optimal temperature; KC, foliar application of 100 nM KAR₁ + optimal temperature; CH, foliar application of water + heat stress; KH, foliar application of 100 nM KAR₁ + heat stress. Data shown are the mean ± SE of four biological replicates. Bars not sharing a common letter indicate significant differences at $p < 0.05$ level.

To counteract the negative impact of excessive ROS induced by stress, plants rely on antioxidant enzymes, such as SOD, APX, and CAT to scavenge ROS and reduce hydrogen peroxide (H₂O₂)^[39]. SOD is particularly important as it serves as the first line of defense against oxidative stress by converting O₂^{•−} into O₂ and H₂O₂^[39–41]. APX then further reduces H₂O₂ to H₂O to decrease its toxicity for the cell^[39–41]. In this study, high-temperature stress reduced SOD and APX activities in creeping bentgrass, while foliar application of KAR₁ significantly increased the activities of both SOD and APX. Shah et al.^[21] also found that KAR₁ could enhance the activities of SOD and APX in *Sapium sebiferum* plants under salt and osmotic stresses. In addition, exogenous KAR₁ induced significantly enhanced expression levels of antioxidant enzyme encoding genes including *AsAPX1*, *AsAPX2*, *AsCATA*, *AsCATB*, *AsCATC*, *AsFe-SOD*, *AsCuZn-SOD*, and

AsMn-SOD under heat stress (Fig. 3). This suggested that KAR₁-regulated antioxidant defense could contribute to improved heat tolerance in creeping bentgrass.

Previous studies have shown that KAR/KL signal transduction is critical for plant growth and survival under abiotic stress^[20,42]. Signal transduction via KAI2 required the binding of an F-box protein known as MAX2 to facilitate the ubiquitination and subsequent degradation of members of the SUPPRESSOR OF MAX2-LIKE (SMXL) protein family, including SMAX1 and SMAX2^[23]. Up-regulated *KAI2* expression activates a positive response mechanism under salt stress, whereas reduced *KAI2* function results in pronounced growth abnormalities in *kai2*-mutant plants^[42]. *DLK2* and *KUF1* are commonly used as transcriptional indicators for KAR/KL signaling as their expressions respond strongly to KAR treatment or the

