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ORIGINAL ARTICLES

The Distribution and Cooperation of Antioxidant (Iso)enzymes and Antioxidants in Different Subcellular Compartments in Maize Leaves during Water Stress

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Abstract The effects of mild water stress induced by polyethylene glycol (PEG) on the activities of antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR)] and their isoenzymes and the antioxidant content [ascorbate (ASC) and glutathione (GSH)] of different subcellular compartments were investigated in maize. For each subcellular compartment, the activities of almost all isoenzymes resolved on native PAGE increased after 4–12 h of exposure to water stress and declined after that, showing concomitant changes with the activities of their respective total enzymes and the antioxidant content. For each subcellular compartment, at least one isoform for the detected antioxidant enzymes was resolved, but different kinds of antioxidant isoenzymes in different subcellular compartments had different responses to water stress. The relative contribution of Fe–SOD in chloroplasts and Mn–SOD in mitochondria was higher than that in other subcellular compartments. However, in apoplasts the activities of Mn–SOD and Fe–SOD declined during the process of water stress, in contrast to those located in other subcellular compartments. The results from the activities of antioxidant (iso)enzymes demonstrated that all antioxidant enzymes in all subcellular compartments were mobilized in cooperation and responded synchronously under mild water stress, with the same trend of changes in their activity. This indicated their orchestrated effects in scavenging reactive oxygen species (ROS) in situ. Additionally, the results suggested that mitochondria and apoplasts, responding most actively, might be targets for improving plant performance under mild water stress.

Keywords Water stress · Antioxidant isoenzymes · Maize

Introduction

Under stress conditions such as salinity, water stress, and extreme temperature, plants produce a surge of reactive oxygen species (ROS), which are harmful to plant growth due to their detrimental effects on the subcellular components and metabolism of the plant (Shim and others 2003). To maintain normal growth, ROS produced as a result of various abiotic stresses are scavenged by antioxidant systems, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR), and antioxidant molecules such as ascorbate (ASC) and glutathione (GSH) (Wang and others 2008b; Balen and others 2009; Hameed and others 2009; Piotrowska and others 2010). These antioxidant enzymes have multiple molecular forms (isoenzymes) and are located in different cellular compartments. The primary antioxidant enzyme, SOD, is found in almost all cellular compartments (Mittler 2002; Wang and others 2008a).

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Three types of SODs, classified by their metal cofactors, are Fe–SOD, Mn–SOD and Cu/Zn–SOD. Fe–SOD is located almost solely in chloroplasts (Mittler and others 2004). Mn–SOD, located predominantly in mitochondria and peroxisomes (Wang and others 2008a), has also been demonstrated to be in apoplasts of several plants (Yamahara and others 1999; Hernández and others 2001; Sgherri and others 2007). Cu/Zn–SOD, the most abundant isoenzyme of SOD, has been resolved in all subcellular fractions (Wang and others 2008a). Regarding CAT, a peroxisomal marker enzyme, its isoenzymes are located mainly in peroxisomes and apoplasts (Wang and others 2008a; Locato and others 2009). In contrast to CAT, APX and GR require an ASC and GSH regeneration system, the ASC–GSH cycle (Mittler 2002; Apel and Hirt 2004; Wang and others 2008b). Because the ASC–GSH cycle is located in almost all cellular compartments (Mittler 2002), the isoenzymes of GR and APX are found in chloroplasts, cytosol, mitochondria, apoplasts, and peroxisomes (Contour-Ansel and others 2006; Locato and others 2009). Moreover, these isoenzymes are regulated by distinct mechanisms in response to various environmental stresses, and they play cooperative roles to protect each organelle and minimize tissue injury (Mittler 2002; Shigeoka and others 2002).

Water stress is one of the most important environmental factors that affect plant growth and development and limit plant production. Plants can respond and adapt to water stress by altering their cellular metabolism and invoking various defense mechanisms (Zhu 2002; Boudsocq and Laurière 2005). The phytohormone abscisic acid (ABA) and ROS accumulate in plants under adverse environmental conditions such as water stress (Jiang and Zhang 2002; Apel and Hirt 2004; Wang and others 2008b; Jubany-Marí and others 2009). Under water stress, the accumulated ABA triggers the increased generation of ROS and upregulates the activities of antioxidant enzymes in maize leaves (Jiang and Zhang 2002; Zhang and others 2006; Jubany-Marí and others 2009).

