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ORIGINAL ARTICLE

Increased dehydrin level decreases leaf rolling grade by altering the reactive oxygen species homeostasis and abscisic acid content in maize subjected to osmotic stress

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Abstract

Dehydrins (DHNs) are stress proteins involved in the development of protective reactions in plants against dehydration. The relationship between DHNs and morphological responses such as leaf rolling in plants exposed to water deficit is not well known. In this study, we detected how variations in DHN levels affect the leaf rolling response in maize exposed to osmotic stress in relation to the antioxidant system and ABA level. In this context, we altered the DHN levels in maize seedlings by treatment with bio-regulators (salicylic acid and abscisic acid) under PEG_{6000}-free and PEG_{6000}-induced osmotic stress. When the DHN levels were increased by the bio-regulators (25 µM SA and 100 µM ABA), the relative expression level of the *Zea mays dehydrin COR410* gene increased in the seedlings, while reactive oxygen species (ROS) and leaf rolling grade decreased. Moreover, induction of DHNs caused increases in the antioxidant enzyme activity and content of antioxidant substances, and very high amounts of endogenous abscisic acid. When DHN level was suppressed by a bio-regulator (200 µM SA) in the maize seedlings, *dehydrin COR410* expression level decreased, while ROS and the leaf rolling grade increased. Moreover, the antioxidant enzyme activity and content of antioxidant substances decreased in the seedlings, while the amount of abscisic acid increased. Taken all together, an increase in DHN level by bio-regulator treatment can stimulate the antioxidant system, enable abscisic acid regulation, and thus reduce leaf rolling through decreased ROS levels. The results also indicated that DHNs may be involved in the signal pathways inducing expression of some genes related to leaf rolling response, possibly by modulating ROS levels, in maize seedlings exposed to osmotic stress.

Keywords Antioxidant system · Dehydrin · Gene expression · Leaf rolling · Osmotic stress

Introduction

Severe water deficiency can dramatically reduce agricultural crop productivity and affect food security. Plants can evolve an array of strategies to adapt to water stress or survive, ranging from morphological and physiological adaptations to biochemical and molecular responses. Some symptoms describing tolerance to water deficit stress include cuticular wax accumulation, stomata closing, enhanced root length, and leaf rolling (Kadioglu et al. 2012; Khazaei et al. 2013).

Leaf rolling, visually observed in maize in agricultural areas, is among the main responses by plants to water deficit stress (Baret et al. 2018). Through this mechanism, maize plants can avoid moderate and severe water deficiency. Knowing how leaf rolling is controlled under conditions of water deficiency is very important in terms of the sustainability of photosynthetic production and grain yield. To understand the molecular mechanisms controlling leaf

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rolling, researchers have studied some genes controlling it. They demonstrated that several transcription factors were involved in the regulation of leaf rolling. For instance, Juarez et al. (2004a) reported that the maize *ROLLED LEAF1* (*RLD1*) gene (Accession no. AY501430) encoded a class III homeodomain-leucine zipper (HD-ZIPIII) protein and controlled the upward rolling of the leaf blade. Similarly, Zhang et al. (2009) demonstrated that *SHALLOT-LIKE1* (*SLL1*) (Accession no. Os09g23200) controlled leaf rolling in rice. Leaf rolling might be due to the failure of the microRNA to pair with mRNA of the target gene, which would cause expression of adaxial genes on the abaxial side of the leaves. Some microRNAs like miRNA166 may produce a movable signal to define the expression domain of *RLD1* (Juarez et al. 2004b). Additionally, some signaling molecules have been proven to play an important role in the regulation of leaf rolling. Application of salicylic acid (SA), a signaling molecule at low concentration (1 µM), delayed leaf rolling by decreasing water loss and activating the antioxidant system in maize seedlings (Kadioglu et al. 2011; Saruhan et al. 2012). Likewise, exogenous H_2O_2 as a signaling molecule delayed leaf rolling by inducing osmotic adjustment in maize (Terzi et al. 2014).

Microarray analysis of differentially expressed genes (DEGs) in rolled maize leaves showed that dehydrin (*DHN*) genes interestingly increased between 10- and 250-fold in comparison with the control leaves. It was reported that there could be an important relationship between leaf rolling and DHNs because of the very high increase in DHNs according to the microarray analyses (Kadioglu et al. 2014; Saruhan-Güler and Terzi 2020). Dehydration (dehydrin) proteins belong to a subfamily within the late-embryogenesis abundant (LEA) protein family, which plays an important role in the tolerance of plants to environmental stresses (Saruhan-Güler and Terzi 2020). DHNs can function as hub proteins and coordinate cross-talk with cellular signals in response to environmental stresses (Zhang et al. 2018). Since the expression of DHNs was significantly induced by abiotic stresses, it was shown that a positive correlation exists between the accumulation of DHN transcripts or DHN proteins and stress tolerance (Verma et al. 2017).

DHNs create abiotic stress tolerance in plants by scavenging reactive oxygen species (ROS) (Liu et al. 2017). Transgenic tobacco lines overexpressing YSK2 type dehydrin *SbDHN1* (Accession no. KT865881) and SK3 type dehydrin *SbDHN2* (Accession no. KT780443) genes isolated and characterized from *Sorghum bicolor* were able to prevent oxidative damage by decreasing ROS levels (Halder et al. 2018). Mehrabad Pour-Benab et al. (2019) examined the expression pattern of genes encoding antioxidant enzymes along with the *DHN* gene *WDHN13* (Accession no. AB863588.1), which is involved in drought stress tolerance in wheat. Furthermore, Liu et al. (2019) reported DHN accumulation and increases in antioxidant enzyme activities in switch grass exposed to drought. However, it is not known whether the induction of enzymatic and non-enzymatic antioxidants is due to an increase in DHN levels. Therefore, the relationship between DHNs and enzymatic and non-enzymatic antioxidants is still unclear.

DHN biosynthesis is considerably influenced by both dehydration stress (Kovacs et al. 2008) and phytohormone signaling (Maryan et al. 2019). Phytohormones are a crucial factor affecting *DHN* expression. ABA is an elicitor of *DHN* gene transcriptions and DHNs are highly expressed in response to ABA. Since DHNs are among the genes stimulated by ABA, they are considered ABA-responsive proteins and also they play a significant role in plants' stress resilience (Eriksson et al. 2011). For example, most of the *CaDHN* genes were induced by abiotic stresses and treatment with the signaling molecules ABA and SA in pepper (Jing et al. 2016). Likewise, treatments with SA at low concentration and ABA caused enhancements in DHN levels in plants under abiotic stresses but high SA concentrations caused suppression of DHN levels (Sun et al. 2009; Lv et al. 2017). Therefore, it was seen that exogenous ABA or SA could alter *DHN* gene expression and DHN levels in plants.

As can be understood from the literature mentioned above, leaf rolling is a complex phenomenon controlled by multiple genes and gene products. Although some genes controlling leaf rolling have been isolated, the signaling networks related to leaf rolling remain to be elucidated. Moreover, an insufficient number of studies have been performed on the interaction between leaf rolling (including morphological responses to environmental stresses) and DHNs in connection with the antioxidant system and phytohormonal regulation in plants exposed to abiotic stresses. The aim of the present study was to determine how variations in DHN level in osmotic stressed maize affect leaf rolling response in relation to the antioxidant system and ABA level. We tested the hypothesis that high DHN levels, created by bio-regulatory treatment, reduce the leaf rolling grade by both inducing the antioxidant system and causing a very high ABA level in maize leaves exposed to osmotic stress. To achieve the aim and to correct the hypothesis of the current study, DHN level was modified by treatment with bio-regulators (ABA and SA) in maize leaves under PEG_{6000}-free and PEG_{6000}-induced osmotic stress. Then the changes in the expression of the *dehydrin COR410* gene (Accession no. NM_001370707.1; Gene ID: 100,281,087), leaf rolling grade, antioxidant system, and amount of ABA were determined in the leaves.

