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Karrikin$_1$ Enhances Drought Tolerance in Creeping Bentgrass in Association with Antioxidative Protection and Regulation of Stress-Responsive Gene Expression

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Abstract: Karrikins are active components of smoke that can promote seed germination and regulate seedling morphogenesis. However, the role of karrikins as alleviators of abiotic stress remains largely elusive. In this study, we examined whether exogenous application of karrikin$_1$ (KAR$_1$) might improve drought tolerance in creeping bentgrass (*Agrostis stolonifera* cv. PennA4), and investigated the underlying mechanism. We found that exogenous application of 100 nM KAR$_1$ enhanced drought tolerance in creeping bentgrass, as manifested by significant increases in leaf relative water content, efficiency of photosystem II, leaf chlorophyll content, proline content, and membrane stability, as well as significantly enhanced activities of antioxidant enzymes. RT–PCR analysis indicated that improved drought stress tolerance by application of KAR$_1$ might be related to upregulation expression of karrikin-responsive genes (*KAI2*, *MAX2* and *AFL1*), transcription factors (*ABF3*, *bHLH148*, *MYB13* and *DREB2A*), antioxidant defense genes (*Cu/Zn-SOD*, *APX2*, *CAT1*, and *POD2*), and downregulation expression of chlorophyll-degradation genes (*PPH* and *Chl-PRX*). These findings suggest that KAR$_1$ may promote the drought tolerance of creeping bentgrass by activating karrikin-responsive genes and transcription factors, enhancing proline accumulation and antioxidant capacity, and suppressing leaf senescence under prolonged drought stress.

Keywords: karrikin; drought stress; antioxidant defense; transcription factors; reactive oxygen species; creeping bentgrass (*Agrostis stolonifera*)

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1. Introduction

Drought stress has become a primary abiotic stress limiting plant growth and development, and the frequency and duration of drought is predicted to increase in the future [1]. Drought stress causes various types of damage to cellular, physiological, and biochemical processes, which ultimately leads to the loss of plant functionality and productivity [2].

Drought directly affects the water uptake of plant roots, reduces the relative water content of leaves, and causes chlorophyll degradation leading to stress-induced leaf senescence [3,4]. Drought stress causes the production of reactive oxygen species (ROS), and ROS result in photosynthesis inhibition, lipid peroxidation, and cellular function interruption, which consequently lead to cell membrane electrolyte leakage (EL), lipid peroxidation, and even to plant death [5]. However, plants adapt to drought stress by developing an effective ROS scavenging system to suppress the production of ROS, such as antioxidant metabolism [6]. Antioxidant enzymes, such as glutathione peroxidase (GPx), peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX), and nonenzymatic antioxidants, such as proline and ascorbic acid, work coordinately to prevent ROS accumulation so as to maintain cell water balance and stabilize the cell membrane [3,7]. SOD converts $O_2^{\cdot -}$ to H_2O_2, then GPX, CAT, and APX detoxifies H_2O_2 to water, while the conversion of H_2O_2 to H_2O by APX and GPX needs ascorbate and glutathione as the

electron donor [8]. It is manifested that the regulation of antioxidant enzymes activity and gene expressions may be crucial to plant survival from drought stress. Therefore, withholding a high level of antioxidative enzyme activities is positively associated to drought tolerance [9].

In addition, many transcription factors (TFs), especially stress-related TFs, are highly activate under drought stress. TFs act as a molecular switchboard, which can either active or repress the expression of the downstream genes whenever necessary. For example, WRKY TFs (*WRKY60*, *WRKY40* and *WRKY18*) were proven to interact with each other to regulate ABA signaling during dehydration stress [10]. Similarly, the expression of *MdMYB10* in apples (*Malus domestica*) was autoregulated to further regulate the accumulation of anthocyanins [11]. The expression of some DREB TFs, such as ICE1, a TF from bHLH family, is cross-regulated by binding to the promoter of *DREB1C* to activate its expression during cold stress [12]. Furthermore, when the abiotic stresses withdraw, TFs may degrade via the ubiquitin–proteasome system (UPS) to prevent useless energy consumption; for example, the high expression of *VuDEREBA2A* in cowpea (*Vigna unguiculata*) upon desiccation may stop by mediating the UPS to degrade the TF [13].

