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Role of Abscisic Acid in Water Stress-induced Antioxidant Defense in Leaves of Maize Seedlings

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Roles of abscisic acid (ABA) in water stress-induced oxidative stress were investigated in leaves of maize (*Zea mays* L.) seedlings exposed to water stress induced by polyethylene glycol (PEG 6000). Treatment with PEG at −0.7 MPa for 12 and 24 h led to a reduction in leaf relative water content (RWC) by 7.8 and 14.1%, respectively. Duration of the osmotic treatments is considered as mild and moderate water stress. The mild water stress caused significant increases in the generation of superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) and the contents of ascorbate (ASC), reduced glutathione (GSH). The moderate water stress failed to further enhance the capacity of antioxidant defense systems, as compared to the mild water stress. The contents of catalytic Fe, which is critical for H_2O_2-dependent hydroxyl radical ($^{\bullet}OH$) production, and the oxidized forms of ascorbate and glutathione pools, dehydroascorbate (DHA) and oxidized glutathione (GSSG), markedly increased, a significant oxidative damage to lipids and proteins took place under the moderate water stress. Pretreatment with ABA caused an obvious reduction in the content of catalytic Fe and significant increases in the activities of antioxidant enzymes and the contents of non-enzymatic antioxidants, and then significantly reduced the contents of DHA and GSSG and the degrees of oxidative damage in leaves exposed to the moderate water stress. Pretreatment with an ABA biosynthesis inhibitor, tungstate, significantly suppressed the accumulation of ABA induced by water stress, reduced the enhancement in the capacity of antioxidant defense systems, and resulted in an increase in catalytic Fe, DHA and GSSG, and oxidative damage in the water-stressed leaves. These effects were completely prevented by addition of ABA, which raised the internal ABA content. Our data indicate that ABA plays an important role in water stress-induced antioxidant defense against oxidative stress.

Keywords: Abscisic acid; Antioxidant defense system; Oxidative damage; Reactive oxygen species; Water stress; *Zea mays*

INTRODUCTION

Oxygen toxicity is an inherent feature of aerobic life. Photosynthetic plant cells are especially at risk from oxidative damage because of their oxygenic conditions and the abundance of photosensitizers and polyunsaturated fatty acids in the chloroplast envelope and thylakoids. Even under optimal conditions, many metabolic processes, including chloroplastic, mitochondrial and plasma membrane-linked electron transport systems, produce reactive oxygen species (ROS) such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxide radical ($^{\bullet}OH$) and $^1O_2$.[1,2] Under stress conditions, reduced CO_2 supply, often as a result of restricted stomatal opening and excessive energy input may give rise to the excessive concentrations of ROS and result in lipid peroxidation, the denaturation of proteins, and the oxidation of DNA. Therefore, antioxidant enzymes and non-enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), ascorbate (ASC), glutathione (GSH), α-tocopherol (α-TOC) and carotenoids (CAR) play important roles in avoiding uncontrolled oxidation in plant cells.[2–4]

Water stress can disturb the balance between prooxidants and antioxidants in plant cells. It has

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been documented that water stress can cause an increase in the generation of ROS, including O_2^-, H_2O_2, $^{\bullet}OH$ and $^1O_2$, and a reduction in the activities of SOD, CAT, ASC–GSH cycle enzymes and the contents of ASC, GSH, α-TOC and CAR in many stressed plant tissues.[5–10] The formation of excessive ROS and the decrease in the capacity of antioxidant defense systems in plants under water stress can result in cellular oxidative damage. This situation may only occur, however, under severe water stress.[5] During water stress, an antioxidative defense mechanism may be induced. Non-lethal water deficit often results in the increase in the activities of antioxidative enzymes and the contents of non-enzymatic antioxidants.[5,9,11] The capacity of the antioxidant systems and their ability to respond to increased ROS generation in plants may be sufficient to prevent an oxidative damage under a normally encountered moderate water stress.

Increasing evidence indicates that water stress-induced antioxidant defense may be related to the action of abscisic acid (ABA). It has been documented that ABA can cause an increased generation of ROS[12–16] and induce the expression of antioxidant genes encoding Cu, Zn-SOD,[17–19] Mn-SOD,[19–21] Fe-SOD[19] and CAT.[12,22–24] Meanwhile, ABA also increases the activities of antioxidant enzymes such as SOD, CAT, APX and GR and the contents of antioxidant metabolites such as ASC, GSH, α-TOC and CAR in plant tissues.[16,21,23,25–27] These observations indicate that the action of ABA may be linked to oxidative stress.

However, whether ABA acts in the upstream of ROS is controversial because one contrary hypothesis has been proposed that an oxidative burst might function as one of the triggers of the water-stress responses and ABA might function in the downstream of ROS to regulate gene expression as well as physiological and biochemical responses during water stress.[28] A recent study has shown that ROS induced by water stress is involved in water stress-induced ABA biosynthesis in root tips of wheat seedlings.[29] Moreover, there might also exist ABA-independent or ROS-independent signal pathways during the process of antioxidant response to water stress.[12,27] Therefore, whether water stress-induced oxidative stress is related to ABA accumulation in plants under water stress remains undefined. A comprehensive study is essential to investigate the role of water stress-induced ABA in oxidative stress induced by water stress in plants.

In this paper, utilizing both exogenous ABA and ABA biosynthesis inhibitor, tungstate, which was shown to block the formation of ABA from ABA-aldehyde by impairing ABA-aldehyde oxidase,[30] to manipulate endogenous levels in water stressed and non-stressed leaves of maize seedlings, we tried to investigate the role of water stress-induced ABA in water stress-induced ROS, antioxidant defense systems and oxidative damage in leaves of maize seedlings exposed to the mild and moderate water stress.

MATERIALS AND METHODS

Plant Materials and Treatments

Seeds of maize (*Zea mays* L.) were sown in trays of sand in a greenhouse at a temperature of 25–30°C, photosynthetic active radiation (PAR) of 400 $\mu mol\, m^{-2}\, s^{-1}$ (enhanced with high-pressure sodium lamps) and photoperiod of 14/10 (day/night), and watered daily. When the second leaf was fully expanded, they were collected and used for all investigations.

Detached leaves were cut into leaf segments (3 cm in length), rinsed with distilled water, and then pretreated with distilled water, 100 μM ABA or 2 mM sodium tungstate for 12 h, respectively. The pretreated segments were then exposed to osmotic stress for 24 h in PEG 6000 solution at −0.7 MPa at 25°C with a continuous light intensity of 150 $\mu mol\, m^{-2}\, s^{-1}$. In order to study the effects of pretreatment on non-stressed segments, the segments pretreated with ABA or sodium tungstate were exposed to distilled water for 24 h under the same conditions as described above. To test whether effects of tungstate could be overcome by exogenously supplied ABA, the segments were pretreated with 2 mM tungstate + 100 μM ABA for 12 h, then exposed to osmotic stress for 24 h under the same conditions as described above. The segments were treated with distilled water under the same conditions during the whole period served as controls for the above. Samples with 12 h or 24 h treatments were taken out and immediately frozen under liquid N_2, and then stored at −80°C for further analysis.

Determination of RWC

Relative water content (RWC), defined as water content of tissue as a percentage of that of the fully turgid tissue, was determined according to the method of Smart and Bingham.[31] Fresh leaf segments were weighted (Wi), floated on distilled water at 20°C in the dark overnight, weighed again (Wf) and dried at 80°C for 48 h for the determination of dry mass (Wd). RWC was calculated as: $(Wi - Wd)/(Wf - Wd) \times 100$.