Materials and methods

Plant materials and treatments

Seeds of *Zea mays* L. 'Burak' were provided by the Black Sea Agricultural Research Institute, Samsun, Turkey. The seeds were sterilized with 0.1% $HgCl_2$ for 1 min and then subsequently washed thoroughly four times with sterilized distilled water (Afridi et al. 2019) and sown in plastic pots (25 × 18 × 12 cm) filled with commercially obtained peat soil. The seedlings were placed in a growth chamber, with 22/18 °C temperature, 16/8 h light/dark period, 60% relative humidity, and 400 µmol m^{-2} s^{-1} photosynthetic photon flux density. When the seedlings reached the fully developed four-leaf stage (after three weeks), they were cut 2 cm above ground level and kept in distilled water for 1 h to minimize the effects of wounding due to excision. The excised seedlings were placed in test tubes containing various concentrations of bio-regulator. First, to determine the concentrations of bio-regulators that decreased and increased DHN levels the most in the leaves, the seedlings were treated with different SA (0, 10, 25, 50, 100, 200, 250, 500, and 1000 µM) and ABA concentrations (0, 10, 25, 50, 100, 150, and 200 µM). After that, the excised seedlings were kept in 25 µM SA and 100 µM ABA, which increased DHN level the most, and 200 µM SA, which decreased it the most under PEG-free conditions and under osmotic stress (-0.5 MPa) supplemented with polyethyleneglycol$_{6000}$ (PEG) addition. Therefore, the seedlings were subjected to the following eight different treatments for 12 h: (1) Control (only distilled water for 12 h), (2) 200 µM SA (pretreated with SA for 6 h and then kept in distilled water for 6 h), (3) 25 µM SA (pretreated with SA for 6 h and then kept in distilled water for 6 h), (4) 100 µM ABA (pretreated with ABA for 6 h and then kept in distilled water for 6 h), (5) PEG (osmotic stress treatment (-0.5 MPa) created by PEG_{6000} treatment), (6) 200 µM SA + PEG (pretreated with SA for 6 h and exposed to PEG_{6000} for 6 h), (7) 25 µM SA + PEG (pretreated with SA for 6 h and exposed to PEG_{6000} for 6 h), (8) 100 µM ABA + PEG (pretreated with SA for 6 h and exposed to PEG_{6000} for 6 h). The second leaf samples were used for the following experiments immediately 12 h after the treatments.

Measurements of leaf rolling grade (%) and plant water status

Leaf rolling grade was measured according to Premachandra et al. (1993). The width of the middle portion of the leaves was measured and the leaf rolling grade was calculated as the percentage reduction in leaf width. Because rolling is a visual indicator of water loss in plants, changes in water status based on relative water content (RWC) were determined according to the method described by Castillo (1996).

Protein extraction and western blot analysis

Protein extraction was performed using 4X protein extraction buffer (PEB, AS08 300 Agrisera Inc.). Samples were dissolved in an equal volume of sample buffer (2X Laemmli sample buffer, Bio-Rad) and heated at 95 °C for 5 min. Total proteins of samples (30 µg from each sample, calculated according to Bradford 1976) were separated by 12% SDS-PAGE using a Bio-Rad TGX Stain-Free FastCast Acrylamide Kit. The marker (Precision Plus Protein Western C Blotting Standards, Bio-Rad) was used to determine the molecular weight of the proteins. Proteins separated by gel electrophoresis were transferred to the PVDF membrane (Trans-Blot Turbo Mini PVDF Transfer Packs, Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad). Blotted membrane for immune determination was washed with TTBS buffer (5% dry skim milk, 24.8 mM Tris (pH 7.4), 150 mM NaCl, 2.7 mM KCl, and 0.5% Tween 20) for 1 h. It was then incubated with polyclonal rabbit anti-dehydrin primary antibody (Agrisera) for 1 night. The membrane was treated with alkaline phosphatase-bound goat anti-rabbit immunoglobulin G (IgG) polyclonal antibody (Sigma) for 4 h after being washed twice with TTBS buffer for 5 min at intervals of 10 min. After this washing, 5-solution of BCIP/NBT containing alkaline phosphatase substrate (Bio-Rad) was added, followed by a wait of approximately 15 min for the reaction to take place. The membrane was washed with water and scanned with the ChemiDoc MP imaging system (Bio-Rad) and the content was determined according to the marker.

RNA isolation and cDNA synthesis

Total RNA was extracted from maize leaves (0.1 g) using an RNeasy Plant Mini Kit (Cat. No: 74904, Quiagen, Germany). The concentration and purity of RNA obtained were determined using a NanoDrop spectrophotometer (Thermo Scientific, NanoDrop 2000, USA). cDNA was synthesized from the isolated total RNA samples (2000 ng RNA) using a high capacity cDNA Reverse Transcription Kit 4,368,814 (Applied Biosystems).

Quantitative real-time (qRT)-PCR analysis

For each qPCR, a total volume of 4 µL of Supermix (5X HOT FIREPol Eva Green qPCR Supermix (08–36-00,008, Solis Biodyne)), 1 µL of primers, 1 µL of cDNA sample,

and 20 µL of gene-specific primers was used and 20 µL of nuclease-free water was added. The analysis was carried out using the CFX Connect Real-Time PCR System (Bio-Rad). The qRT-PCR was performed according to Solis BioDyne's instructions, applying the following thermal protocol: 95 °C for 12 min, 45 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s, and melting curve analysis was performed in the range of 60 °C to 95 °C with 0.5 °C increments. Each biological repeat was analyzed as three technical repeats and the average technical error was considered in the form of 0.5 (± 1) Cq values. The primers used are listed in Table 1.

Analyses of chemical compounds

Leaf samples (0.5 g) were extracted with 0.1% (w/v) trichloroacetic acid (TCA). After centrifugation for 5 min at 15,000 × *g*, 0.5% (w/v) thiobarbituric acid (TBA) prepared in 20% (w/v) TCA was added to a 1-mL aliquot of the supernatant. To measure the thiobarbituric acid reagent (TBARS) content of the supernatant, the homogenate was heated at 95 °C for 30 min, followed by a rapid ice-cooling step ($\varepsilon = 155$ mmol^{-1} cm^{-1}) (Heath and Packer 1968). TBARS concentration was expressed as nmol TBARS per g^{-1} FW.

Hydrogen peroxide (H_2O_2) content was assayed according to Velikova et al. (2000) by extracting leaves in 0.1 g of activated charcoal in 5 mL of 0.1% trichloroacetic acid at 0 °C. To 1 mL of the supernatant were added 1 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1.5 mL of 1 M KI. The absorbance was measured at 390 nm and H_2O_2 concentration was expressed as µmol g^{-1} FW.

Superoxide radical production was measured spectrophotometrically using the tetrazolium salt XTT [(2,3-bis (2methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H tetrazolium hydroxide] according to Frahry and Schopfer (2001) with a minor modification. Increase in reduced XTT was read at 470 nm in a spectrophotometer.

Antioxidant enzyme assays

Frozen leaf segments (0.5 g) were homogenized in 5 mL of 50 mM of potassium phosphate (K_2HPO_4) buffer (pH 7.0) containing 1% polyvinylpolypyrrolidone and 1 mM EDTA mix. Additionally, 5 mM ascorbic acid (ASC) was added to the extraction buffer for the APX assay. The Bradford protein assay was used to measure the concentration of total protein (Bradford 1976).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined based on the method described by Beauchamp and Fridovich (1971). Catalase (CAT, EC 1.11.1.6) activity was measured by following the consumption of H_2O_2 ($\varepsilon = 39.4$ mM^{-1} cm^{-1}) at 240 nm (Aebi 1983). Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined by monitoring the decrease in ascorbate at 290 nm (25 °C, $\varepsilon = 2.8$ mM^{-1} cm^{-1}) (Nakano and Asada 1987). Glutathione reductase (GR, EC 1.6.4.2) activity was measured by monitoring the oxidation of NADPH at 340 nm ($\varepsilon = 6.22$ mM^{-1} cm^{-1}) (Foyer and Halliwell 1976). The activities of all antioxidant enzymes were expressed as µmol min^{-1} mg^{-1} protein.

Analysis of antioxidant substances

For glutathione analysis, leaves (0.5 g) were extracted in 5 mL of 5% metaphosphoric acid containing 1 mM EDTA. The extract was centrifuged for 10 min at 10,000 × *g* at 4 °C. Glutathione content was determined using the total glutathione assay kit (Northwest Life Sci. Spec) according to the manufacturer's instructions. Glutathione was measured using a reaction mixture containing 250 mM K_2HPO_4/KH_2PO_4 (pH 7.5), 200 µM NADPH, 600 µM DTNB, 25 µL of extract, and 0.3 U of GR. The change in absorbance at 412 nm was observed for 3 min. Glutathione content was calculated on the GSH standard curve at 0–5 µM concentrations.