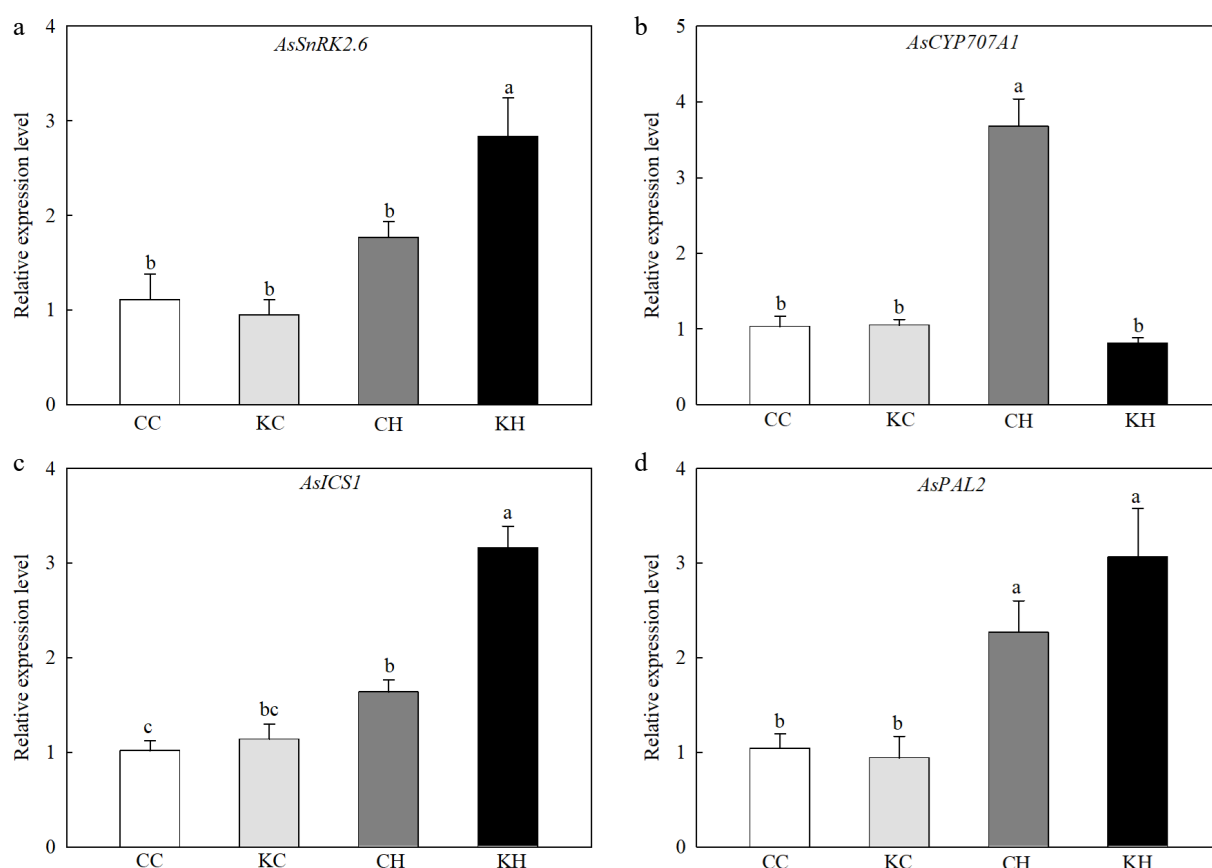


Fig. 5 Effects of KAR₁ on expression of (a) *AsSnRK2.6*, (b) *AsCYP707A1*, (c) *AsICS1*, and (d) *AsPAL2* in leaves of creeping bentgrass exposed to optimal temperature and heat stress. CC, foliar application of water + optimal temperature; KC, foliar application of 100 nM KAR₁ + optimal temperature; CH, foliar application of water + heat stress; KH, foliar application of 100 nM KAR₁ + heat stress. Data shown are the mean \pm SE of four biological replicates. Bars not sharing a common letter indicate significant differences at $p < 0.05$ level.

disruption of core KAR/KL signaling components^[43]. DLK2, a KAI2 paralog, exhibits transcriptional up-regulation in response to SLs^[44]. KUF1 is considered to hinder the conversion of KAR₁ into a functional ligand for KAI2 and the biosynthesis of KAR/KL^[45]. In the present study, foliar application of KAR₁ significantly increased the transcript levels of *AsKAI2* and *AsDLK2* and decreased the transcript levels of *AsKUF1* under heat stress (Fig. 4a, c, d), which was consistent with previous research that KAR₁ may promote the heat tolerance of creeping bentgrass by activating KAR/KL signaling pathway^[20,21].

Phytohormones, such as ABA, SL, SA, JA, and ETH, are actively involved in plant response to heat stress^[46]. Previous studies have shown that KAR-regulated plant growth and development under salt/drought stress was related to phytohormonal biosynthesis, catabolism, and signaling pathways^[25,42]. In the present study, exogenous application of KAR₁ increased the expression levels of *AsD27* while decreasing the expression levels of *AsMAX1* and *AsMAX2* in heat-stressed plants (Fig. 4b, e, f), suggesting an interaction between KAR/KL and SL signaling under heat stress in creeping bentgrass.

The KAR/KL signal transduction pathway has been demonstrated to enhance ABA sensitivity through multiple regulatory mechanisms, including the modulation of key genes involved in ABA biosynthesis and signal transduction pathways^[17,21]. In the present study, KAR₁ treatment significantly elevated the expression of the ABA signaling gene *AsSnRK2.6* and decreased the expression of the ABA catabolic gene *AsCYP707A1* (Fig. 5a–b), indicating that KAR₁ might inhibit ABA catabolism, leading to accumulated ABA, and promote ABA signaling to enhance heat tolerance in creeping

bentgrass. SA is also a hormone that plays a crucial role in promoting plant heat tolerance^[47]. ICS is a key enzyme in SA synthesis, and the *salicylic acid induction deficient 2-1* (*sid2-1*) mutant of *Arabidopsis*, which lacks functional ICS1, accumulated higher levels of ROS under heat stress compared to WT plants^[48]. In this study, exogenous KAR₁ significantly increased the expression of the SA biosynthesis gene (*AsICS1*) (Fig. 5c), suggesting that KAR₁ application may activate SA biosynthesis and affect heat tolerance in creeping bentgrass.