Karrikins (KARs) are small butenolides that are discovered in the smoke of burning plant material. Six compounds (KAR_1–KAR_6) have been identified, of which KAR_1 was generally proven to be most abundant and most biologically active [14,15]. According to previous studies, KARs can induce seed germination [15–17] and promote seedling vigor in plants [18]. In Arabidopsis, KAR_1 enhanced light-dependent germination and seedling development [19], and induced the expression of numerous light-responsive genes, resulting in an increased sensitivity to light [19]. Recent discoveries have shown that KARs can also play a key role in defending against abiotic stresses. Under salt, osmoticum or temperature stress conditions, KAR-treated (100 nM) seeds produced stronger seedlings than water-treated seeds of tomato (*Lycopersicon esculentum*) [20]. Supplementation of 1 nM KAR_1 in growth medium could significantly alleviate drought and salt stress in *Sapium sebiferum*, as proven by increased seed germination, enhanced seedling biomass, improved taproot length, and number of lateral roots under drought and salt stresses [18]. Under cadmium stress, KAR_1-treated seedlings displayed higher stomatal conductivity and intercellular carbon dioxide concentration in *Brassica oleracea* [21]. Previous studies of KARs have mainly focused on the improvement of seed germination and seedling establishment, however, the effects of KARs on regulating plant drought tolerance and how to regulate plant performance under stress conditions remain largely unknown.

With the growth of human population, the demand for water is dramatically increasing; therefore, how to save water resources has become a worldwide research topic [3]. Creeping bentgrass (*Agrostis stolonifera*) is a widely used cool-season perennial grass species and is sensitive to drought stress [22]. Exogenous application of biostimulants to plants, including chemicals and hormone mimetics, has been proven to be an efficient way to reduce the adverse effects caused by abiotic stresses [2,23]. KAR_1 has been proven to be a positive regulator of seed germination and seedling establishment under abiotic stresses, thus, we suppose that it may also function as an alleviator of prolonged drought stresses in grass plants. The objective of this study was to investigate how KAR_1 might be involved in membrane stability, antioxidant enzymatic activity, and the expression level of stress-responsive genes, including TFs, contributing to drought tolerance in creeping bentgrass.

2. Materials and Methods

2.1. Plant Materials and Growing Conditions

Creeping bentgrass (*Agrostis stolonifera* cv. PennA4) seeds were sown in PVC tubes (diameter: 11 cm, height: 25 cm) filled with a mixture of potting media (Pindstrup Mosebrug, Pindstrup, Denmark) and vermiculite (V:V, 3:1) in a research greenhouse at Nanjing Agricultural University, China. Plants were fertilized twice a week by using Miracle-Gro fertilizer (The Scotts Company LLC; Marysville, OH, USA) and trimmed every three days to maintain a canopy height of 3–5 cm. After two months of establishment, plants were

moved to a controlled growth chamber (XBQH-1, Xubang, Jinan, China) with the settings of a day/night temperature of 23/18 °C, a 14-h photoperiod with photosynthetically active radiation of 600 μmol m^{-2} s^{-1} and 70% relative humidity during the experimental period. Prior to foliar application and drought treatment, plants were acclimated in the growth chamber for 1 week.

2.2. KAR_1 Preparation and Experimental Treatments and Design

KAR_1 (3-methyl 2H-furo [2,3-c] pyran-2-one) was ordered from the Toronto Research Chemicals (Toronto, ON, Canada). The KAR_1 stock and working solution was prepared according to Shah et al. [18]. Prior to the initiation of drought stress (once each day for 3 days) and every subsequent 5 days of drought, plants were treated with 20 mL of water (untreated control) or 100 nM KAR_1 solution by foliar-spraying the canopy. The concentration for KAR_1 was selected on the basis of a pre-experiment with five-concentration gradients (1, 10, 50, 100, or 1000 nM KAR_1), which showed that 100 nM KAR_1 was the most effective treatment for improving the drought stress tolerance of creeping bentgrass, as demonstrated by less electrolyte leakage of leaves in 100 nM KAR_1-treated plants under drought stress (Figure S1). KAR_1-treated plants and water-treated plants were exposed either to control conditions (plants were irrigated every 2 d, soil relative water content (RWC) was kept at approximately 90%) or drought stress (stopping irrigation for 30 d, soil RWC decreased to 8.13%) (Figure S2). Leaf samples were collected at 20 d and 30 d of drought stress treatment, and each treatment had three biological replicates. Plants were randomly distributed and relocated in chambers every two days.

2.3. Measurements of Physiological Parameters

Plant physiological parameters were measured at 30 d of treatment. Leaf RWC was measured and calculated by using the method described by Barrs and Weatherley [24]. Leaf cell membrane stability was tested by measuring electrolyte leakage (EL). Leaf EL was detected and calculated according to the method described by Jespersen and Huang [25]. The leaf chlorophyll (Chl) content was measured and calculated according to the method described by Barnes et al. [26] and Zhang et al. [27]. The variable to maximum fluorescence ratio (Fv/Fm) is a general way to measure leaf photosystem II effectiveness. Leaves were maintained in the dark for 30 min and Fv/Fm was measured with a fluorescence induction monitor (OPTI-Sciences, Hudson, NY, USA).