Ascorbate concentration was determined according to the method described by Liso et al. (1984). For this, 0.25 g of leaves was homogenized with 5 mL of 5% (w/v) m-phosphoric acid. The homogenate was centrifuged for 4 min at 10,000 × *g* at 4 °C. After centrifugation, 70 µL of the sample was added to 3 mL of reaction medium containing 0.1 M citrate–0.2 M phosphate buffer (pH 6.2). The initial absorbance was read at 265 nm. Ascorbate concentration was determined by the decrease 5 min after the addition of two units of ascorbate oxidase to the reaction medium. After the ascorbate oxidation was complete, ascorbate oxidase was inhibited with 10 mM sodium azide. Subsequently, 2.5 mM dithiothreitol (DTT) was added to the medium. After the reduction with DTT (3 min), the absorbance was read again

Table 1 The sequence of *dehydrin COR410* gene-specific primers used for qRT-PCR analysis

Target Gene	NCBI accession no	Gene ID	Primer name and its sequence
Actin 1	NM_001155179.1	100,282,267	ACT1Zm_F: "GAAGATCACCCTGTGCTGCT"
			ACT1Zm_R: "ACCAGTTGTTCGCCCACTAG"
dehydrin COR410	NM_001370707.1	100,281,087	DHRNZm_F: "GAAGGTAGCTAGCGTTGGCA"
			DHRNZm_R: "ACCACGTCCTACACAAGCAG"

at 265 nm and the ascorbate concentration of the samples was determined on the standard curve.

Extraction and determination of ABA

Leaves were frozen in liquid nitrogen and extracted in distilled water at a ratio of 1:7 (w/v) and then left overnight in the dark at 4 °C. The extract was centrifuged at 10,000 × *g* for 10 min at 4 °C and the supernatant was diluted four times in standard Tris-buffered saline. ABA content was quantified using a Phytodetek ABA immunoassay kit (ELISA) (Agdia/Linaris).

Statistical analysis

All experiments were performed in triplicate. Ten samples were used for each treatment group. The results were analyzed with Duncan's multiple comparison test (one-way ANOVA) using SPSS for Microsoft Windows (Ver. 15.0, SPSS Inc., USA) and the significance level was determined as 5% ($P < 0.05$). For qRT PCR analysis, the relative gene expression level was calculated by the Bio-Rad CFX Manager 3.1. Expression levels were assayed by SPSS.

Results

When the DHN levels were altered by exogenous treatment with bio-regulators, the DHN protein fraction revealed the presence of bands with a molecular weight of approximately 62.5 kDa by western blot analysis under PEG-free conditions and PEG-induced osmotic stress (Fig. 1A). According to the densitometry (DHN band density) data, the 200 µM SA treatments suppressed DHN levels compared with their controls under PEG-free conditions and PEG-induced osmotic stress. Conversely, the 25 µM SA and 100 µM ABA treatments enhanced DHN levels under both conditions. The DHN level increased in the seedlings treated with PEG, but this increase was not as great as that in the 100 µM ABA + PEG and 25 µM SA + PEG treatments. In addition, the highest increase compared with the control was in the seedlings treated with 100 µM ABA + PEG (Fig. 1B).

The expression level of the *dehydrin COR410* gene

Changes in the expression levels of *dehydrin COR410* were consistent with the findings for DHN protein levels. The relative expression of the *dehydrin COR410* gene was suppressed by the 200 µM SA and 200 µM SA + PEG treatments in comparison with the control and the PEG treatments, respectively. Gene expression showed enormous increases in the 25 µM SA + PEG- and 100 µM ABA + PEG-treated seedlings compared with the PEG treatment. The *dehydrin COR410* gene expression displayed a more dynamic induction pattern with the 100 µM ABA + PEG treatment, while it was generally less affected in the 25 µM SA + PEG-treated seedlings (Fig. 1C).

Leaf rolling grade (%) and relative water content (RWC)

Leaf rolling was not observed in the control seedlings. The maximum leaf rolling (22.2%) was observed in the 200 µM SA-treated seedlings under the PEG-free conditions, while the leaf rolling grades were 20.9% and 19.04% in the seedlings treated with 25 µM SA and 100 µM ABA, respectively (Fig. 2A, B).

Leaf rolling grades (%) significantly changed with the PEG-induced osmotic stress in comparison with PEG-free conditions. The rolling grades decreased as the level of DHN increased and vice versa. For instance, compared with the PEG treatment (rolling grade 41.4%), the 200 µM SA + PEG treatment increased the leaf rolling grade (48.9%). Conversely, the 25 µM SA and 100 µM ABA treatments decreased the leaf rolling grades. The values of leaf rolling grades recorded were 36.8% and 32.5% for the 25 µM SA + PEG and 100 µM ABA + PEG treatments, respectively (Fig. 2A, B).

Compared with the control, the 25 µM SA and 100 µM ABA treatments did not significantly change RWC, an indicator of water status, under PEG-free conditions. Conversely, the 200 µM SA treatment decreased RWC under PEG-free conditions. RWC also decreased in the seedlings under the PEG-induced osmotic stress in comparison with PEG-free conditions, and RWC was lowest after the 200 µM SA + PEG treatment. The RWC of the 25 µM SA + PEG- and 100 µM ABA + PEG-treated seedlings were greater than that of the PEG seedlings, while the RWC of the seedlings treated with 200 µM SA + PEG was lower than that of the PEG seedlings (Fig. 2C).

Lipid peroxidation, H_2O_2 and superoxide contents

The 200 µM SA treatment under the PEG-free conditions increased TBARS content in comparison with the control treatment. The 25 µM SA and 100 µM ABA treatments did not affect the TBARS content. Similarly, the 200 µM SA treatment under PEG-induced osmotic stress increased TBARS content in comparison with the PEG treatment. The TBARS values after the 25 µM SA + PEG and 100 µM ABA + PEG treatments were lower than that after the PEG treatment (Fig. 3A).

Under the PEG-free conditions, H_2O_2 content was enhanced in the leaves treated with 200 µM SA, but it was not changed significantly by the 25 µM SA and 100 µM ABA treatments compared with the control treatment.