Determination of Superoxide Radical, Hydrogen Peroxide and Catalytic Fe

The production rate of O_2^- was measured as described by Elstner and Heupel[32] by monitoring

the nitrite formation from hydroxylamine in the presence of O_2^-. The absorbance was read at 530 nm. Corrections were made for the background absorbance in the presence of 50 units SOD. A standard curve with NO_2^- was used to calculate the production rate of O_2^- from the chemical reaction equation of O_2^- and hydroxylamine. The content of H_2O_2 was measured by monitoring the A_{415} of the titanium-peroxide complex following the method described by Brennan and Frenkel.[33] Absorbance values were calibrated to a standard graph generated with known concentrations of H_2O_2. Recovery was checked by adding various amounts of H_2O_2 to the plant extracts as internal standard.

Catalytic Fe represents the fraction of Fe in a tissue that is active in the generation of $^{\bullet}OH$ and possibly other AOS through Fenton chemistry, and its concentration was measured as described by Evans and Halliwell.[34] Leaf segments were extracted with Chelex-treated 50 mM potassium phosphate buffer (pH 7.0) and the homogenate was centrifuged at 15,000*g* for 20 min and further fractionated using Centricon-3 membranes (Amicon). The concentration of catalytic Fe in the <3 kDa fraction was estimated as bleomycin-dependent DNA damage using 50 μl of sample, incubation at 37°C for 1 h, and free Fe^{3+} as standard. Controls are run in which water replaces the sample and also in which sample is added in the absence of bleomycin to correct for any other TBA-reactive material that might be present in the samples. Recovery was checked by adding 2 μM free Fe^{3+} to the plant extracts as internal standard.

Enzyme Assays

Frozen leaf segments (0.5 g) were homogenized in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone, with the addition of 1 mM ASC in the case of APX assay. The homogenate was centrifuged at 15,000*g* for 20 min at 4°C and the supernatant was immediately used for the following enzyme assays. Protein content was determined according to the method of Bradford[35] with bovine serum albumin as a standard.

Total SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium according to the method of Giannopolitis and Ries.[36] One unit of SOD activity was defined as the amount of enzyme that required to cause 50% inhibition of the reduction of nitro blue tetrazolium as monitored at 560 nm. Activities of different forms of SOD were identified by using 3 mM KCN or 5 mM H_2O_2 as inhibitors. CAT activity was assayed by measuring in the rate of decomposition of H_2O_2 at 240 nm, as described by Aebi.[37] APX activity was measured by monitoring the decrease in absorbance at 290 nm as ASC was oxidized, as described by Nakano and Asada.[38] GR activity was measured by following the change in A340 as GSSG-dependent oxidation of NADPH, according to the method of Schaedle and Bassham.[39]

Determination of Non-enzymatic Antioxidants

Total ascorbate (ASC plus DHA) and total glutathione (GSH plus GSSG) were extracted from 0.5 or 1.0 g leaf segments with 10 ml of 5% trichloroacetic

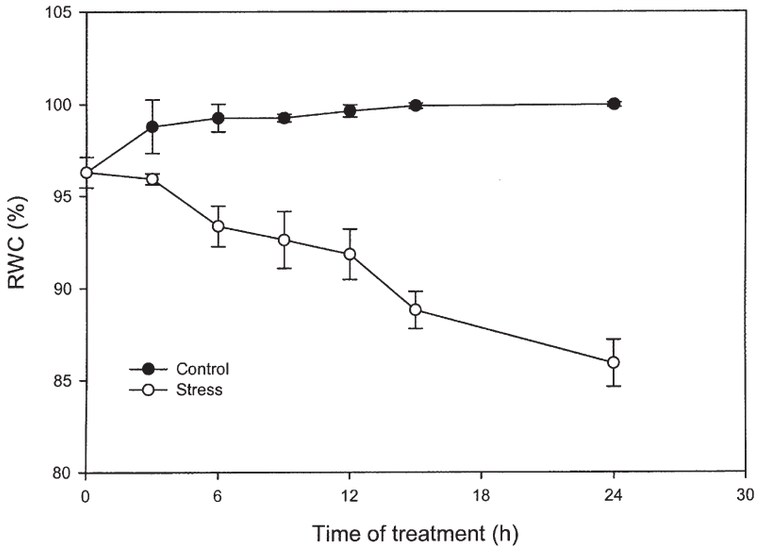

FIGURE 1 Changes in RWC (%) in leaves exposed to −0.7 MPa osmotic stress for 24 h. Values are means ± SE ($n = 6$).

acid and 5% sulfosalicylic acid, respectively. ASC content was determined as described by Arakawa *et al.*,[40] using a method based on the reduction of ferric ion to ferrous ion with ASC in acid solution followed by formation of the red chelate between ferrous ion and bathophenanthroline, which absorbs at 534 nm. Total ascorbate content was determined through a reduction of DHA to ASC by dithiothreitol. DHA content was estimated on the basis of the difference between total ascorbate and ASC values. Total glutathione was measured by an enzymatic cycling assay method, according to Griffiths.[41] GSSG was determined after removal of GSH by 2-vinylpyridine derivatization. GSH was determined by subtraction of GSSG from the total glutathione content. α-TOC was extracted as described by Munné-Bosch *et al.*[42] and its concentration was determined by HPLC. UV detection was carried out

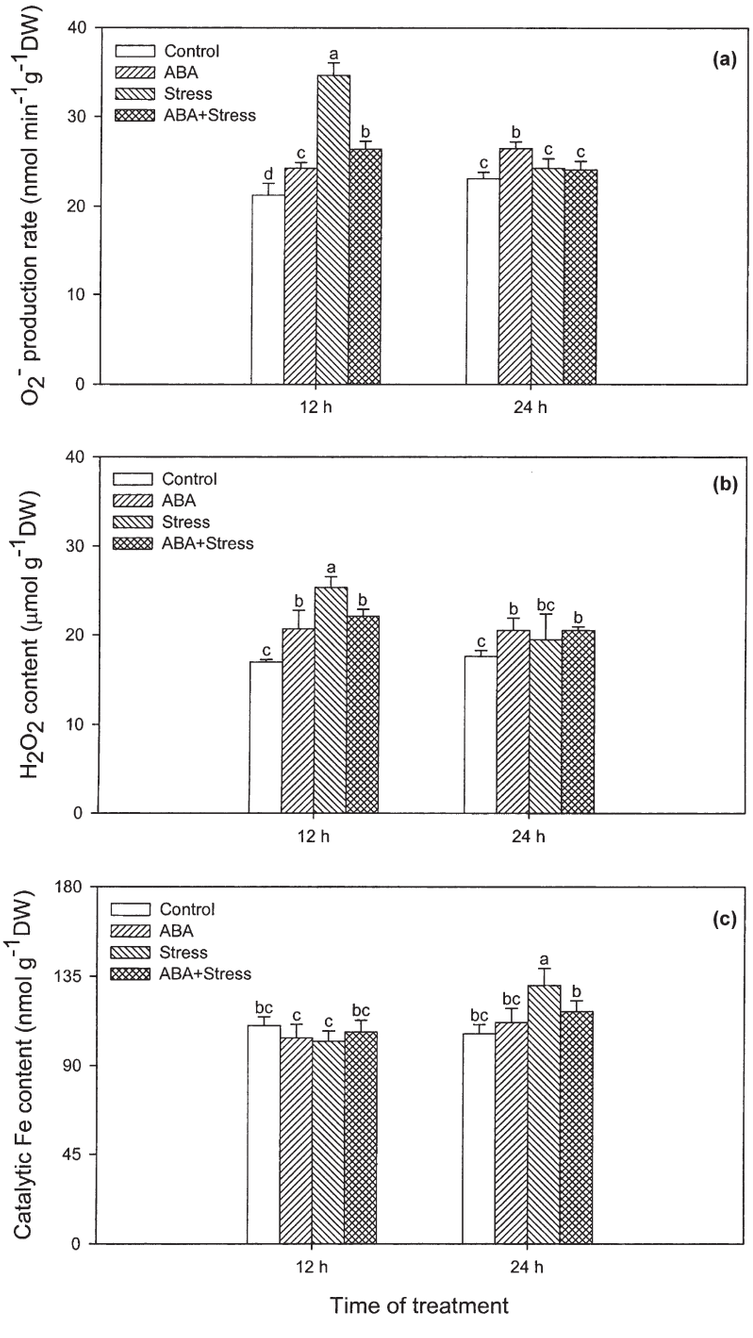

FIGURE 2 Effects of pretreatment with 100 μM ABA on the production of superoxide radicals (a), hydrogen peroxide (b) and the content of catalytic Fe (c) in leaves exposed to −0.7 MPa osmotic stress for 12 and 24 h. Values are means ± SE ($n = 6$). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