2.4. Quantification of Antioxidant Enzyme Activity

Approximately 0.3 g of fresh leaf tissue was weighed, and then ground in liquid nitrogen into fine powder. Leaf tissue was extracted with 6 mL of 50 mM phosphate buffer (pH 7.8) and centrifuged at 15,000× *g* for 30 min at 4 °C. The supernatant was prepared to measure antioxidant enzyme activities, proline content, and MDA content.

SOD, CAT, and POD activities were measured following the methods of Du et al. [28]. APX activity was detected following the procedure provided by Nakano and Asada [29], by reading the absorbance at 290 nm on a spectrophotometer (GE Healthcare Life Sciences, Cambridge, UK) at 30 s intervals for 5 min [30]. The MDA content was measured through the thiobarbituric acid (TBA) reaction assay of Heath and Packer [31] and Dhindsa and Matowe [32]. The MDA content was calculated by using the equation described by Heath and Packer [31]. To measure proline content, a reaction mixture was prepared by making up 1 mL enzyme extract, 2 mL acetic acid, and 2 mL acid-ninhydrin solution. Acid-ninhydrin solution was compounded by dissolving 2.5 g ninhydrin in 60 mL glacial acetic acid and 40 mL 6 M phosphoric acid. The reaction mixture was vortexed thoroughly and boiled well for 30 min, and then cooled immediately in an ice-water bath. Four milliliters of methylbenzene was added to each reaction tube and mixed fully to extract proline. The absorbance at 520 nm of the upper organic phase was read using a spectrophotometer (GE Healthcare Life Sciences, Cambridge, UK), and the calculation was based on a standard curve with a known concentration of L-proline.

2.5. RNA Isolation and qRT–PCR Analysis

Total RNA was isolated from leaf samples collected at 20 d. Total RNA extraction and qRT–PCR were performed following the methods described by Zhang el al. [27]. *AsACTIN* was selected as the reference gene in creeping bentgrass [33]. Each biological sample was repeated two times, and the calculation of all genes was based on the formula: $2^{-\Delta\Delta Ct}$ [34]. The full sequences of *Agrostis stolonifera* genes were obtained through blasting against the local transcriptome database using amino acid sequences of Arabidopsis and rice as queries. Primer Premier 6.0 software (Premier Biosoft International, Palo Alto, CA, USA) was used to design the specific primers, which are listed in the Supplementary Table S1; the RT–PCR melting curves of all genes are shown in the Supplementary Figure S3.

2.6. Statistical Analysis

SPSS 13.0 (SPSS, Chicago, IL) was used to analyze all data. ANOVA analysis was used to determine differences among the water and KAR_1 treatments under control and drought conditions. Least significant difference (LSD) was used to examine the significance of means ($p < 0.05$).

3. Results

3.1. Effects of KAR_1 on Physiological Responses of Creeping Bentgrass

Drought stress inhibited shoot growth, caused leaf senescence and leaf abscission of creeping bentgrass plants, while KAR_1-treated plants maintained more vigorous growth compared with water-treated plants (Figure S4). Drought stress caused significant declines in leaf RWC, Fv/Fm and chlorophyll content (Figure 1). Under control conditions, no significant differences were found in those physiological parameters between water- and KAR_1-treated plants. Under drought stress, KAR_1-treated plants maintained significantly higher leaf RWC, Fv/Fm, chlorophyll and proline content (increased by 43.19%, 7.55%, 29.34%, and 8.88%, respectively) than water-treated plants (Figure 1B–E). The EL and MDA contents of creeping bentgrass leaves significantly increased due to drought stress, while KAR_1-treated plants had significantly lower EL and MDA contents (decreased by 24.75 and 34.53%, respectively) than water-treated plants under drought stress (Figure 1A,F).