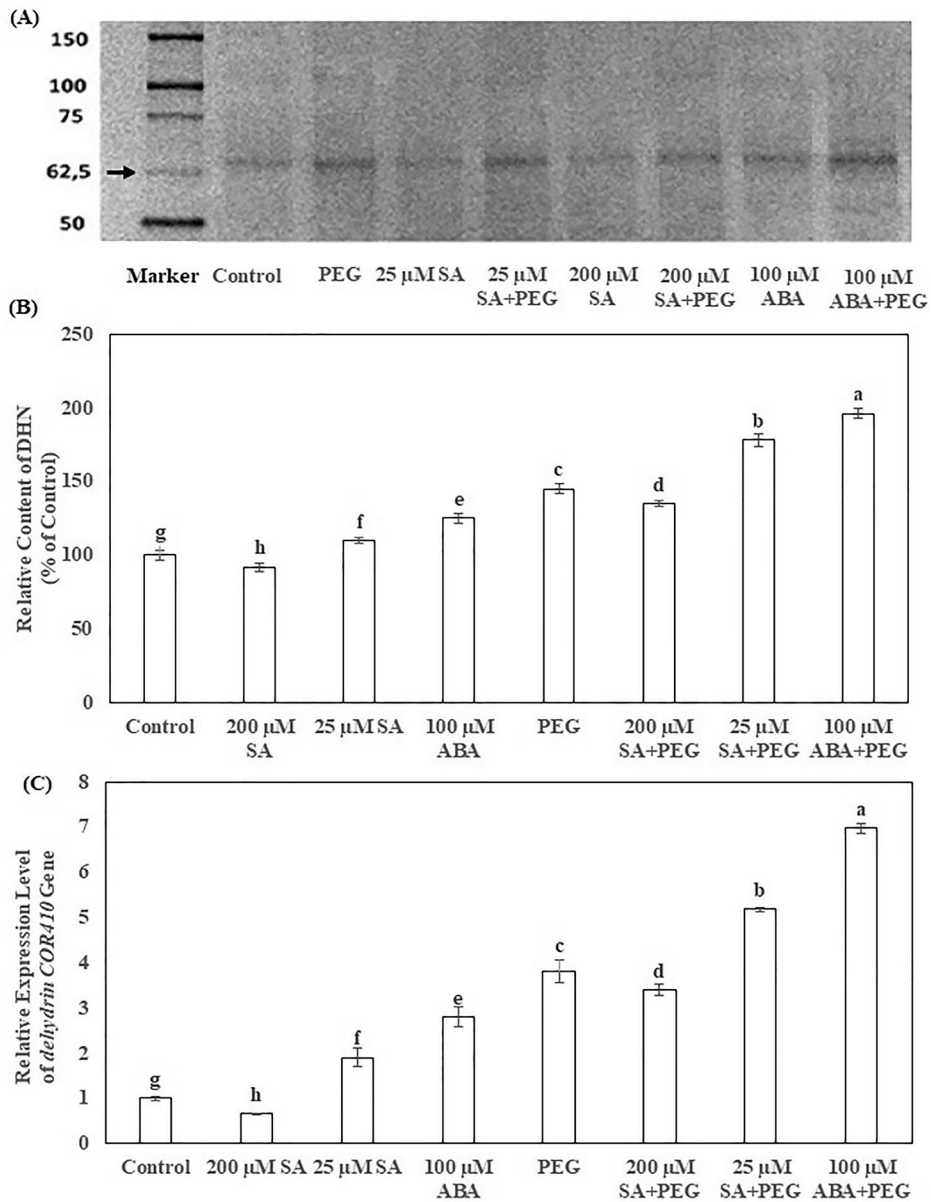

Fig. 1 Effect of exogenous bio-regulators on dehydrin accumulation under PEG-free conditions and PEG-induced osmotic stress. Representative western blot **A**, densitometric analysis of DHN bands expressed as a percentage of values for corresponding control **B**, changes in expression level of *dehydrin COR410* gene **C**. Data are means ± SD of three replicates. Different letters indicate significant differences according to a Duncan's multiple range test ($P < 0.05$)

H_2O_2 content increased under PEG-induced osmotic stress in comparison with the control. Moreover, the highest increase in H_2O_2 content was after the 200 µM SA + PEG treatment. The H_2O_2 content after the 25 µM SA + PEG and 100 µM ABA + PEG treatments was lower than that after the PEG treatment (Fig. 3B).

The superoxide level increased after the 200 µM SA treatment compared with the control treatment, while there were no significant changes in superoxide content after the 25 µM SA and 100 µM ABA treatments. In comparison with the PEG treatment, the superoxide content increased after the 200 µM SA + PEG treatment but it decreased after 25 µM SA + PEG and 100 µM ABA + PEG treatments (Fig. 3C).

Antioxidant enzyme activities

All treatments caused significant differences in the activities of antioxidant enzymes. SOD activity was higher after the 25 µM SA and 100 µM ABA treatments, whereas the activity did not differ after the 200 µM SA treatment from that of the control treatment. On the other hand, SOD activity was significantly lower after the 200 µM SA + PEG treatment in comparison with the PEG treatment but the activity was higher after the 25 µM SA + PEG and 100 µM ABA + PEG treatments (Fig. 4A).

CAT activity was not changed by the 200 µM SA treatment in comparison with the control treatment, whereas activity was higher after the 25 µM SA and 100 µM ABA

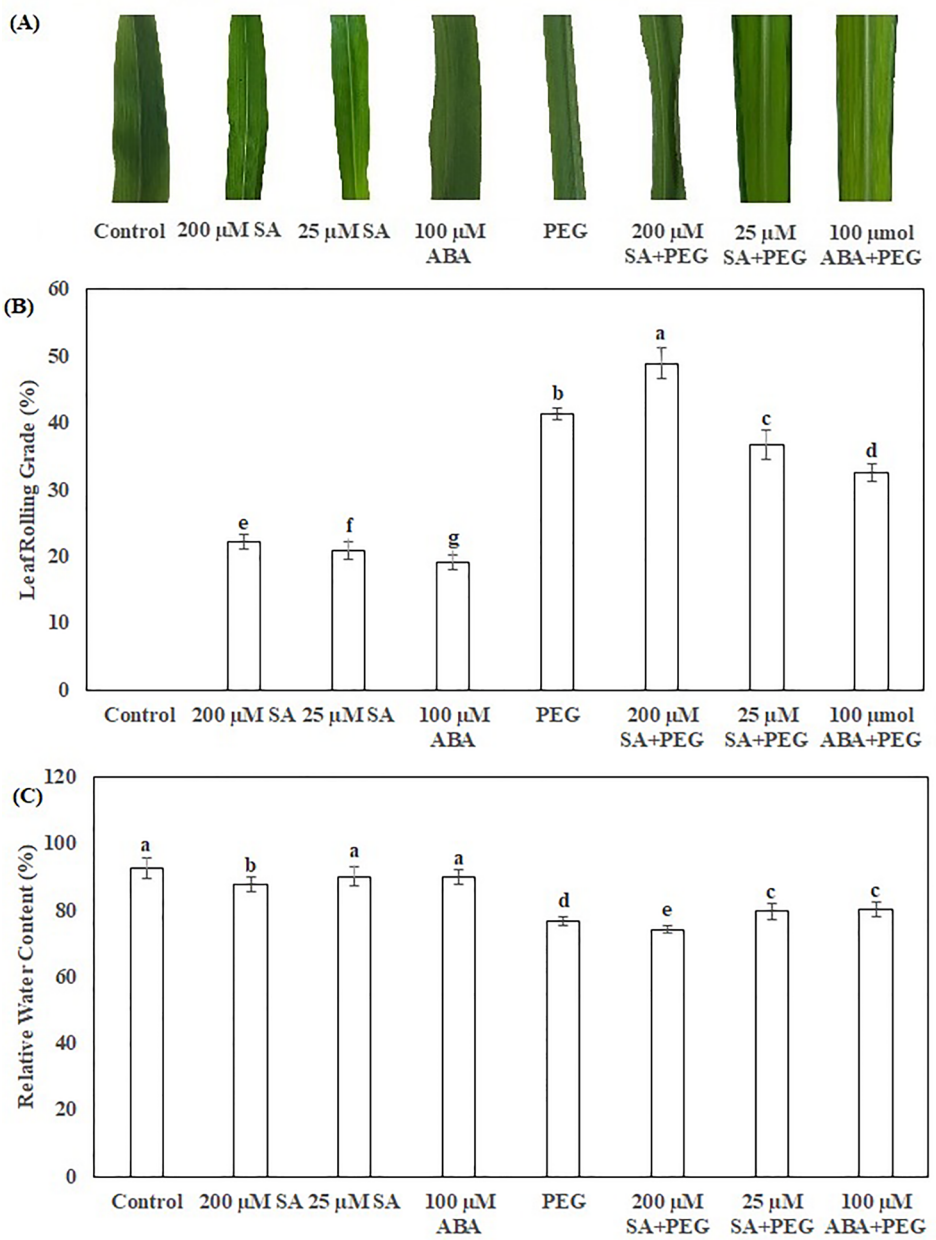

Fig. 2 Changes in leaf rolling grade (%) (**A**, **B**) and RWC (**C**) in the maize seedlings with modified the DHN levels by exogenous bio-regulators under PEG-free conditions and PEG-induced osmotic stress. Data are means ± SD of three replicates. Different letters indicate significant differences according to a Duncan's multiple range test ($P < 0.05$)

treatments. CAT activity after the 200 µM SA + PEG treatment was lower than that after the PEG treatment but activity was higher after the 25 µM SA + PEG and 100 µM ABA + PEG treatments (Fig. 4B).

In comparison with the control, APX activity increased in parallel with DHN accumulation in the leaves treated with 25 µM SA and 100 µM ABA, whereas the activity did not change in the 200 µM SA-treated leaves. In addition, while APX activities were higher after the 25 µM SA + PEG and 100 µM ABA + PEG treatments compared with the PEG treatment, the activity was lower after 200 µM SA + PEG treatment. The strongest effect compared with the PEG treatment was in leaves treated with 100 µM ABA + PEG, with an approximately twofold increase (Fig. 4C).