TFs are classified into various families, including MYB, NAC, WRKY, and bZIP, which have been extensively documented to play critical roles in regulating plant growth, development, and stress responses^[49]. NAC TF is one of the largest families of plant-specific TFs that have been shown to regulate seed and embryo development, shoot apical meristem formation, cell division, and leaf senescence^[50]. In addition, many NAC genes are involved in plant responses to abiotic stresses, such as drought, salinity, cold, and heat stresses^[50]. For instance, overexpression of *ZmNAC074* increased antioxidant enzyme activities (SOD and CAT) and proline content, while decreasing ROS accumulation and malondialdehyde content, conferring enhanced heat tolerance in *Arabidopsis*^[51]. WRKY TFs may have similar functions and play a key role in plant growth, development, and biotic and abiotic stresses in different plant species^[52]. Several WRKY members, including *WRKY25*, *WRKY26*, *WRKY40*, and *WRKY70*, showed increased expression under heat stress, and overexpression of *LIWRKY22* increased heat tolerance in *Lilium longiflorum*^[53]. Similarly, studies in model species such as *Arabidopsis thaliana* and *Oryza sativa* have shown that bZIP

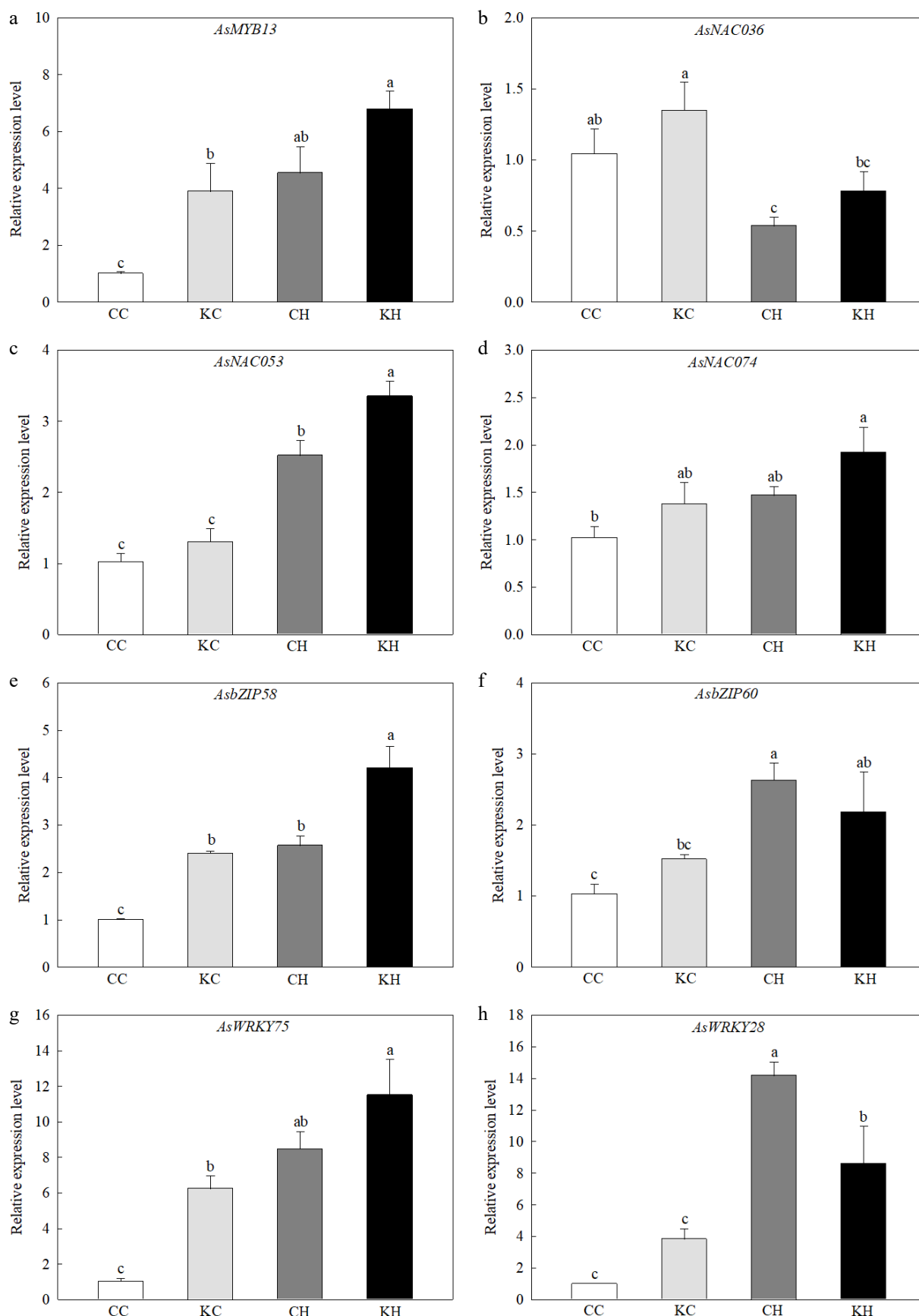


Fig. 6 Effects of KAR₁ on expression of (a) *AsMYB13*, (b) *AsNAC036*, (c) *AsNAC053*, (d) *AsNAC074*, (e) *AsbZIP58*, (f) *AsbZIP60*, (g) *AsWRKY75*, and (h) *AsWRKY28* in leaves of creeping bentgrass exposed to optimal temperature and heat stress. CC, foliar application of water + optimal temperature; KC, foliar application of 100 nM KAR₁ + optimal temperature; CH, foliar application of water + heat stress; KH, foliar application of 100 nM KAR₁ + heat stress. Data shown are the mean \pm SE of four biological replicates. Bars not sharing a common letter indicate significant differences at $p < 0.05$.

family members play essential roles in plant responses to abiotic stresses^[54]. For instance, *OsZIP58* and *TaZIP60* have been demonstrated to regulate thermotolerance in plants^[54,55]. In this study, exogenous application of KAR₁ up-regulated the expression of three NAC TFs (*AsNAC036*, *AsNAC053* and *AsNAC074*), *AsMYB13*, *AsbZIP58*, and *AsWRKY75* under heat stress, which indicated a positive association of specific TFs with KAR₁-enhanced heat tolerance in creeping bentgrass.