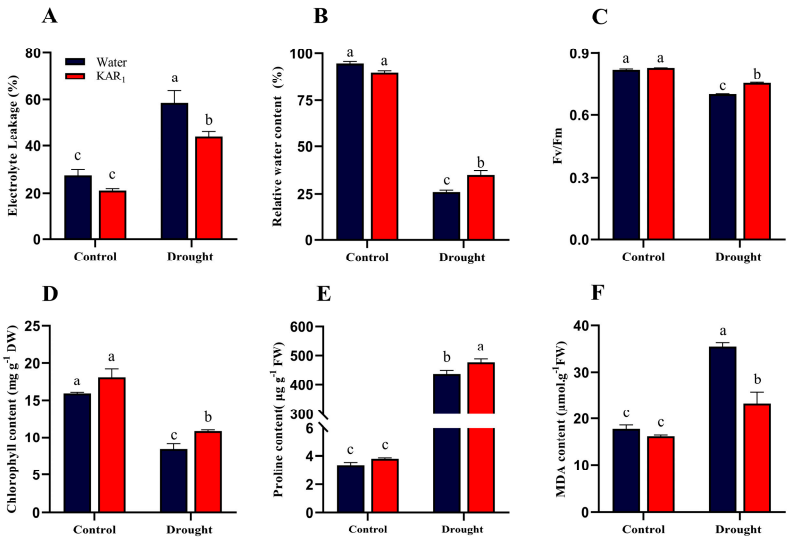

Figure 1. Effects of KAR_1 on physiological responses of creeping bentgrass under control or drought stress for 30 d. (**A**): Leaf electrolyte leakage; (**B**): Leaf relative water content; (**C**): Fv/Fm; (**D**): Chlorophyll content; (**E**): Proline content; (**F**): MDA content. Small letters indicate significant differences between the water and KAR_1 treatment under a given condition based on LSD values ($p < 0.05$).

3.2. Effects of KAR_1 on Antioxidant Enzyme Activities

Under drought stress, KAR_1-treated plants had significantly higher activities of POD, SOD, and APX (by 30.53%, 16.11%, and 221.1%, respectively) compared with the water-treated plants, while the activity of CAT was not significantly affected by KAR_1 application under drought stress (Figure 2A–D). Under control conditions, KAR_1 application resulted in significantly higher POD and CAT activities, which were 49.51% and 44.85% higher than those in the water-treated plants at 30 d, respectively (Figure 2A,C). The activity of SOD was significantly lower in KAR_1-treated plants than in the water-treated plants at 30 d of control conditions (Figure 2B). No significant differences were observed in APX activity between water- and KAR_1-treated plants under the control conditions (Figure 2D). Overall, these results suggested that exogenous application of KAR_1 could enhance the antioxidant capacity and scavenge the ROS, thus improving membrane stability and integrity under drought stress.

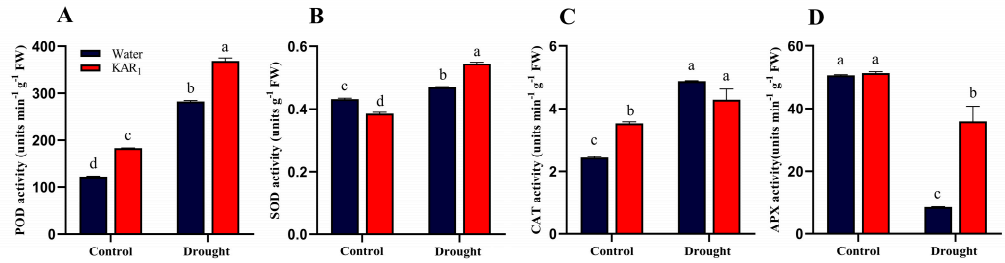

Figure 2. Effects of KAR_1 on antioxidant enzyme activities of creeping bentgrass under control or drought stress for 30 d. (**A**): POD activity; (**B**): SOD activity; (**C**): CAT activity; (**D**): APX activity. Small letters indicate significant differences between the water and KAR_1 treatment under a given condition based on LSD values ($p < 0.05$).

3.3. Effects of KAR_1 on the Expression Level of Genes Related to Karrikin-Responsive Signaling

The expression levels of *MAX2*, *KAI2*, and *AFL1* were all significantly higher (6.9-, 6.9-, and 1.31-fold, respectively) in KAR_1-treated plants than in water-treated plants at 20 d under drought stress, while the expression levels of *DLK2*, *KUF1*, and *SMAX1* were significantly lower in KAR_1-treated plants than in water-treated plants at 20 d of drought stress (Figure 3). Most of these karrikin-related signaling genes (except *AFL1* and *KUF1*) showed no significant differences between water- and KAR_1-treated plants under control conditions (Figure 3). Compared with water-treated plants, foliar application of KAR_1 significantly induced transcription of *AFL1* (4.05-fold) at 20 d under control condition (Figure 3C). Moreover, the expression level of *KUF1* was 38.34% lower in KAR_1-treated plants than in water-treated plants (Figure 3E). These results revealed that karrikins might defend against abiotic stresses by regulating the specific karrikin signaling pathways.