As for GR, the activity was higher after the 25 µM SA and 100 µM ABA treatments under PEG-free conditions compared with the control treatment, while the activity was lower after the 200 µM SA treatment. GR activity increased in parallel with the DHN accumulation in the leaves treated with 25 µM SA + PEG and 100 µM ABA + PEG compared with the PEG treatment. In contrast, the activity after the 200 µM SA + PEG treatment was lower than that after the PEG treatment (Fig. 4D).

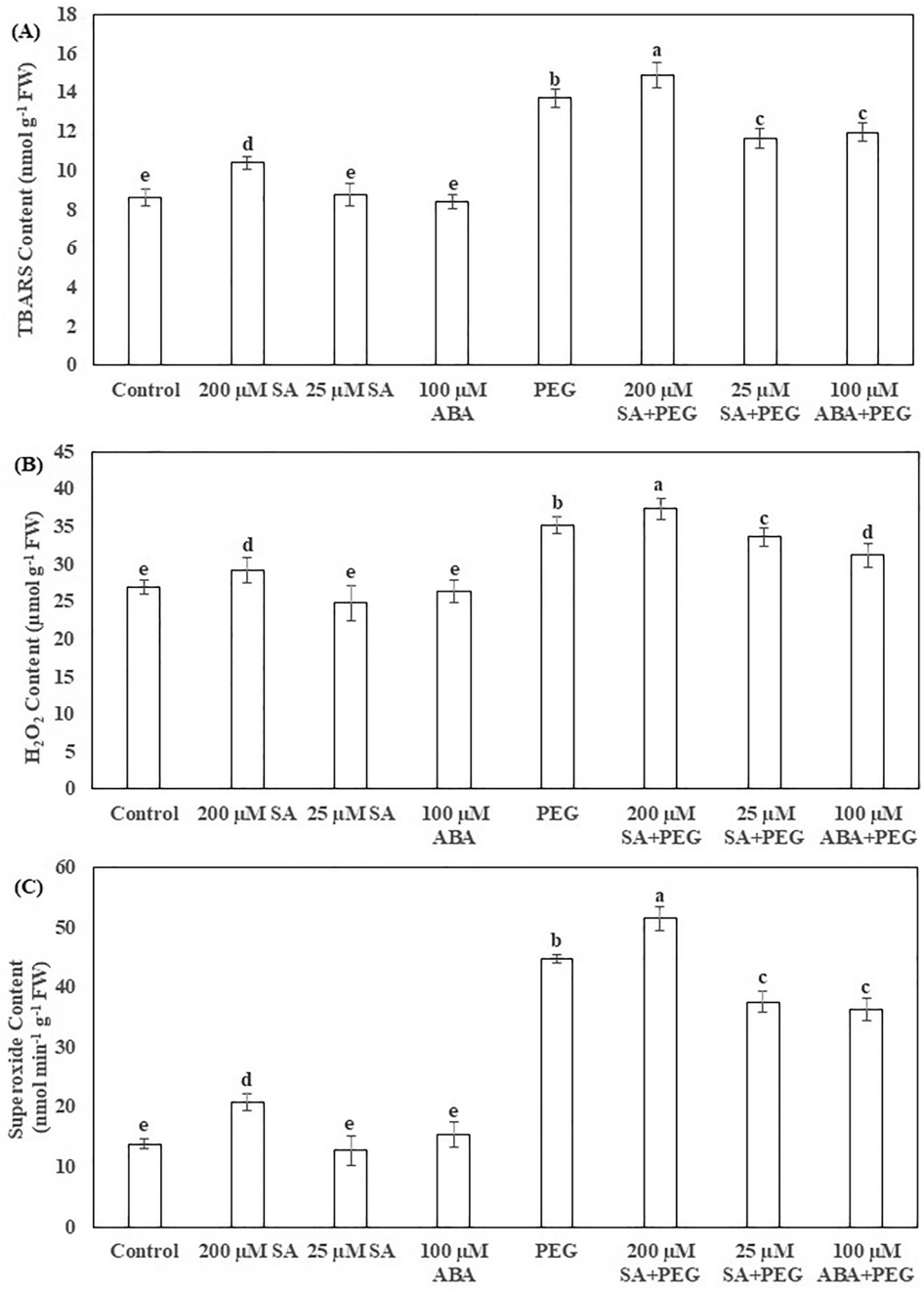

Fig. 3 Changes in TBARS (**A**), H_2O_2 (**B**) and superoxide (**C**) contents in the maize seedlings with modified the DHN levels by exogenous bio-regulators under PEG-free conditions and PEG-induced osmotic stress. Data are means ± SD of three replicates. Different letters indicate significant differences according to a Duncan's multiple range test ($P < 0.05$)

Content of antioxidant compounds

Compared with the untreated leaves, ASC content was not affected by the 200 µM SA treatments under the PEG-free conditions or under PEG-induced osmotic stress. However, ASC content was higher in the 25 µM SA and 100 µM ABA groups compared with the untreated groups under both PEG-free conditions and PEG-induced osmotic stress (Fig. 5A).

As for DHA, it did not differ between the 200 µM SA-treated leaves and the untreated leaves under PEG-free or osmotic stress conditions. Compared with the control treatment, DHA content was lower after the 25 µM SA and 100 µM ABA treatments. Similar decreases were detected after the 25 µM SA + PEG and 100 µM ABA + PEG treatments in comparison with the PEG treatment (Fig. 5B).

Total GSH content increased in parallel with DHN accumulation in the 25 µM SA and 100 µM ABA treatments compared with the control treatment. However, it was lower after the 200 µM SA treatment. Compared with the PEG treatment, the GSH content increased in parallel with the DHN accumulation after the 25 µM SA + PEG