Water stress induces H_2O_2 accumulation in cell walls, xylem vessels, chloroplasts, mitochondria, and peroxisomes; however, the apoplast is the major source of H_2O_2 production in the leaves of plants exposed to water stress (Bartoli and others 2004; Hu and others 2006; Jubany-Marí and others 2009). Signal propagation is accompanied by the accumulation of ROS in the apoplasts (Miller and others 2009), and apoplastic ROS accumulation upregulates the activities of chloroplastic and cytosolic antioxidant enzymes in maize leaves under water stress (Hu and others 2005, 2006). However, what other subcellular compartments do under water stress is not clear. Furthermore, the response of several major antioxidant enzymes and their isoenzymes in different subcellular compartments to water stress also remains to be elucidated.

In this study, an effort was made to elucidate different subcellular antioxidant responses to mild water stress. The activities of antioxidant enzymes and the antioxidant contents in subcellular compartments were investigated. The relative contribution of these isoenzymes was also investigated by analyzing antioxidant enzyme isoforms through native PAGE profiling. The results suggest that antioxidant enzymes, isoenzymes, and antioxidant molecules, broadly distributed in all subcellular compartments, were mobilized to scavenge ROS in coordination under mild water stress, also with their own characterization of subcellular distribution and relative contribution.

Materials and Methods

Plant Material and Treatments

Seeds of maize (*Zea mays* L. cv Nongda 108) were sown in trays and grown in a greenhouse at a temperature of 22–28°C, with photosynthetic active radiation (PAR) of 400 μmol m^{-2} s^{-1} and a photoperiod of 14/10 h (day/night). When the second leaves were fully expanded, they were collected and used for all investigations.

The plants were excised at the base of the stem and placed in distilled water for 1 h to eliminate wound stress. After treatment, the cut ends of the stems were placed in beakers wrapped with aluminum foil and containing polyethylene glycol (PEG) 6000 solution at –0.7 MPa (a mild water stress [Jiang and Zhang 2002; Hu and others 2006]) for various times up to 24 h at 25°C, with a continuous light intensity of 200 μmol m^{-2} s^{-1}. Detached plants were treated with distilled water under the same conditions for the whole period and served as controls for the above. After treatment of detached maize plants, the second leaves were sampled and immediately frozen under liquid N_2, and then stored at –80°C for further analysis.

Isolation of Chloroplasts

Chloroplasts were isolated from maize leaves using the method described by Hu and others (2005). Briefly, after grinding 5 g of leaves in isolation buffer [0.33 M sorbitol, 2 mM EDTA, 50 mM Hepes–KOH (pH 7.5), 0.1% (w/v) BSA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT], the homogenate was filtered through four layers of cheesecloth and centrifuged at 2°C and 200 *g* for 1 min. The pellet was resuspended in isolation buffer and then centrifuged at 2°C and 2500 *g* for 5 min. Chloroplasts were purified by resuspending the pellets in isolation buffer, layering on 12.5 ml of 25% (v/v) of Percoll (in isolation buffer), and centrifuging at 2°C and 15,800 *g* for 20 min. The intact chloroplasts in the lower layer were resuspended in

isolation buffer without BSA and $MnCl_2$ and centrifuged at 2°C and 2500 *g* for 5 min. The chloroplast pellets were lysed in lyses buffer [50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 1 mM $MgCl_2$] and used immediately for analyses. On the basis of the specific activities of subcellular marker enzymes, the contaminations of other fractions to the respective subcellular compartments were estimated (Supplementary Table S1).

Preparation of Cytosolic Fraction

The cytosolic fraction was prepared according to the method described by Hu and others (2005). Leaf segments (0.5 g) were homogenized in a mortar and pestle in 5 ml homogenization buffer containing 50 mM phosphate buffer (pH 7.5), 0.25 M sucrose, 10 mM EGTA, 1 mM DTT, and 1 mM PMSF. The homogenates were centrifuged at 10,000 *g* for 10 min, the supernatants were centrifuged at 100,000 *g* for 1 h, and the cytosolic fraction was obtained by collecting the supernatant.

Isolation of Mitochondria and Peroxisomes

Mitochondria and peroxisomes were collected as described by Busi and others (2006) with modifications. Leaves (10 g) were ground in 30 ml extraction buffer containing 20 mM HEPES (pH 7.5), 5 mM *o*-caproic acid, 20 mM sodium ascorbate, 0.3% BSA (w:v), 0.4 M sucrose, 10 mM NaCl, 10 mM mercaptoethanol, 2 mM EDTA, and 1% PVP. The homogenates were filtered through four layers of cheesecloth and centrifuged at 4°C and 3000 *g* for 10 min. The supernatant of the 3000 *g* centrifugation was recentrifuged at 12,000 *g* for 15 min and the pellet (crude mitochondria and peroxisomes) was collected. Mitochondria and peroxisomes were fractionated on a 25–45% sucrose gradient and centrifuged at 4°C and 27,000 *g* for 20 min. Fractions were collected from the bottom and analyzed for the respective enzyme marker activities. The fraction showing the highest activity of the mitochondrial or peroxisomal marker enzymes SDH and HPR, respectively, and the two fractions adjacent to the peak from both sides were collected and pooled for further study.