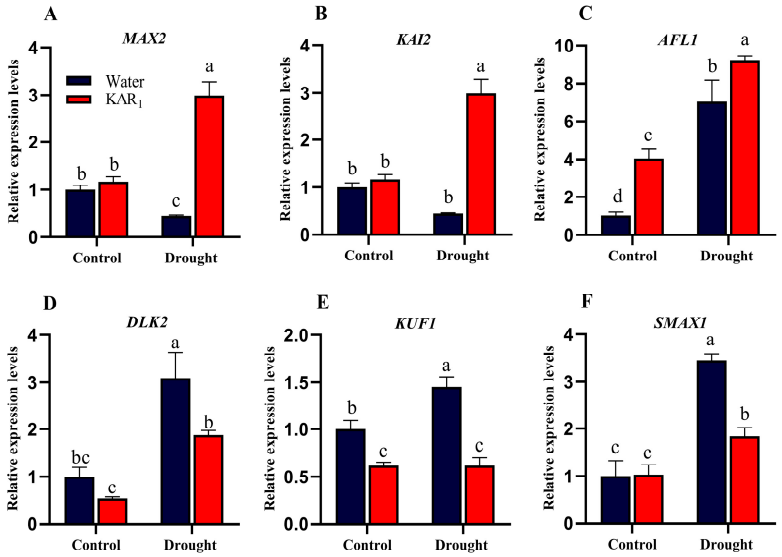

Figure 3. Effects of KAR_1 on the transcriptional level of genes related to karrikin responsive signaling in creeping bentgrass under control or drought stress for 20 d. (**A**): *MAX2*; (**B**): *KAI2*; (**C**): *AFL1*; (**D**): *DLK2*; (**E**): *KUF1*; (**F**): *SMAX1*. Small letters indicate significant differences between the water and KAR_1 treatment under a given condition based on LSD values ($p < 0.05$).

3.4. Effects of KAR_1 on the Expression Level of Transcription Factors Related to Stress Tolerance

The transcript levels of *ABF3*, *bHLH148*, *MYB13*, and *DREB2A* in KAR_1-treated plants were 2.76-, 2.77-, 1.77-, and 2.14-fold, higher than those in water-treated plants at 20 d of drought stress, respectively (Figure 4A–D). The expression levels of *WRKY75* and *WRKY28* in KAR_1-treated plants was 40.53% and 41.55%, respectively, lower than those in the water-treated plants at 20 d of drought stress (Figure 4E,F). Under control conditions, most of those genes were not responsive to KAR_1-treatment except *WRKY28*, which was downregulated by KAR_1-treatment (Figure 4).

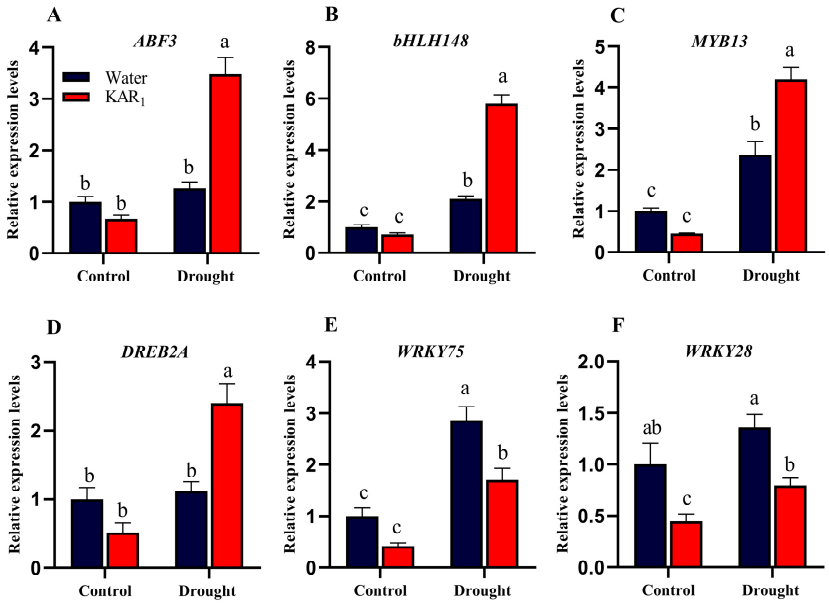

Figure 4. Effects of KAR_1 on the expression level of transcription factors related to stress tolerance in creeping bentgrass under control or drought stress for 20 d. (**A**): *ABF3*; (**B**): *bHLH148*; (**C**): *MYB13*; (**D**): *DREB2A*; (**E**): *WARKY75*; (**F**): *WARKY28*. Small letters indicate significant differences between the water and KAR_1 treatments under control or drought conditions based on LSD values ($p < 0.05$).