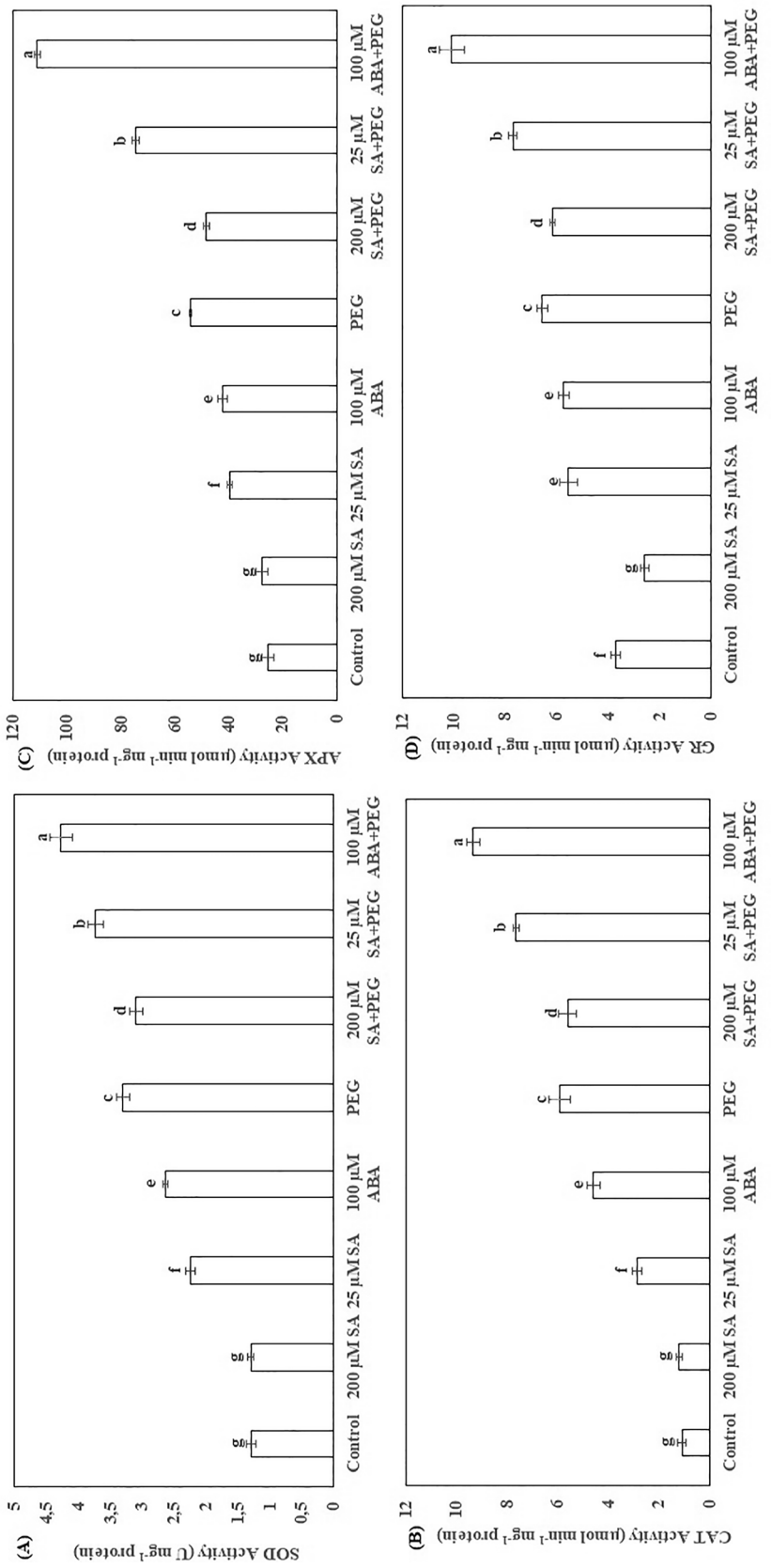

Fig. 4 Changes in activities of antioxidant enzymes SOD (**A**), CAT (**B**), APX (**C**) and GR (**D**) in the maize seedlings with modified the DHN levels by exogenous bio-regulators under PEG-free conditions and PEG-induced osmotic stress. Data are means ± SD of three replicates. Different letters indicate significant differences according to a Duncan's multiple range test ($P < 0.05$)

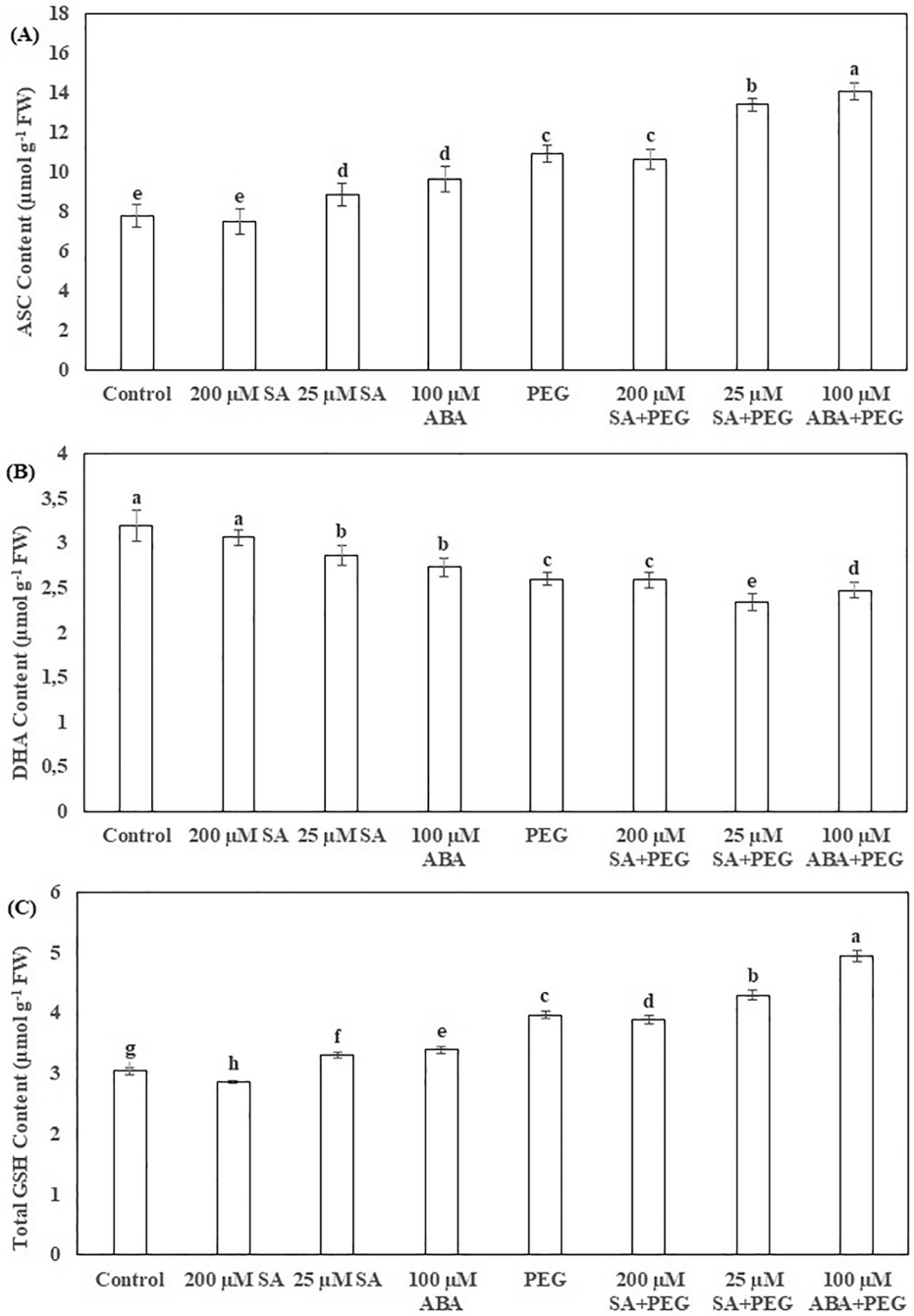

Fig. 5 Changes in ASC (**A**), DHA (**B**) and GSH (**C**) contents in the maize seedlings with modified the DHN levels by exogenous bio-regulators under PEG-free conditions and PEG-induced osmotic stress. Data are means ± SD of three replicates. Different letters indicate significant differences according to a Duncan's multiple range test ($P < 0.05$)

and 100 µM ABA + PEG treatments, while it was lower after the 200 µM SA + PEG treatment (Fig. 5C).

ABA content

ABA content was high in the leaves treated with 100 µM ABA under PEG-free and PEG-induced osmotic stress conditions. Moreover, all SA treatments caused increases in ABA content in parallel with DHN accumulation. ABA content after the 25 µM SA treatment and the 25 µM SA + PEG treatment were higher than that after the 200 µM SA and 200 µM SA + PEG treatment, respectively (Fig. 6).

Discussion

Studies investigating the relationship between DHNs and plant stress responses have attracted considerable attention in recent years, as it is very important to gain a better understanding of the mechanisms regulating stress tolerance and

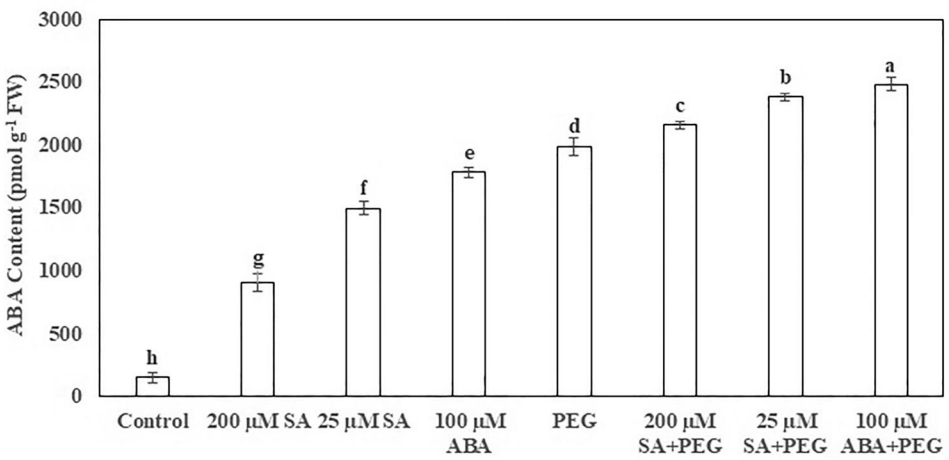

Fig. 6 Changes in ABA content in the maize seedlings with modified the DHN levels by exogenous bio-regulators under PEG-free conditions and PEG-induced osmotic stress. Data are means ± SD of three replicates. Different letters indicate significant differences according to a Duncan's multiple range test ($P < 0.05$)