Preparation of the Apoplastic Fluid

Preparation of the apoplastic fluid was performed according to the method described previously (Hernández and others 2001; del Carmen Córdoba-Pedregosa and others 2007) with minor modifications. For the acquisition of the infiltrated washing fluid (IWF), 10 g of leaves were soaked in deionized water and subsequently vacuum infiltrated for 5 min at 1.0 kPa and 4°C with 50 mM K-phosphate buffer (pH 6.5) containing 0.2 M KCl and 0.1 mM $CaCl_2$. After having been wiped, the leaves were showered by distilled water twice and centrifuged in a 25-ml syringe barrel placed in a tube at 1500 *g* for 10 min at 4°C to obtain the IWF.

Antioxidant Enzymes Assays

The activities of antioxidant enzymes were determined as previously described (Jiang and Zhang 2002; Zhang and others 2006; del Carmen Córdoba-Pedregosa and others 2007; Wang and others 2008b; Hameed and others 2009; Sreedhar and others 2009; Piotrowska and others 2010).

Total SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium. One unit of SOD activity was defined as the amount of enzyme that was required to cause 50% inhibition of the reduction of nitroblue tetrazolium as monitored at 560 nm. Total CAT activity was assayed by measuring the rate of decomposition of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) at 240 nm for 3 min. Total GR activity was measured by following the change in A340 (extinction coefficient 6.2 mM^{-1} cm^{-1}) for 3 min as oxidized glutathione (GSSG)-dependent oxidation of NADPH.

To detect the activity of APX isoenzymes, an independent organelle-isolation procedure was used (20 mM sodium ascorbate added to the extraction medium), and all other solutions also contained 2 mM ascorbic acid to prevent the possible inactivation of APX. Total APX activity was measured by monitoring the decrease in absorbance at 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}) for 1 min as ascorbate was oxidized.

Nonenzymatic Antioxidants Assays

Nonenzymatic antioxidants were determined as previously described (Jiang and Zhang 2002). ASC content was determined using a method based on the reduction of the ferric ion to the ferrous ion with ASC in acid solution, followed by formation of the red chelate between the ferrous ion and bathophenanthroline, which absorbs at 534 nm (Jiang and Zhang 2002).

Total GSH and GSSG were determined by the 5′,5′-dithiobis-2-nitrobenzoic acid (DTNB)-GR recycling procedure (Jiang and Zhang 2002; Wang and others 2008b). Changes in absorbance were measured at 412 nm and total glutathione content was calculated from a standard curve with GSH. GSSG was determined after removal of GSH by 2-vinylpyridine derivation and the standard curve with GSSG was used. Then, GSH was determined by subtraction of GSSG from the total glutathione content.

Subcellular Antioxidant Enzymes Isoforms Assays

Isoenzymes of CAT, APX, GR, and SOD in native PAGE were visualized by the activity staining procedure, basically following the methods described previously (Parida and others 2004; Balen and others 2009; Sreedhar and others 2009) with minor modifications. Samples containing 40 μg of proteins were subjected to native PAGE in 4% stacking and separating gels at proper concentration under constant current (20 mA for 50 min, then 35 mA for 1 h) at 4°C.

SOD isoenzymes were resolved on 10.5% polyacrylamide gels and their activity was detected by photochemical staining with riboflavin and NBT as described by Seckin and others (2009). The different isoenzymes of SOD were differentiated by adding inhibitors to staining solution, such as 2 mM KCN to inhibit Cu/Zn–SOD activity and 3 mM H_2O_2 to inhibit Cu/Zn–SOD and Fe–SOD activities, whereas Mn–SOD activity is resistant to both inhibitor treatments (Seckin and others 2009) (Supplementary Fig. S1).

CAT isoenzymes were resolved on non-denaturing polyacrylamide gels (7.5%). Immediately after electrophoresis, the gels were incubated in a solution containing 1% soluble starch for 2 h at 4°C and then soaked in 0.3% H_2O_2 for 3 min at room temperature. The gel was then rinsed with distilled water and flooded with 90 mM potassium iodide solution acidified with 0.5% glacial acetic acid. Negative bands of CAT enzymes appeared on the blue background of the gel.