3.5. Effects of KAR_1 on the Expression Level of Antioxidant Defense Genes

Under drought stress, exogenous application of KAR_1 significantly upregulated the transcript level of genes encoding antioxidant enzymes. The expression levels of *Cu/Zn-SOD*, *APX2*, *CAT1*, and *POD2* were significantly upregulated in KAR_1-treated plants (1.42-, 3.39-, 2.48-, and 2.6-fold, respectively) compared to the water-treated plants at 20 d of drought stress, respectively (Figure 5). However, no significant differences in the expression levels of those genes were found between water- and KAR_1-treated plants under control conditions (Figure 5).

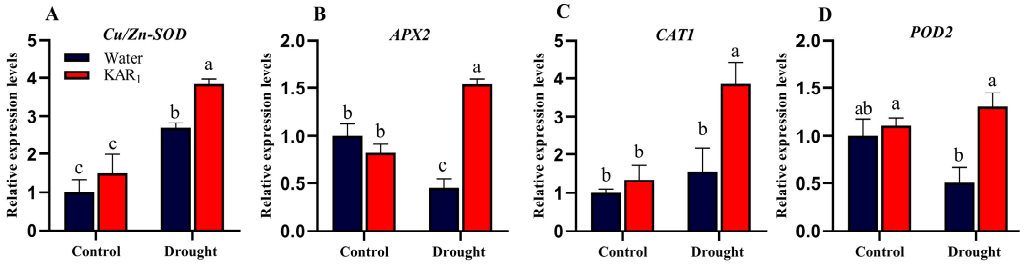

Figure 5. Effects of KAR_1 on the expression level of antioxidant defense genes in creeping bentgrass under control or drought stress for 20 d. (**A**): *Cu/Zn-SOD*; (**B**): *APX2*; (**C**): *CAT1*; (**D**): *POD2*. Small letters indicate significant differences between the water and KAR_1 treatment under a given condition based on LSD values ($p < 0.05$).

3.6. Effects of KAR_1 on the Expression Level of Chlorophyll-Degradation Genes

The expression of two chlorophyll-degradation genes (*Chl-PRX* and *PPH*) was significantly induced after 20 d of drought stress, while the application of KAR_1 inhibited their expression compared with the water-treated plants (Figure 6). Under control conditions, the expression level of *Chl-PRX* was significantly higher in water-treated plants (5.78-fold) than in KAR_1-treated plants (Figure 6B). No significant difference was observed in the expression level of *PPH* between water- and KAR_1-treated plants under control conditions (Figure 6A).

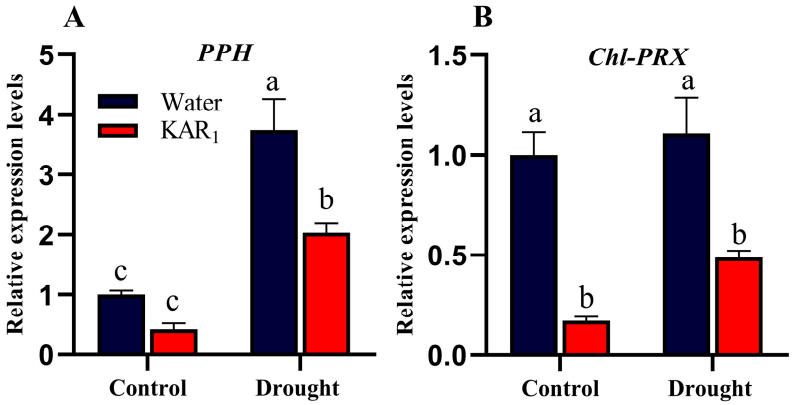

Figure 6. Effects of KAR_1 on the expression level of chlorophyll-degradation genes in creeping bentgrass under control or drought stress for 20 d. (**A**): *PPH*; (**B**): *Chl-PRX*. Small letters indicate significant differences between the water and KAR_1 treatment under a given condition based on LSD values ($p < 0.05$).

4. Discussion

Karrikins, discovered in smoke after fire, are bioactive components that stimulate the regrowth of many plants. Recently, karrikins have been found to be associated with the alleviation of abiotic stresses during seed germination and seedling establishment stages [35,36]. Nevertheless, the mechanism by which karrikins regulate plant stress

adaptation remains largely elusive. Our results indicated that exogenous application of KAR_1 significantly enhanced drought tolerance in creeping bentgrass, as manifested by the increased leaf RWC, photochemical efficiency, Chl content, and membrane stability (decreased EL and MDA content). The positive physiological effects of KAR_1 were associated with the enhanced antioxidant-defense and transcriptional regulation of genes involved in chlorophyll catabolism, karrikin responsive signaling, and stress-related transcription factors, as discussed in detail below.