how plants respond to environmental stresses. In this sense, the interaction between DHNs and leaf rolling, a morphological response to water deficit, may be an essential factor revealing the stress tolerance mechanisms in plants subjected to environmental stresses. Furthermore, to the best of our current knowledge, there is no record of how variations in DHN levels modify antioxidant system components and abscisic acid level in plants under water deficit. Here, we first determined the concentrations of bio-regulators (SA and ABA) altering the DHN levels in maize seedlings based on the studies reported by other researchers. For instance, Lv et al. (2017) reported that DHNs are highly expressed in response to 50 µM ABA application in Bermuda grass under drought stress. Sun et al. (2009) reported that exogenous SA at low concentration increased DHN levels but its high concentrations caused suppression of DHN levels in water-stressed barley seedlings. Similarly, we detected that the levels of DHN proteins with a molecular weight of 62.5 kDa in the 25 µM SA- and 100 µM ABA-treated seedlings were higher than those of the untreated seedlings under PEG-free conditions and PEG-induced osmotic stress, while DHN levels in the 200 µM SA-treated seedlings were low. It was reported that *dehydrin COR410* expression is highly induced by drought stress in *Zea mays* (Ahmad et al. 2019). Moreover, we previously conducted microarray analysis of differentially expressed genes in rolled maize leaves and reported that there were significant increases in dehydrin genes, especially in *dehydrin COR410* (Kadioglu et al. 2014; Saruhan-Güler and Terzi 2020). Therefore, we determined the expression level of the *dehydrin COR410* gene in the present study. According to the western blot analysis and RT-PCR data, the changes in the expression levels of *dehydrin COR410* were consistent with the findings for DHN protein levels. In addition, we detected that *dehydrin COR410* gene expression was induced more by the 100 µM ABA + PEG treatment than by the 25 µM SA + PEG treatment (Fig. 1C). These findings indicated that *dehydrin COR410* gene expression might be stimulated more effectively by ABA-dependent signal pathway than by SA signaling. As known, the expression of many *DHN* genes was upregulated via ABA-dependent and ABA-independent pathways in plants (Saruhan-Güler and Terzi 2020).

We evaluated changes in leaf rolling grades in the leaves by altering the DHN levels using exogenous bio-regulators. Leaf rolling was not observed in the control seedlings due to no water loss. However, leaf rolling grades of the seedlings with increased DHN levels (the 25 µM SA and 100 µM ABA treatments) were lower than those of the seedlings with suppressed DHN levels (the 200 µM SA treatments) under the both PEG-free and PEG conditions. Moreover, leaf rolling grade was low in the ABA + PEG seedlings with the highest DHN level (Fig. 2B). Therefore, we can conclude that induction of DHN levels by bio-regulator treatment alleviates increased leaf rolling grade in maize exposed to osmotic stress. In addition, the roles of ABA and SA at low concentration in protecting against stress damage may contribute to the reductions in leaf rolling grades. On the other hand, there are only a few studies on the effects of DHN level on morphological responses. For instance, Hassan et al. (2015) suggested that the wheat variety capable of expressing high *DHN* genes showed the greatest decrease in leaf area under drought conditions.

We also detected changes in water status in maize seedlings because the rolling is a visual indicator of water loss in plants. The decreases in leaf rolling grades were consistent with the increases in leaf RWC in the maize seedlings (Fig. 2A-C). RWC and TBARS content did not differ significantly between the treatments with induced DHN levels under PEG-free conditions and the control treatment. A high SA concentration (200 µM) had a negative influence on RWC, TBARS content, and leaf rolling compared with the control treatment. Similar negative findings in these parameters were determined with the 200 µM SA + PEG treatment, compared with the PEG treatment. Indeed, in a similar study,

excessive SA suppressed the accumulation of DHN proteins and triggered oxidative damage in water-stressed barley seedlings (Sun et al. 2009). While low concentrations of SA enhanced water stress tolerance, high SA concentrations decreased stress tolerance and even led to susceptibility to abiotic stresses or cell death (Miura and Tada 2014). Conversely, RWC in the treatments with induced DHN levels under osmotic stress was higher than that with the PEG treatment, and the TBARS content, which indicated lipid peroxidation levels, was lower. The reason there was less membrane damage in the seedlings with induced DHN levels under osmotic stress may be the hydrophilic properties of DHNs and their functions as a possibly osmotic regulator. As known, structurally DHNs are rich in amphiphilic α-helix and random coils, can interact with intracellular molecules, especially proteins and membranes, by hydrophobic interaction, and thus inhibit their aggregation under water stress conditions. The water-binding activity of the randomized α-helices can also ensure that adequate water is preserved in the cells with water deficiency (Shekhawat et al. 2011).

We determined that the ROS level increased in line with the increases in TBARS content, an indicator of lipid peroxidation. Moreover, the 200 µM SA treatment under the PEG-free conditions increased TBARS content compared with the control treatment because of the increase in ROS level and decrease in DHN level. On the other hand, the exposure of plants to certain environmental stresses often leads to the generation of ROS. Increases in endogenous ROS levels induced leaf rolling in plants such as *Ctenanthe setosa* under water deficit (Saruhan et al. 2010). We observed low ROS (H_2O_2 and superoxide) accumulation and a reduced leaf rolling grade in the treatments with increased DHN level under osmotic stress in comparison with the PEG treatment. Some researchers have pointed out how DHNs mitigate oxidative damage by scavenging hydroxyl and peroxyl radicals and binding metals in stressed plants (Hara et al. 2003; Zhang et al. 2006). In particular, several SK3-type DHNs were shown to act as radical-scavenging proteins to protect membrane systems under stress conditions (Liu et al. 2017). We analyzed the main components of the antioxidant system, including ROS, antioxidant enzymes, and antioxidant substances. Therefore, we determined whether the bio-regulator-induced alterations in DHN levels affect the leaf rolling response in conjunction with the efficiency of the antioxidant defense system in maize seedlings under osmotic stress. ROS levels were high in the seedlings with decreased DHN level under osmotic stress and PEG-free conditions. Therefore, it appears that decreased DHN levels under osmotic stress may cause increases in free radicals playing a signaling role in leaf rolling response in maize seedlings. Conversely, ROS content was lower in the seedlings with induced DHN levels under osmotic stress in comparison with the PEG treatment. Our findings indicated that DHN levels induced by bio-regulator treatment could scavenge free radicals by stimulating the activities of antioxidant enzymes. Thereby, they could reduce oxidative damage caused by water deficit and reduce leaf rolling.

We determined that the activities of all antioxidant enzymes in the seedlings with induced DHN levels under PEG-free and osmotic stress conditions were higher than those of the control and PEG-treated seedlings. In previous studies, it was stated that exogenously applied SA and ABA increased the activities of antioxidant enzymes (Kadioglu et al. 2011; Saruhan et al. 2012; Sezgin et al. 2018). Additionally, it was reported that DHNs could scavenge ROS, and there was a close interaction between DHNs and abiotic stress tolerance (Liu et al. 2017). The results indicated that a suitable increase in DHN level can provide moderate leaf rolling response in maize seedlings under osmotic stress due to the tolerance mechanism provided by antioxidant enzymes reducing ROS to a non-toxic level. On the other hand, some researchers showed that exogenous SA at high concentration was toxic, suppressing plant growth and drought tolerance in wheat seedlings (Kang et al. 2012). It could also promote ROS formation in photosynthetic tissues and increase oxidative damage during osmotic stress (Miura and Tada 2014). According to our results, suppression of DHN levels via SA treatment at high concentrations caused decreases in antioxidant enzyme activities and thus increases in free radical levels and leaf rolling grade.