at 295 nm and fluorescence detection was carried out at an excitation wavelength of 298 nm and emission at 328 nm. CAR and chlorophyll were spectrophotometrically measured in 80% acetone extract, as described by Lichtenthaler.[43]

Determination of Oxidative Damage and Electrolyte Leakage

Oxidative damage to lipids was estimated by measuring the content of thiobarbituric acid-reactive substances (TBARS) in leaf segment homogenates, prepared in 10% trichloroacetic acid containing 0.65% 2-thiobarbituric acid (TBA) and heated at 95°C for 25 min, as in Hodges *et al.*[44] TBARS content was calculated by correcting for compounds other than TBARS which absorb at 532 nm by subtracting the absorbance at 532 nm of a solution containing plant extract incubated without TBA from an identical solution containing TBA. Oxidative damage to proteins was quantified as total protein carbonyl content by reaction with 2,4-dinitrophenylhydrazine after the removal of possible contaminating nucleic acids with 1% of streptomycin sulfate.[45]

The leakage percentage of electrolyte was determined as previously described.[16] After the initial electrical conductivity (EC_1) and the final electrical

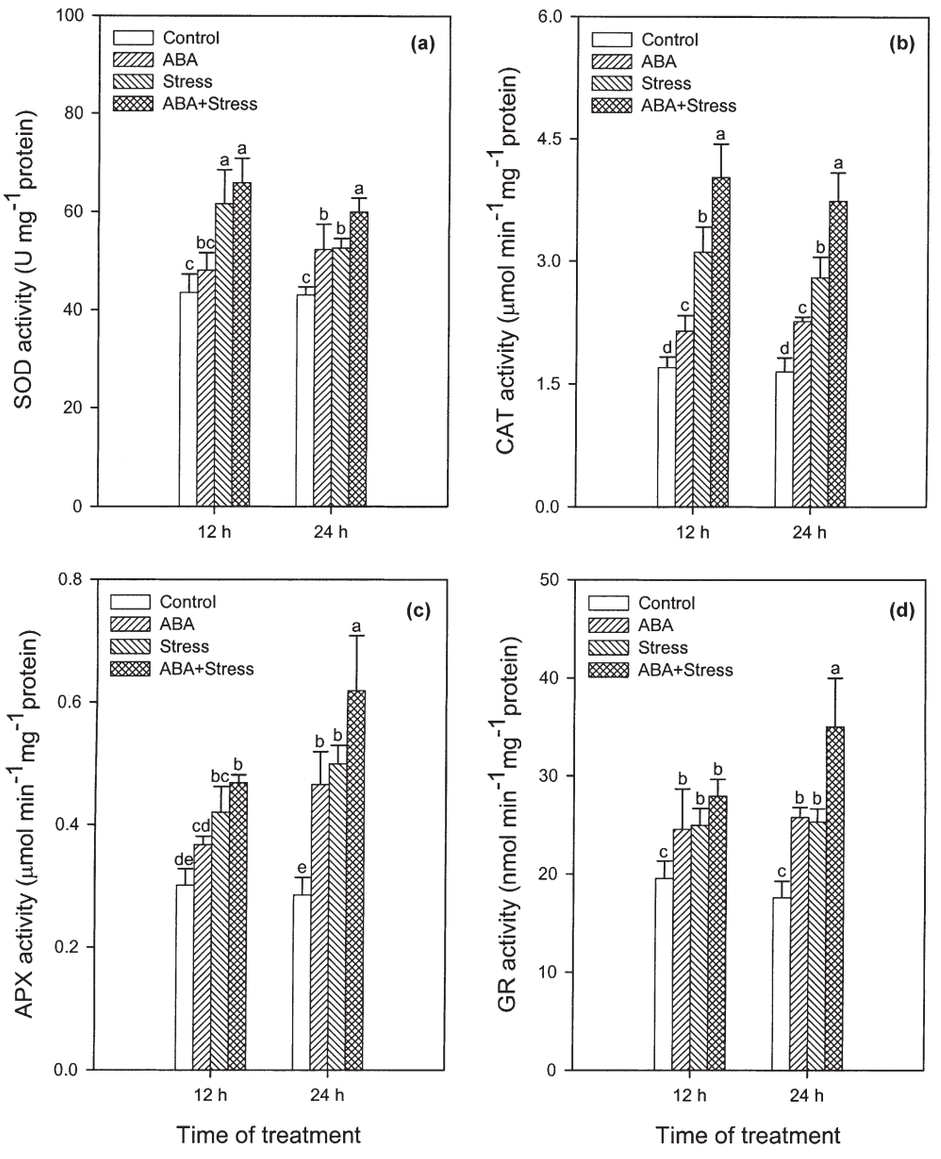

FIGURE 3 Effects of pretreatment with 100 μM ABA on the activities of SOD (a), CAT (b), APX (c) and GR (d) in leaves exposed to −0.7 MPa osmotic stress for 12 and 24 h. Statistical analyses are as for Fig. 2.

conductivity (EC_2) of the medium were measured, respectively, the leakage percentage of electrolytes was calculated by using the formula: $EC_1/EC_2 \times 100$.

ABA Analysis

ABA analysis was carried out using the radioimmunoassay method as described by Quarrie *et al.*[46] The highly specific monoclonal antibody (AFRC MAC 225) was provided by Dr S.A. Quarrie (Cambridge Lab, IPSR, John Innes Center, UK). 100 mg freeze-dried and powdered leaf mass was shaken in 5 ml distilled water for 24 h at 4°C in darkness. Supernatant was obtained as crude extracts through centrifuging. 50 μl of crude extracts was mixed with 200 μl phosphate-buffered saline (pH 6.0), 100 μl diluted antibody solution and 100 μl ^{3}H-ABA (about 8000 cpm) solution. Reaction mixture was incubated at 4°C for 45 min and the bound radioactivity was measured in 50% saturated $(NH_4)_2SO_4$-precipitated pellets with a liquid scintillation counter. The extraction efficiency and immunoreactive contamination in crude extracts of maize samples were tested earlier by Zhang and Davies[47] and proved to be reliable.

Statistical Analysis

The results presented were the mean of six replicates. Means were compared by one-way analysis of variance and Duncan's multiple range test at the level of 5% level of significance.

RESULTS

Leaf Water Stress Status

RWC is a commonly used indicator of plant water stress status. Figure 1 shows that −0.7 MPa osmotic stress caused a considerable reduction in leaf RWC during 24 h of osmotic stress treatment. At 12 and 24 h of osmotic stress treatment, leaf RWC reduced by 7.8 and 14.1%, respectively, compared with the control leaves. According to the definition of Hsiao,[48] −0.7 MPa osmotic stress for 12 h can be considered as a mild water stress, and −0.7 MPa osmotic stress for 24 h can be considered as a moderate water stress.