Drought stress directly affects the water uptake of plants and causes a significant production of ROS, which can severely damage the proteins and DNA, resulting in peroxidation of lipid membranes and interruption of cellular functions [5]. In the present study, EL and MDA content were significantly lower in KAR_1-treated plants than in the water-treated plants under drought stress (Figure 1), indicating that foliar application of KAR_1 had a positive effect in preserving membrane structure and integrity under drought stress. Plant tolerance to abiotic stress may be positively related to the accumulation of endogenous antioxidants, as stress-resistant plants may produce a higher amount of enzymatic antioxidants to eliminate the adverse effects caused by ROS [37,38]. Previous studies have shown that KAR_1 can increase the antioxidant activity of SOD and CAT in embryos to eliminate the oxidative stress caused by ROS accumulation during the *Avena fatua* caryopses seed dormancy, and the increase of glutathione reductase (GR) and APX activities were essential for ROS scavenge to maintain optimal ratio of reduced and oxidized forms of glutathione [39]. Shah et al. [18] found that supplementation of 1 nM KAR_1 in growth medium increased the level of enzymatic antioxidants in *Sapium sebiferum* under osmotic and drought stresses. In accordance with these studies, results from the present study showed that KAR_1-treated plants had significantly higher levels of enzymatic antioxidants, including SOD, POD, and APX at 30 d of drought stress (Figure 2). In addition, it has to be noticed that the transcript levels of genes encoding antioxidant enzymes (*Cu/Zn-SOD*, *APX2*, *CAT1*, and *POD2*) were all significantly higher in KAR_1-treated plants than in water-treated plants at 20 d of drought stress (Figure 5) and showed more dramatic rises than antioxidant enzyme activities. The changes of transcript abundance could affect enzyme activities through regulation of cellular potential to synthesize new proteins, and has been considered to be more important for long-term adaptation of plants to stresses [40]. In addition to ROS-scavenging enzymes, proline act as a ROS scavenger stabilizing the integrity of proteins and antioxidant enzymes [41], also accumulated more in KAR_1 treated plants under drought stress. Overall, the enhanced antioxidant system corresponded to KAR_1-reduced EL and MDA content under drought stress, indicating that foliar application of KAR_1 could activate the antioxidant defense system and alleviate lipid peroxidation of creeping bentgrass under drought stress.

In addition to ROS accumulation, decreased chlorophyll content is also considered as a symptom of oxidative stress, and a higher chlorophyll content is strongly associated with enhanced drought tolerance [42]. Previous studies have indicated that drought stress increases the activities of chlorophyll catabolic enzymes, which contribute to the leaf senescence [43,44]. In the present study, the transcript level of genes encoding key chlorophyll catabolic enzymes, *Chl-PRX* and *PPH*, was significantly induced by drought, but KAR_1 could significantly suppress the expression level of *Chl-PRX* and *PPH* under drought condition, resulting in increased chlorophyll content (Figure 1D; Figure 6). These results demonstrated that KAR_1 could suppress drought-induced chlorophyll-degradation and maintain higher chlorophyll levels in creeping bentgrass under drought stress.

Karrikin draws the attention of scientists and emerges as a plant biostimulator due to its high similarity in chemical structure with an important phytohormone, strigolactone (SL) [45]. Two genes, *KARRIKIN INSENSITIVE2* (*KAI2*) and *MORE AXILLARY GROWTH2* (*MAX2*), are required for karrikin responses in *Arabidopsis thaliana* [46]. *KAI2* functions in the initial step of karrikin signal transduction through the binding of karrikin and plays a key role in drought stress adaptation, including stomatal closure, membrane integrity, and cuticle formation [47,48]. *MAX2* is also an important component of the SL

signaling pathway, and participates in various biological processes, such as plant growth, development and abiotic stress responses [49]. Accordingly, *SUPPRESSOR of MAX2 1* (*SMAX1*) has been proven to be a negative regulator of karrikin and SL signaling for drought resistance in *Arabidopsis thaliana* [50]. In this study, foliar application of KAR_1 significantly upregulated the transcript levels of *KAI2* and *MAX2* and downregulated the transcript level of *SMAX1* in creeping bentgrass under drought stress (Figure 3). These results indicate that KAR_1 may trigger the expression of karrikin-responsive genes that activate downstream pathways and enhance the tolerance of creeping bentgrass under drought stress.