GR isoenzymes were also resolved on non-denaturing polyacrylamide gels (7.5%). Activity of GR isoenzymes was detected based on the activity of GR catalyzing GSSG-dependent oxidation of NADPH. After electrophoresis, the gels were washed in distilled water and incubated in 50 ml of 25 mM Tris–HCl buffer (pH 7.5) containing 10 mg MTT, 10 mg DCPIP, 3.4 mM GSSG, and 0.5 mM NADPH until the bands of GR appeared on the gel.

APX isoenzymes were resolved on polyacrylamide gels (9%) with 2 mM ascorbic acid in buffer and gels, and their activity was detected based on the ability of APX to prevent the ascorbate-dependent reduction of nitroblue tetrazolium in the presence of H_2O_2. After electrophoresis, the gels were incubated in 10 mM potassium phosphate (pH 7.0) solution containing 2 mM ASC for 30 min, then incubated in 2 mM H_2O_2 in the same buffer for 20 min at room temperature. Gels were washed with water and then dipped into a solution of 50 mM potassium phosphate (pH 7.0) containing 28 mM TEMED and 2.45 mM NBT for 15 min until the band appeared.

The quantity of isoenzyme activity was obtained by recording the density of the different bands in native PAGE through Quantity One 1-D analysis, and then the relative contribution of isoenzymes was expressed as the percentage of total activity per lane by integrating the activity data of the isoenzymes (Pérez-López and others 2009). All experiments were performed at least three times and the displayed data are typical results.

Statistical Analysis

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

Results

Effects of Water Stress on the Activities of Antioxidant (Iso)enzymes and the Antioxidant Contents in Chloroplasts

PEG treatment led to significant increases in the activities of antioxidant enzymes such as SOD, the enzyme for catalyzing the dismutation of O_2^- to O_2 and H_2O_2, and APX and GR, the two key enzymes of the ASC–GSH cycle for the removal of H_2O_2 in chloroplasts (Fig. 1A). A significant increase in the activities of SOD, GR, and APX occurred within 4 h, maximized at 12 h of PEG treatment, and then declined. In particular, PEG treatment for 12 h enhanced the activities of chloroplastic SOD, GR, and APX by 12.9, 40.7, and 116%, respectively, compared with the control values (Fig. 1A). The activity of APX in chloroplasts was more sensitive than that of SOD and GR in response to PEG treatment.

Also in chloroplasts, the contents of antioxidants such as ascorbic acid (ASC) and glutathione (GSH) displayed the same trend of change as antioxidant enzymes. PEG treatment for 12 h upregulated the contents of chloroplastic ASC and GSH by 60.3 and 141.1%, respectively, compared with the control values (Fig. 1B).

To investigate the effects of water stress on the activities of antioxidant isoenzymes, the native PAGE profile for chloroplastic antioxidant enzymes isoforms was analyzed. Four SOD isoenzymes, three GR isoenzymes, and two APX isoenzymes were detected, and most of them exhibited the same change in activity tendency as the total enzymes (Figs. 1A, 2), except for GRI and GRIII. The activities of Fe–SOD, Cu/Zn–SODI–III, GRII, and APXI–II increased during 12 h of PEG treatment, and then they declined. However, the activities of GRI and GRIII kept increasing during the whole process of water stress (Fig. 2). The activities of three SOD isoenzymes were inhibited by H_2O_2 or KCN, suggesting the presence of three isoenzymes of Cu/Zn–SOD and another SOD