Effects of ABA and Osmotic Stress on AOS and Catalytic Fe

Osmotic stress for 12 h led to a significant increase in the production of ROS in leaves of maize seedlings (Fig. 2). The production rate of O_2^- (Fig. 2a) and the content of H_2O_2 (Fig. 2b) increased by 63 and 49%, respectively, compared with the control leaves. Pretreatment with ABA markedly reduced the generation of O_2^- and H_2O_2 under osmotic stress, although pretreatment with ABA alone caused an apparent increase in the production of O_2^- and H_2O_2 when compared with the control. In contrast, leaf segments subjected to osmotic stress for 24 h exhibited O_2^- and H_2O_2 production similar to those of the control, and pretreatment with ABA did not affect the generation of these ROS in leaves exposed to osmotic stress.

In order to ascertain whether other ROS are produced under osmotic stress, we determined the content of catalytic Fe, which is a stringent requirement for H_2O_2-dependent $^{\bullet}OH$ radical production. Figure 2c shows that osmotic stress for 24 h enhanced the content of catalytic Fe by 23%, but osmotic stress for 12 h did not affect the content of catalytic Fe when compared to the control. Pretreatment with ABA markedly reduced the increase in the content of catalytic Fe induced by osmotic stress, although pretreatment with ABA alone had no effect

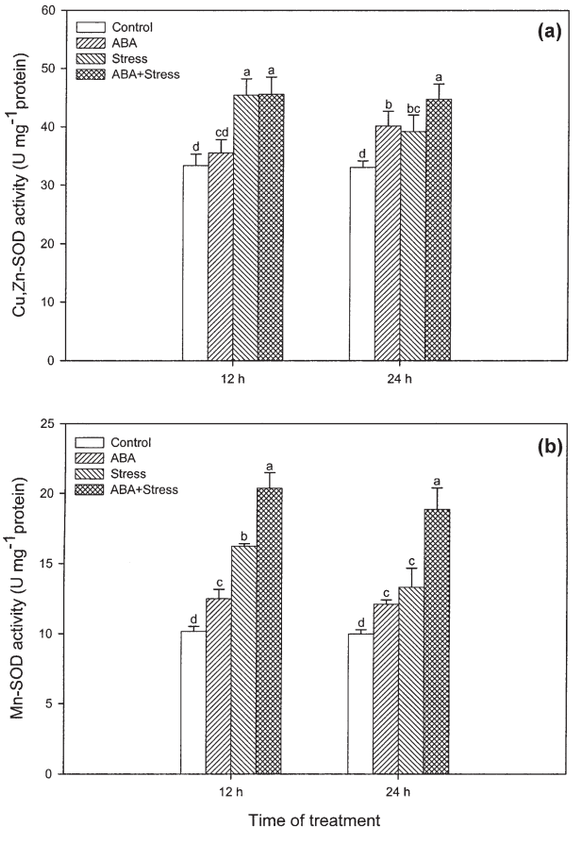

FIGURE 4 Effects of pretreatment with 100 μM ABA on the activities of SOD isozymes such as Cu, Zn-SOD (a) and Mn-SOD (b) in leaves exposed to −0.7 MPa osmotic stress for 12 and 24 h. Statistical analyses are as for Fig. 2.

on the content of catalytic Fe when compared to the control.

Effects of ABA and Osmotic Stress on Antioxidant Enzymes

Osmotic stress led to some significant changes in the activities of SOD, CAT, APX and GR in leaves of maize seedlings (Fig. 3). Treatment with −0.7 MPa osmotic stress for 12 h enhanced the activities of SOD (Fig. 3a), CAT (Fig. 3b), APX (Fig. 3c) and GR (Fig. 3d) by 42, 83, 40 and 28%, respectively, compared with the control values. However, at the 24 h of osmotic stress treatment, the activities of these antioxidant enzymes did not increase further when compared to the 12 h treatment, and the activity of SOD even decreased (Fig. 3a). Pretreatment with ABA significantly enhanced the activities of SOD, CAT, APX and GR in the stressed leaves for 24 h, but only resulted in a slight increase in the activities of these antioxidant enzymes in the stressed leaves for 12 h, except for CAT activity, compared with the stressed leaves alone. Pretreatment with ABA also led to a significant increase in activities of antioxidant enzymes in non-stressed leaves during 24 h of treatment.

The activities of SOD isozymes such as Cu, Zn-SOD, Mn-SOD and Fe-SOD were determined in stressed and non-stressed leaves (Fig. 4). The changes in the activities of Cu, Zn-SOD (Fig. 4a) and Mn-SOD (Fig. 4b) in the stressed or pretreated with ABA leaves were similar to the activity of total SOD (Fig. 3a). Mn-SOD was more sensitive to osmotic stress or ABA treatment than Cu, Zn-SOD.

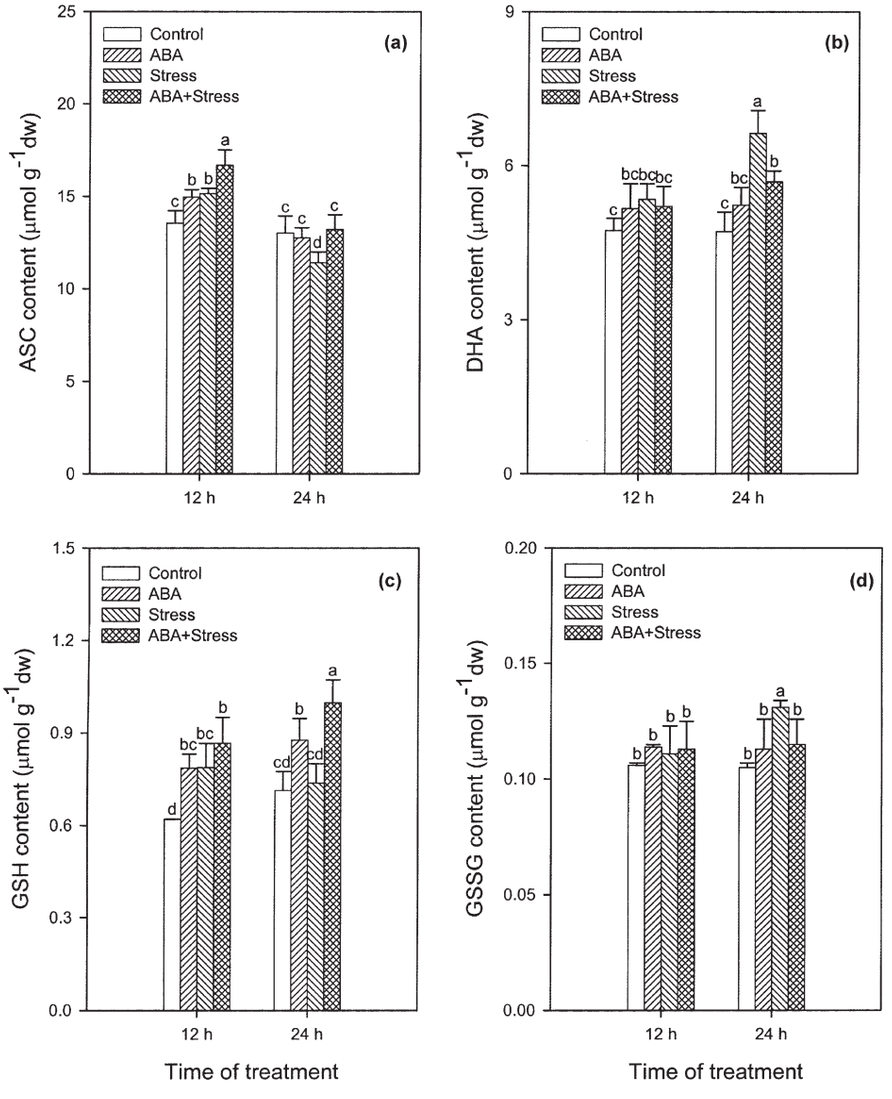

FIGURE 5 Effects of pretreatment with 100 μM ABA on the contents of ASC (a), DHA (b), GSH (c) and GSSG (d) in leaves exposed to −0.7 MPa osmotic stress for 12 and 24 h. Statistical analyses are as for Fig. 2.