HSPs function as molecular chaperones to facilitate the repair of denatured/misfolded proteins, which are significantly induced by heat stress^[56]. For instance, overexpression *ZjHsp70* has been shown to enhance heat tolerance in *Arabidopsis*, as evidenced by reduced malondialdehyde content and increased antioxidant enzyme activities^[57]. HSFs are crucial components of the transcriptional regulatory networks that control the transcription of heat-HSPs^[58]. In potato (*Solanum tuberosum*), *StHsfB5* functions as a coactivator that enhances heat resistance by directly stimulating the expression of HSPs, including *sHsp17.6*, *sHsp21*, *sHsp22.7*, and *Hsp80*^[59]. In addition, previous research has shown that HSF family members serve as primary regulators of plant thermotolerance^[56,59]. For instance, in *Arabidopsis*, *AtHsfA6b* acts as a downstream regulator of the ABA-mediated stress response and positively regulates *Arabidopsis* heat tolerance^[60]. Furthermore, transgenic tobacco (*Nicotiana benthamiana*) plants overexpressing *FaHsfA2a* exhibited significantly

reduced cell membrane damage and leaf senescence under heat stress^[56]. In this study, foliar application of KAR₁ significantly up-regulated the expression levels of HSFs (*AsHsFB-2b* and *AsHsFB-4d*) and HSPs (*AsHSP12*, *AsHSP16*, *AsHSP70*, and *AsHSP90.5*) in creeping bentgrass under heat stress (Fig. 6), suggesting that KAR₁ application may enhance thermotolerance through a regulatory mechanism involving specific HSFs, which subsequently activate the transcription of downstream HSPs, thereby facilitating the maintenance of protein homeostasis and cellular functions under heat stress.

Conclusions

Heat stress significantly reduced in leaf RWC and increased electrolyte leakage in creeping bentgrass. Exogenous application of 100 nM KAR₁ effectively mitigated heat-induced stress damage, as demonstrated by improved maintenance of water balance and membrane stability, along with enhanced antioxidant enzyme activities. KAR₁-mediated thermotolerance was associated with the regulation of key genes involved in multiple physiological pathways, including heat stress response, antioxidant defense, phytohormone biosynthesis, and signaling, as well as KAR/KL signaling and TFs (Fig. 8). These findings suggested that KAR₁ might serve as an effective bioregulator for enhancing heat tolerance of cool-season