Exogenous SA and ABA have been shown to affect levels of several antioxidant substances such as ASC and GSH in plants and ensure plant tolerance to abiotic stresses (Kadioglu et al. 2011; Liu et al. 2011; Saruhan et al. 2012). On the other hand, treatment with low concentrations of SA or ABA could increase the DHN levels in plants, while high SA concentrations could reduce them (Sun et al. 2009). Moreover, several studies have revealed a powerful relationship between DHNs and ASC in plants subjected to various stress conditions. For instance, it was found that transgenic *Arabidopsis* plants expressing the wheat dehydrin genes *DH-4* and *DH-2* had higher ASC content than the wild type under osmotic stress. Thus, both transgenic lines showed enhanced tolerance to oxidative stress (Brini et al. 2011). Similarly, Zhang et al. (2020) reported that transgenic *Arabidopsis thaliana* expressing the *CdDHN4-S* gene (Accession no. KJ000690) isolated from Bermuda grass exhibited higher ASC formation than the wild type under salt or drought stress. The present study investigated how variations in DHN levels mediated by a bio-regulator such as SA or ABA affect leaf rolling response in relation to antioxidant substances. It was determined that ASC content in the leaves with increased DHN levels under osmotic stress were higher than those treated with PEG and the leaf rolling grades of these seedlings were low. Conversely, ASC content did not significantly change in the seedlings with suppressed DHN levels

in comparison with the PEG treatment. As a ROS scavenger, higher amounts of ASC have shown that the seedlings could tolerate ROS-mediated oxidative stress better. Similar to the ASC results, GSH content in the leaves with increased DHN levels under osmotic stress was higher than that with PEG treatment. Such a rise in the level of GSH may be related to the increases in the activity of GR catalyzing the reduction of oxidized glutathione (GSSG) to GSH. An opposite pattern in GSH content was observed in the leaves with suppressed DHN levels. Our findings supported the idea that suitable increases in DHN levels through exogenous ABA and SA treatment at low concentration can effectively stimulate the antioxidant defense system and reduce leaf rolling due to an antioxidant system in maize under osmotic stress.

Plants could also develop endogenous defense strategies that increase or decrease endogenous hormone levels to prevent damage caused by ROS (Horváth et al. 2007). We tried to determine whether altering the DHN level influenced the leaf rolling grade in relation to the changes in endogenous ABA level. As mentioned above, phytohormones are crucial factors affecting DHN accumulation. In some of the previous studies it was demonstrated that exogenous ABA and SA affected leaf rolling behavior in plants. For instance, Sezgin et al. (2018) reported that exogenous ABA caused a decrease in leaf rolling by providing osmotic regulation through the accumulation of proline, polyamine, and total soluble sugars in maize under drought. We found that the SA treatments at low concentrations caused greater ABA content than the SA treatments at a high concentration under PEG-free conditions and PEG-induced osmotic stress. Based on our findings on endogenous ABA content in maize seedlings under osmotic stress and PEG-free conditions, we can conclude that bio-regulator-mediated DHN accumulation can reduce leaf rolling in conjunction with abscisic acid regulation in maize under osmotic stress. Actually, exogenous ABA caused more dehydrin accumulation than SA at low concentration, resulting in a lower leaf rolling grade in ABA-treated leaves. Furthermore, we suggest that a low concentration of SA can stimulate the expression of *DHN* genes, leading to increased ABA biosynthesis. Similar to our findings, it was reported in a review paper that SA treatments triggered the accumulation of ABA in cultivated plants, leading to improved acclimation to abiotic stresses (Miura and Tada 2014).

Conclusion

We investigated a new aspect of DHNs thought to be related to leaf rolling response, which is a morphological response in plants exposed to stress. We also examined how variations in DHN levels affect antioxidant system components and abscisic acid level in plants. The data we obtained showed that induction of *dehydrin COR410* gene expression and DHN level through low concentration SA (25 µM) and 100 µM ABA treatments reduced leaf rolling grades. Conversely, leaf rolling grade increased when *dehydrin COR410* gene expression and DHN level were suppressed through SA treatment at high concentration (200 µM). The increased DHN level during osmotic stress could scavenge ROS by stimulating antioxidant enzyme activities. Furthermore, DHN accumulation could trigger an increase in ABA content. These changes in ROS and ABA levels may reduce leaf rolling grade in maize. The bio-regulator-mediated DHN accumulation could result in moderate leaf rolling by increasing the antioxidant enzyme activities and antioxidant compound and cause related high endogenous ABA levels in maize leaves (Fig. 7). We obtained evidence that DHNs can work as a regulatory protein to control leaf rolling response, possibly by modulating ROS level along with some protective mechanisms including activation of the antioxidant system and regulation of ABA level under stress conditions. Further studies in the near future on *DHN* gene expression associated with morphological responses and hormones in plants exposed to environmental stresses will be useful for understanding stress tolerance mechanisms.

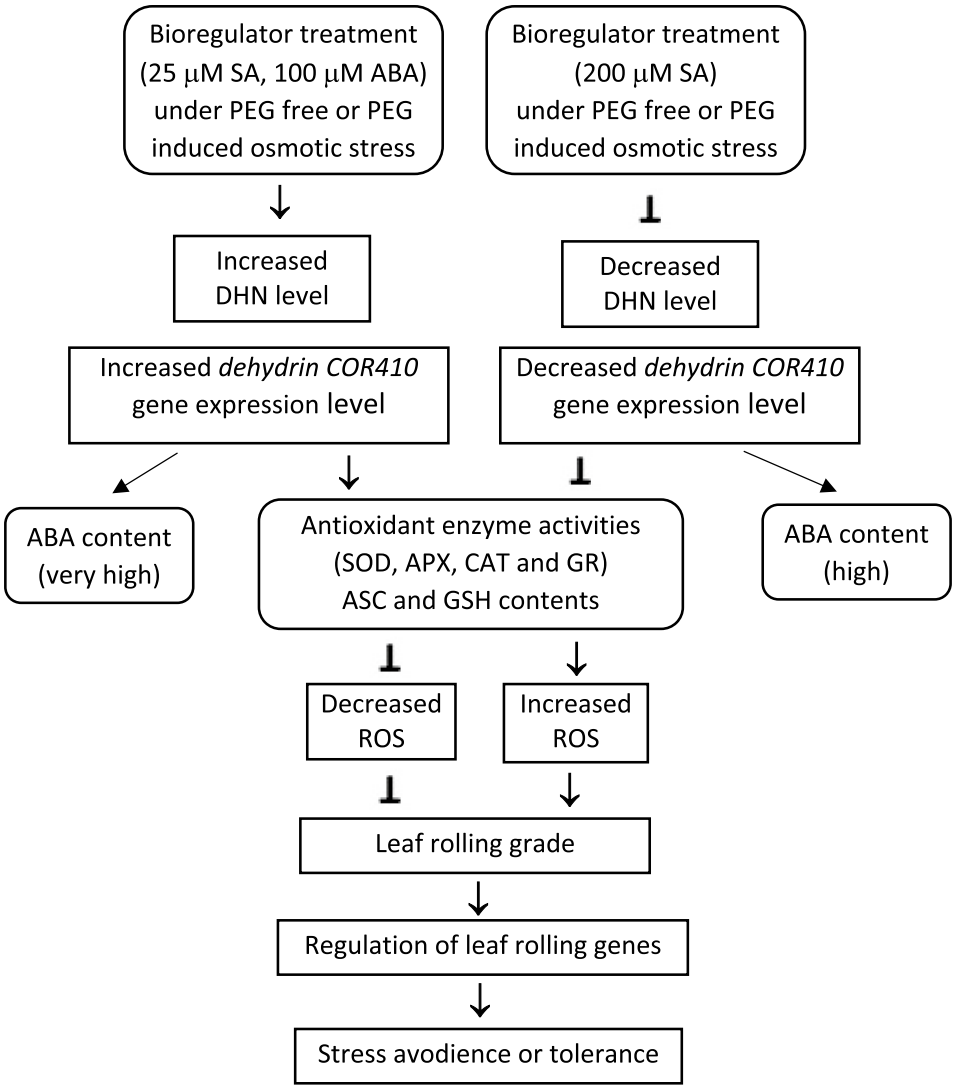

Fig. 7 DHN role in leaf rolling response as connected by antioxidant system and phytohormonal regulation

Authors' contributions Neslihan Saruhan Guler: the design of the study, analyzed all data and wrote the manuscript. Rabiye Terzi: the design of the study, analyzed all data and wrote the manuscript. Asim Kadioglu: review and editing of manuscript. Mehmet Demiralay and Kamil Ozturk performed the experiments.

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Declarations

Conflict of interest All the authors declare that there is no conflict of interest.

Availability of data and material Not applicable.

Code availability Not applicable.

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