No activity of Fe-SOD was detected in all of these situations.

Effects of ABA and Osmotic Stress on Antioxidant Metabolites

The contents of ASC and GSH, two major water-soluble antioxidants, and its oxidized forms, DHA and GSSG, and α-TOC and CAR, two major lipid-soluble antioxidants, were determined in non-stressed and stressed leaves and a distinctive profile was observed (Figs. 5 and 6). Treatment with −0.7 MPa for 12 h led to a significant increase in the contents of ASC (Fig. 5a) and GSH (Fig. 5c). The contents of ASC and GSH enhanced by 12 and 27%, respectively, compared with the control values. But the contents of DHA (Fig. 5b) and GSSG (Fig. 5d) were not affected. At the 24 h treatment, ASC content significantly decreased and GSH content also returned to the control level, but the contents of DHA and GSSG enhanced by 40 and 25%, respectively, compared with the controls. Treatment with 24 h osmotic stress significantly enhanced the contents of two lipid-soluble antioxidants. The contents of α-TOC (Fig. 6a) and CAR (Fig. 6b) increased by 19 and 24%, respectively, as compared to the control values. However, treatment with osmotic stress for 12 h did not affect the contents of α-TOC or CAR. Pretreatment with ABA significantly elevated the contents of ASC, GSH, α-TOC and CAR, and reduced the contents of DHA and GSSG in leaves exposed to osmotic stress for 24 h, and also increased the contents of ASC, GSH and α-TOC to a lesser extent under the 12 h osmotic stress, when compared to those of osmotic stress treatment alone. Pretreatment with ABA also led to a significant

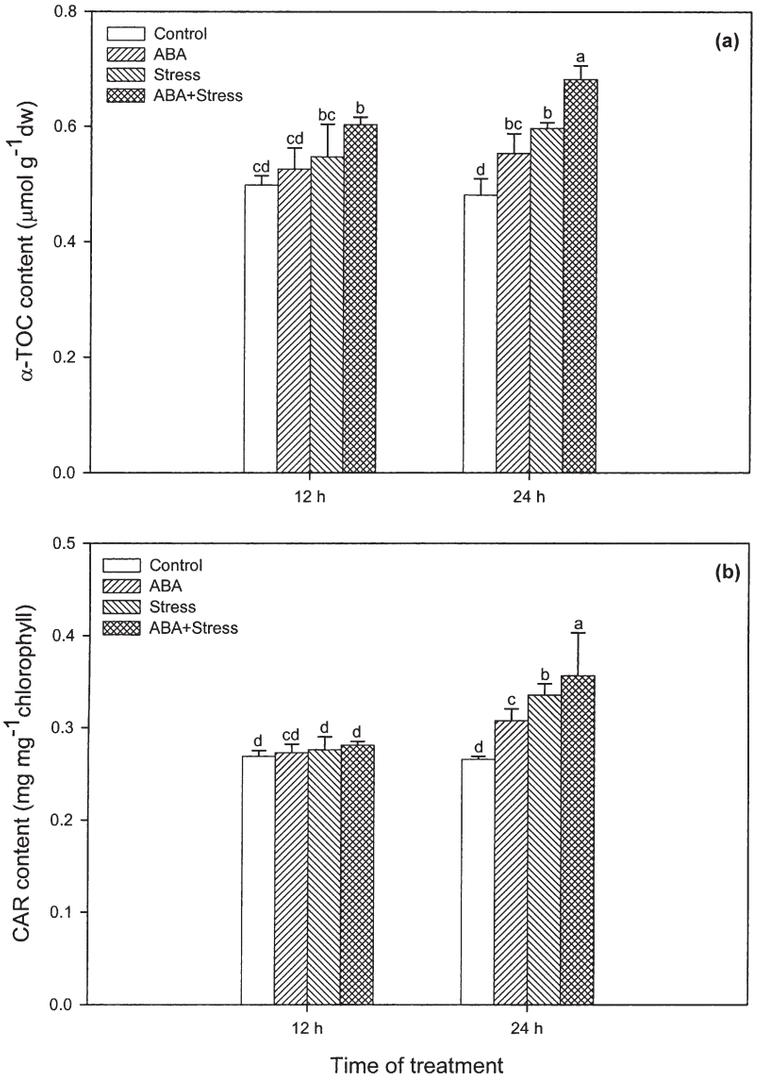

FIGURE 6 Effects of pretreatment with 100 μM ABA on the contents of α-TOC (a) and CAR (b) in leaves exposed to −0.7 MPa osmotic stress for 12 and 24 h. Statistical analyses are as for Fig. 2.

increase in the contents of these antioxidants in non-stressed leaves within 12 or 24 h, but did not affect the contents of DHA and GSSG.

Effects of ABA and Osmotic Stress on Oxidative Damage and Electrolyte Leakage

Oxidative damage to lipids (expressed as TBARS) and proteins (in terms of carbonyl groups) did not take place in leaves exposed to osmotic stress for 12 h (Fig. 7a,b). Pretreatment with ABA also did not change the contents of TBARS or carbonyl groups in leaves subjected to osmotic stress or in the control leaves. However, a marked oxidative damage was observed when the osmotic stress was prolonged. At the 24 h of osmotic stress treatment, the contents of TBARS (Fig. 7a) and carbonyl groups (Fig. 7b) increased by 20 and 30%, respectively, as compared