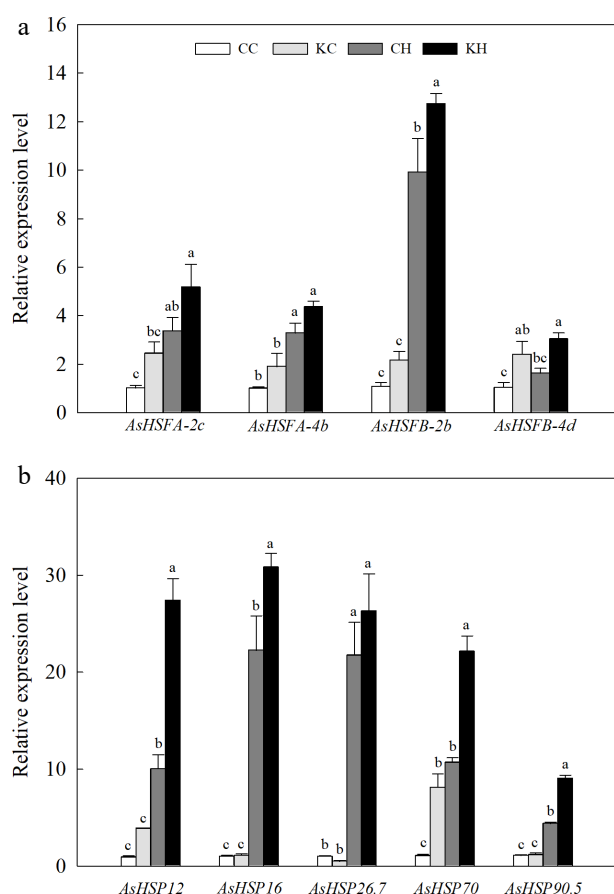


Fig. 7 Effects of KAR₁ on expression levels of (a) HSFs and (b) HSPs in leaves of creeping bentgrass exposed to optimal temperature and heat stress. CC, foliar application of water + optimal temperature; KC, foliar application of 100 nM KAR₁ + optimal temperature; CH, foliar application of water + heat stress; KH, foliar application of 100 nM KAR₁ + heat stress. Data shown are the mean ± SE of four biological replicates. Bars not sharing a common letter indicate significant differences at *p* < 0.05.

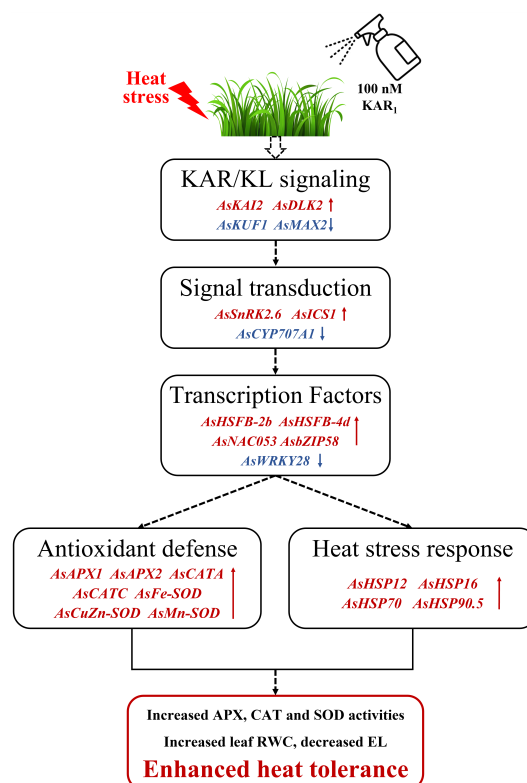


Fig. 8 Schematic diagram illustrating the mechanism of KAR₁ enhances the heat tolerance of creeping bentgrass. Under heat stress, foliar application of KAR₁ up-regulated the expression of KAR/KL signal transduction genes (*AsKAI2*, *AsDLK2*), hormone biosynthesis and signaling genes (*AsSnRK2.6*, *AsICS1*), transcription factors (*AsHsFB-2b*, *AsHsFB-4d*, *AsNAC053*, *AsbZIP58*), antioxidant defense genes (*AsAPX1*, *AsAPX2*, *AsCATA*, *AsCATC*, *AsFe-SOD*, *AsCuZn-SOD*, *AsMn-SOD*), and heat shock proteins (*AsHSP12*, *AsHSP16*, *AsHSP70*, *AsHSP90.5*), while down-regulated the expression of KAR/KL signal transduction genes (*AsMAX2*, *AsKUF1*), phytohormone catabolism gene (*AsCYP707A1*), and transcription factor (*AsWRKY28*), resulting in enhanced heat tolerance (increased leaf RWC, decreased EL, and increased activities of APX, CAT and SOD) of creeping bentgrass.

turfgrass, providing a new perspective for cool-season lawn maintenance under high-temperature conditions.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Zhang X, Yang Z, Tan Z; data collection: Wang Y, Han Y, Zhu M, Li X; analysis and interpretation of results: Wang Y, Tan Z, Zhang X; draft manuscript preparation: Wang Y; manuscript revision: Zhang X, Tan Z, Yang Z. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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