In response to drought stress, multiple TFs are induced or accumulated to impart plant stress adaptation [51]. MYB and bHLH are two large TF families in plants and participate in plant drought resistance by stimulating the antioxidant system and regulating plant hormone signal transduction, including the ABA-mediated signaling pathway [52–56]. ABA-responsive element (ABRE)-binding factors (ABFs) have been proven to be master TFs and are involved together with MYB and bHLHs in ABA-mediated drought stress tolerance, such as transpiration reduction and leaf senescence inhibition [57,58]. Overexpression of *Populus euphratica PeABF3* in *Populus tomentosa* maintained higher photosynthetic activity and promoted cell membrane integrity, resulting in increased water-use efficiency and enhanced drought tolerance compared with wild-type controls [59]. In addition, *FtMYB13* act as positive regulators of salt and drought response in transgenic Arabidopsis, as manifested by the improved photosynthetic efficiency, higher transcript level of some stress-related genes, and the lower of ROS and MDA in the transgenic lines [53]. In addition to ABA-dependent signaling, ABA-independent signal transduction is also an important pathway involved in drought stress tolerance [60]. *DREB2A* is a key transcription factor of drought stress tolerance in many plants and induces the expression of many drought-related genes [61]. WRKY TFs are a large family of plant-specific TFs and participate in plant defense responses either as positive or negative regulators [62]. Previous studies have shown that overexpression of *OsWRKY28* in rice significantly decreased the transcript level of peroxidase [62], and transcription factor *PagWRKY75* may participate the accumulation of H_2O_2 to negative regulate salt and osmotic tolerance by regulating various physiological processes [56,63]. In this study, the application of KAR_1 significantly upregulated the expression levels of *ABF3*, *bHLH148*, *MYB13,* and *DREB2A*, and downregulated the expression levels of two negative regulators of stress adaptation, *WRKY75* and *WRKY28* [62,63], in creeping bentgrass exposed to drought stress (Figure 4). These results suggest that KAR_1 may activate stress signal transduction pathways to improve drought tolerance in creeping bentgrass.

5. Conclusions

Here, we concluded that exogenous application of 100 nM KAR_1 significantly enhanced the drought tolerance of creeping bentgrass, which was manifested by a significant increase in leaf RWC, efficiency of photosystem II, leaf Chl content, proline content, and membrane stability, and significantly elevated activities of antioxidant enzymes, expression of karrikin-responsive genes (*KAI2*, *MAX2*, and *AFL1*), transcription factors (*MYB13*, *ABF3*, *DREB2A*, and *bHLH148*), and the antioxidant defense system (increased transcript level and activities of antioxidant enzymes) (Figure 7). These results indicate that KAR_1 may act as an effective agent to improve turfgrass performance in water-limited environments by activating stress signaling and antioxidant protection against drought stress.

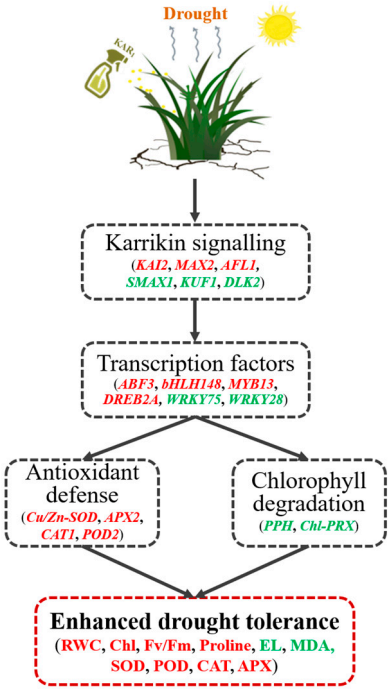

Figure 7. Schematic diagram illustrating the mechanism of KAR_1 enhances the drought tolerance of creeping bentgrass. Foliar application of KAR_1 regulated the expression level of karrikin signaling genes (*KAI2*, *MAX2*, *AFL1*, *SMAX1*, *DLK2*, and *KUF1*), stress-related TFs (*ABF3*, *bHLH148*, *MYB13*, *DREB2A*, *WRKY75*, and *WRKY28*) and antioxidant defense genes (*Cu/Zn-SOD*, *APX2*, *CAT1*, and *POD2*), while suppressed the expression of chlorophyll-degradation genes (*PPH* and *Chl-PRX*), resulting in enhanced drought tolerance (increased leaf RWC, Fv/Fm, Chl content, proline content; decreased EL and MDA content, increased activities of SOD, POD, CAT, and APX).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13030675/s1, Figure S1: Effects of KAR_1 on electrolyte leakage of creeping bentgrass under control and drought-stressed conditions; Figure S2: Temporal trend of soil relative water content under control and drought-stressed conditions; Figure S3: The melting curves of 19 genes by RT-PCR; Figure S4: Effects of KAR_1 on plant performance of creeping bentgrass under control or drought stress for 30 d; Table S1: Primer sequences used for RT–PCR analysis.

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