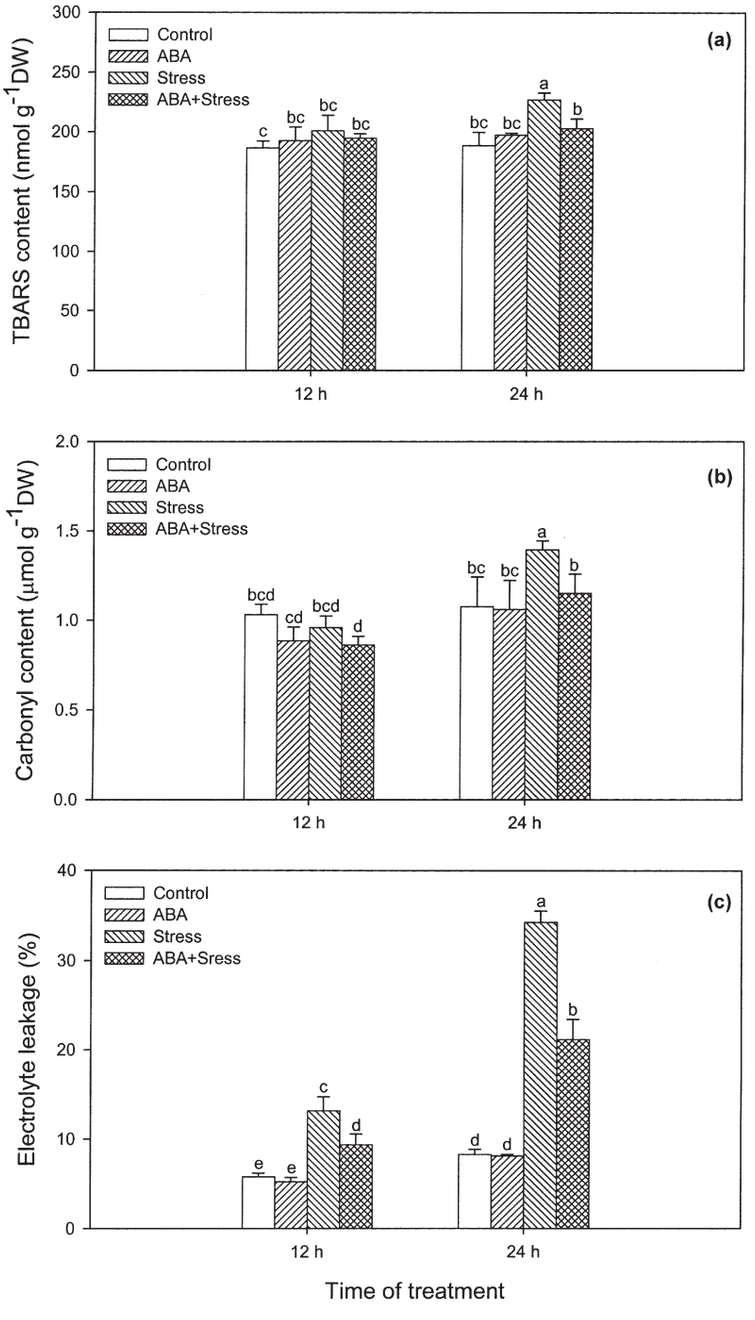

FIGURE 7 Effects of pretreatment with 100 μM ABA on the levels of lipid peroxidation (TBARS content) (a), protein oxidation (carbonyl content) (b) and leakage percentage of electrolytes (c) in leaves exposed to −0.7 MPa osmotic stress for 12 and 24 h. Statistical analyses are as for Fig. 2.

to the control values. Pretreatment with ABA significantly prevented the increase in the contents of TBARS and carbonyl groups induced by the osmotic stress.

As the oxidative damage to lipids and proteins took place, the leakage of electrolytes from cells increased markedly in leaves exposed to the osmotic stress. At the 24 h stress treatment, the percentage of electrolyte leakage increased 3-fold, compared with the control value. Pretreatment with ABA significantly reduced the leakage in the stressed leaves (Fig. 7c).

Effects of Tungstate on ABA, Antioxidant Systems and Oxidative Damage

In order to confirm the up-regulation of antioxidant defense systems was related to the accumulation of ABA in the leaves exposed to osmotic stress, the effects of pretreatment with ABA biosynthesis inhibitor, tungstate, which impairs ABA-aldehyde oxidase, on antioxidant systems and oxidative damage in leaves subjected to osmotic stress were investigated. Meanwhile, the exogenous ABA was added at a final concentration of 100 μM together with tungstate so as to test whether the effects of tungstate could be overridden by exogenously supplied ABA. Figure 8 shows that osmotic stress for 24 h enhanced the content of ABA by 5.5-fold, and pretreatment with 2 mM tungstate inhibited the increase by 82%. This pretreatment did not affect the content of ABA in the non-stressed leaves. At the same time, pretreatment with tungstate almost fully arrested the increase in the activities of SOD (Fig. 9a), CAT (Fig. 9b), APX (Fig. 9c) and GR (Fig. 9d) induced by osmotic stress for 24 h, and led to a significant reduction in the contents of ASC (Fig. 10a) and GSH (Fig. 10c) and an obvious increase in the contents of DHA (Fig. 10b) and GSSG (Fig. 10d) in leaves exposed to osmotic stress for 24 h. This pretreatment also did not affect the activities of these antioxidant enzymes and the contents of ASC, GSH, DHA and GSSG in the non-stressed leaves. Accordingly, pretreatment with tungstate led to significant increases in the contents of catalytic Fe (Fig. 11a) and carbonyl groups (Fig. 11b), and the percentage of electrolyte leakage (Fig. 11c) in the stressed leaves, when compared to the stressed leaves alone, and this pretreatment did not cause any changes in these parameters under the non-stressed condition. The application of 100 μM ABA substantially restored the level of ABA inhibited by tungstate (Fig. 8), and fully prevented the reduction in the activities of antioxidant enzymes (Fig. 9a–d) and the contents of ASC (Fig. 10a) and GSH (Fig. 10c), and the increase in catalytic Fe (Fig. 11a), DHA (Fig. 10b), GSSG (Fig. 10d), protein oxidation (Fig. 11b) and electrolyte leakage (Fig. 11c) in the stressed leaves.

DISCUSSION

Water stress is related to the oxidative stress in plants.[9,10] Water stress can disturb the balance between cellular prooxidants and antioxidants. If the former prevails over the latter, cellular oxidative

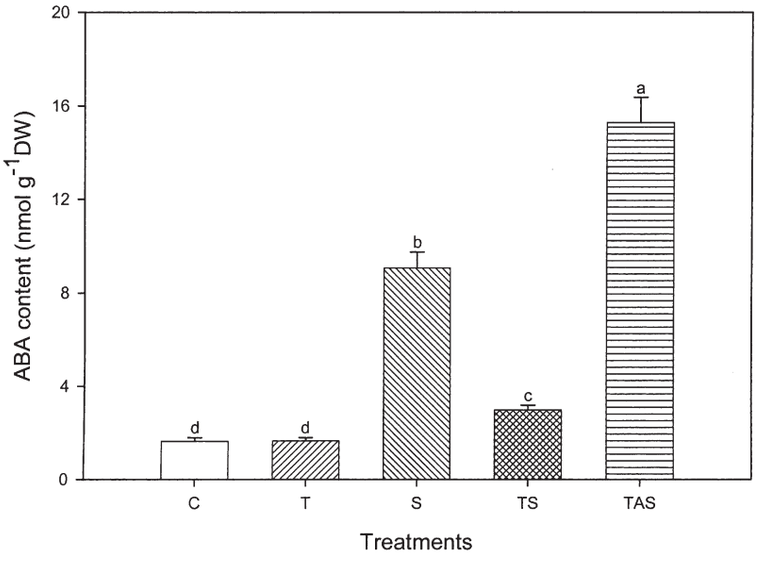

FIGURE 8 Effects of pretreatment with ABA biosynthesis inhibitor, tungstate, on the content of ABA in leaves exposed to −0.7 MPa osmotic stress for 24 h. The leaf segments were treated as follows: C, control (distilled water); T, 2 mM sodium tungstate; S, osmotic stress (−0.7 MPa); TS, 2 mM sodium tungstate + osmotic stress; TAS, 2 mM sodium tungstate + 100 μM ABA + osmotic stress. The leaf segments were pretreated with sodium tungstate, sodium tungstate + ABA for 12 h, respectively, and then exposed to osmotic stress or distilled water for 24 h. Statistical analyses are as for Fig. 2.

damage may occur, although it is generally believed that this situation only occurs under severe water stress.[5] Our results showed that a mild water stress induced a significant increase in the generation of O_2^- and H_2O_2 (Fig. 2a,b), and enhanced the activities of SOD, CAT, APX and GR (Fig. 3a–d) and the contents of ASC and GSH (Fig. 5a,c). Such an enhancement in the antioxidant defenses can efficiently control ROS level, an oxidative damage to lipids and proteins did not take place under the mild water stress (Fig. 7a,b). Under the moderate water stress, the capacity of antioxidant defense systems was still maintained at a higher level, but no longer further enhanced when compared to the mild water stress. After 24 h of water stress, SOD activity decreased (Fig. 3a), GSH content decreased to the level of the control (Fig. 5c), and ASC content was even significantly lower than the level of the control (Fig. 5a). At the same time, the oxidized forms of ascorbate and glutathione pools, DHA and GSSG, significantly increased (Fig. 5b,d). The build-up of DHA and GSSG can be considered as an index of oxidative stress.[49,50] Although the generation of O_2^- and H_2O_2 were reduced to the control levels, the content of catalytic Fe, which is critical for H_2O_2-dependent $^{\bullet}OH$ production, was significantly increased under the moderate water stress (Fig. 2c). Catalytic Fe may interact in turn with O_2^- and H_2O_2 through a Haber–Weiss reaction to produce $^{\bullet}OH$, which is a major ROS responsible for modifications of macromolecules and cell damage.[5–8] This may explain the oxidative damage to lipids and proteins and plasma membrane damage observed during the moderate water stress (Fig. 7a–c). These results indicate that an oxidative stress induced by the moderate water stress could not be effectively controlled by the enhanced antioxidative defense

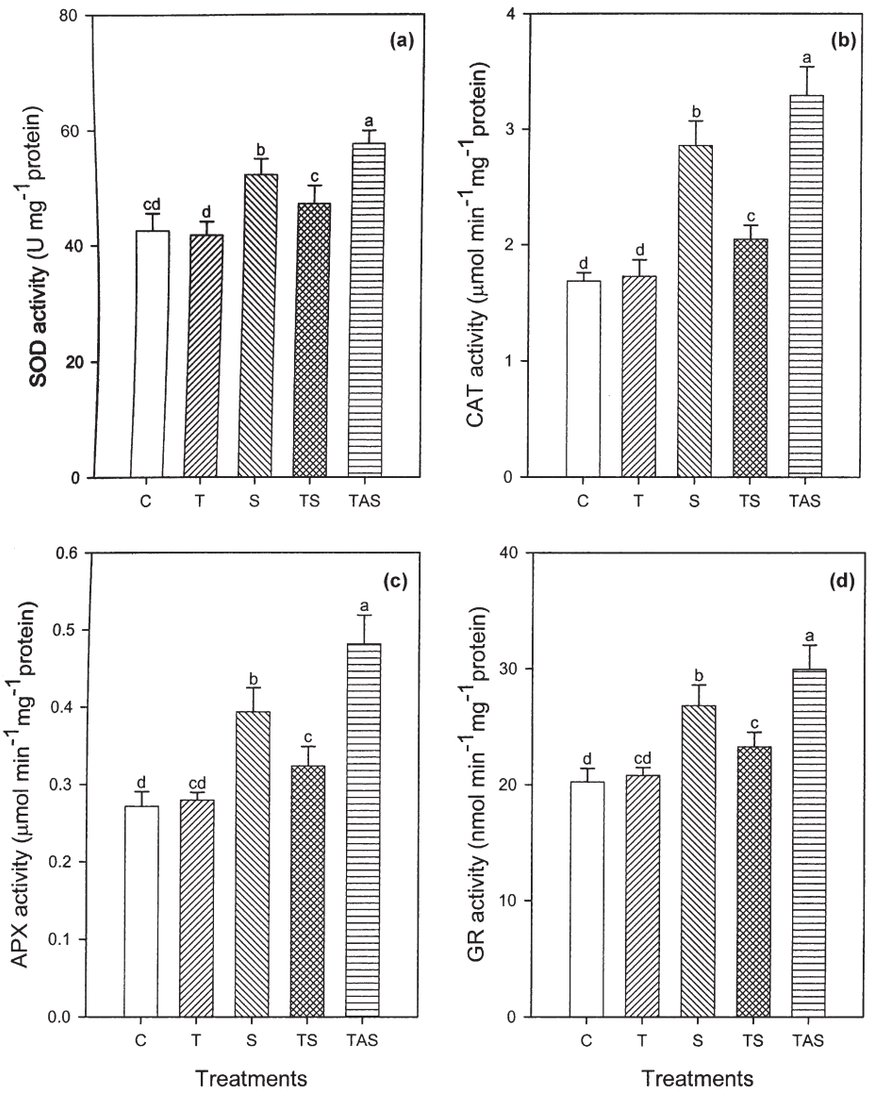

FIGURE 9 Effects of pretreatment with ABA biosynthesis inhibitor, tungstate, on the activities of SOD (a), CAT (b), APX (c) and GR (d) in leaves exposed to −0.7 MPa osmotic stress for 24 h. Plant treatments are as described in Fig. 8. Statistical analyses are as for Fig. 2.

systems and resulted in some cellular oxidative damage. The similar results had been reported in leaves of pea[8] and wheat[9] exposed to moderate water stress. Therefore, a moderate water stress may be the turning point where plant cells lose the control of ROS and an oxidative damage occurs.

It has been shown that water stress-induced antioxidant defense may be related to the action of ABA. ABA-dependent and -independent signal pathways are involved in the expression of some antioxidant genes or up-regulation of antioxidant enzymes under osmotic stress or NaCl stress.[12,16,18,21,24,27] Our results showed that water stress induced ABA accumulation (Fig. 8) and induced a significant increase in the production of ROS and the activities of SOD, CAT, APX and GR, and the contents of ASC, GSH, α-TOC and CAR, and pretreatment with ABA further enhanced the activities of these antioxidant enzymes and the contents of these antioxidant metabolites in water stressed leaves and then improved stress tolerance, and ABA alone also induced an increased generation of ROS and an enhancement in the capacity of antioxidant defense systems in the non-stressed leaves (Figs. 2–7). Furthermore, a substantial reduction in the content of ABA caused by ABA biosynthesis inhibitor, tungstate, was accompanied by a significant decrease in the activities of antioxidant enzymes and the contents of antioxidant metabolites, and an obvious increase in catalytic Fe, DHA and GSSG, and

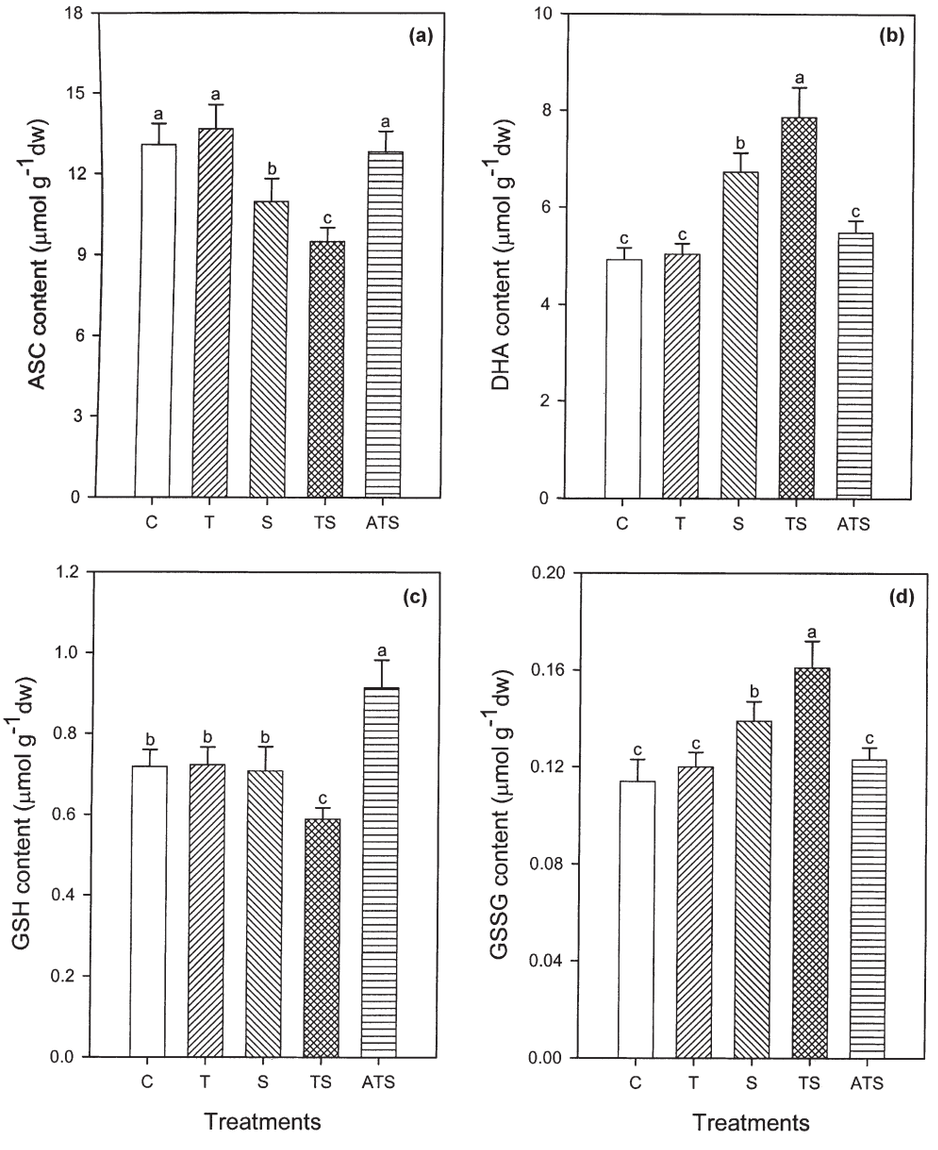

FIGURE 10 Effects of pretreatment with ABA biosynthesis inhibitor, tungstate, on the contents of ASC (a), DHA (b), GSH (c) and GSSG (d) in leaves exposed to −0.7 MPa osmotic stress for 24 h. Plant treatments are as described in Fig. 8. Statistical analyses are as for Fig. 2.

oxidative damage in water-stressed leaves, and these effects were completely prevented by addition of ABA, which raised the internal ABA content (Figs. 8–11). These data not only further support that an ABA-dependent signal pathway is involved in water stress-enhanced antioxidant defense systems against oxidative stress, but also strongly suggest that ABA is an essential mediator in triggering water stress-induced antioxidative defense response against oxidative damage in plants.

It is generally thought that ROS that are inevitably produced under water stress have adverse effects and cause cellular oxidative damage. Compared with O_2^- and H_2O_2, however, $^{\bullet}OH$ is more reactive, and reacts with virtually any molecule, including sugars, lipids, proteins, and DNA, at, or close to, its site of formation.[2,7] It has been demonstrated that water stress led to the accumulation of catalytic Fe in pea leaves[6,8] and pea nodules,[7] and the production of $^{\bullet}OH$ in wheat leaves.[9] The accumulation of

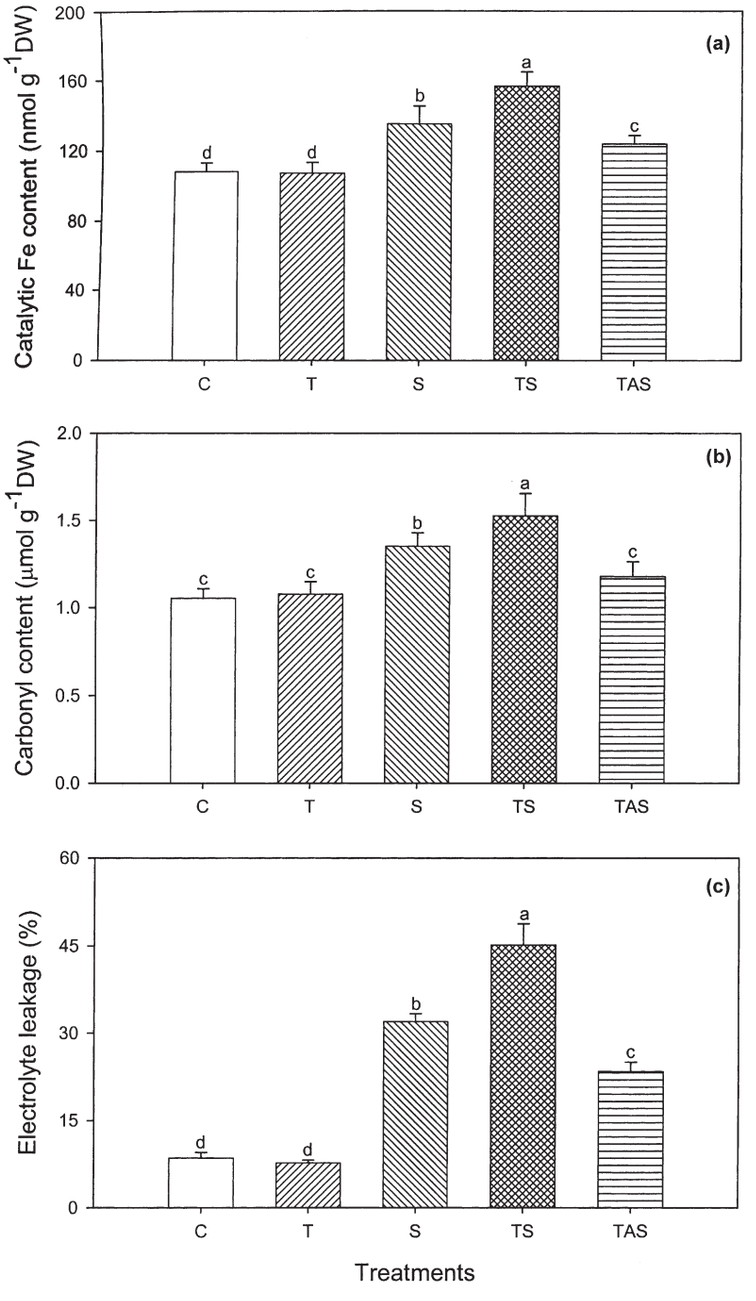

FIGURE 11 Effects of pretreatment with ABA biosynthesis inhibitor, tungstate, on the contents of catalytic Fe (a) and carbonyl groups (b), and leakage percentage of electrolytes (c) in leaves exposed to −0.7 MPa osmotic stress for 24 h. Plant treatments are as described in Fig. 8. Statistical analyses are as for Fig. 2.

catalytic metals may play a critical role in the toxicity of ROS in stressed tissues.[6,7] Our data showed that osmotic stress for 24 h led to an increase in the content of catalytic Fe (Fig. 2c). Pretreatment with ABA significantly reduced the increase of catalytic Fe in the stressed leaves (Fig. 2c), and pretreatment with tungstate markedly increased the accumulation in catalytic Fe in the stressed leaves (Fig. 11a). These results indicate that water stress-induced ABA accumulation can reduce the increase in the content of catalytic Fe caused by water stress in plants. The reduction of catalytic Fe caused by ABA may be related to the enhancement in the binding of Fe to protein, or the reduction in the levels of O_2^- and H_2O_2, which reduces the release of catalytic Fe from Fe-protein such as phytoferritin, in plants under water stress.[8] In addition, the increase in the contents of ASC, GSH, α-TOC induced by ABA under water stress may enhance the scavenging for $^{\bullet}OH$.

In conclusion, the present study provides comprehensive evidence for supporting that an ABA-dependent signal pathway is involved in water stress-induced antioxidant defense systems, including enzymatic and non-enzymatic constituents. ABA-enhanced water stress tolerance is, at least in part, due to the induction of antioxidative defense systems, and the reduction in the content of catalytic Fe, which is critical for Fenton reactions to proceed *in vivo*, in plants under water stress. Although, we cannot rule out the possibility that an ABA-independent signal pathway may also be involved in the induction of antioxidant defense under water stress,[12] our results clearly indicate that the ABA-dependent signal pathway is an important one during this process.

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