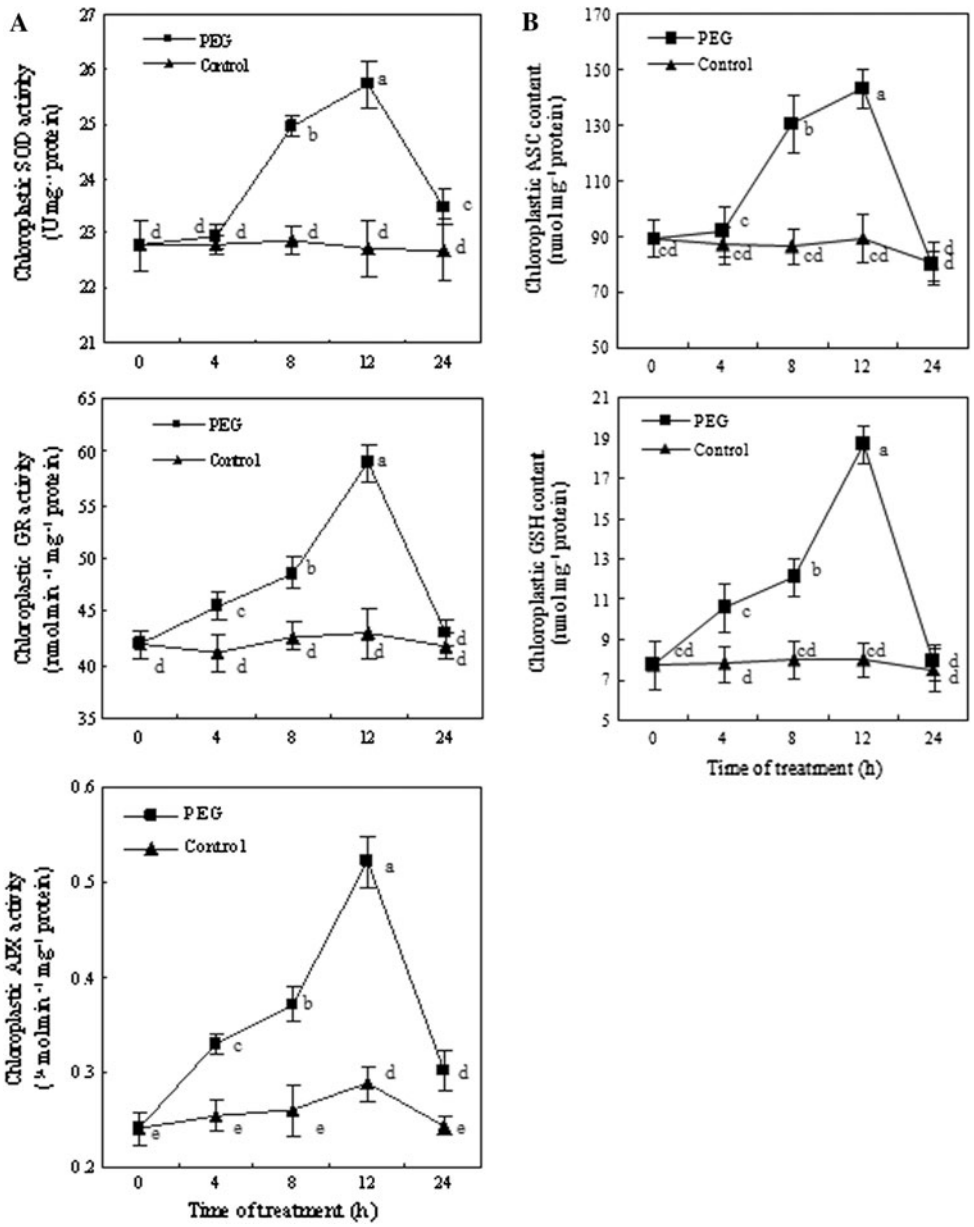

Fig. 1 Effects of water stress on the activities of antioxidant enzymes and the contents of ASC and GSH in chloroplasts. Time course of changes in the activities of SOD, GR, and APX (**A**) and the ASC and GSH contents (**B**) in chloroplastic fractions from leaves of detached maize plants exposed to –0.7 MPa osmotic stress. The values are given as mean ± SE ($n = 6$) of six different experiments. Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Ducan's multiple-range tests

isoenzyme shown to be KCN insensitive, indicating the presence of one Fe–SOD isoform (Supplementary Fig. S1). Moreover, Fe–SOD activity accounted for over 37% whereas the three Cu/Zn–SODs equally shared the remaining 63% of the total SOD isoenzyme activity in chloroplasts throughout the process of water stress (Fig. 2). Thus, Fe–SOD exhibited higher activity than each of the Cu/Zn–SOD isoenzymes. Regarding GR isoenzymes, three well-resolved bands were identified and the most abundant one, GRIII, accounted for more than 45% of the total GR isoenzyme activity (Fig. 2). For the two kinds of APX isoenzymes resolved, the activity of APXI was about twofold greater than that of APXII, and the priority of APXI was maintained throughout the time of PEG treatment (Fig. 2).

Effects of Water Stress on the Activities of Antioxidant (Iso)enzymes and the Antioxidant Contents of Mitochondria

Under water stress, the activities of antioxidant enzymes (SOD, GR, and APX) and the antioxidant molecules (ASC and GSH) in mitochondria (Fig. 3), peroxisomes (Fig. 4), and cytosol (Fig. 5) had similar change trends to those in the chloroplastic fractions (Fig. 1), exhibiting a significant increase from 4 to 12 h, maximizing at 12 h, and then declining. PEG treatment for 12 h enhanced the activities of SOD, GR, and APX by 29.4, 132, and 174% in mitochondria, respectively, compared with the control values (Fig. 3A). PEG treatment for 12 h also enhanced the contents of ASC and GSH by 156.5 and 136.9% in mitochondria, respectively (Fig. 3B).

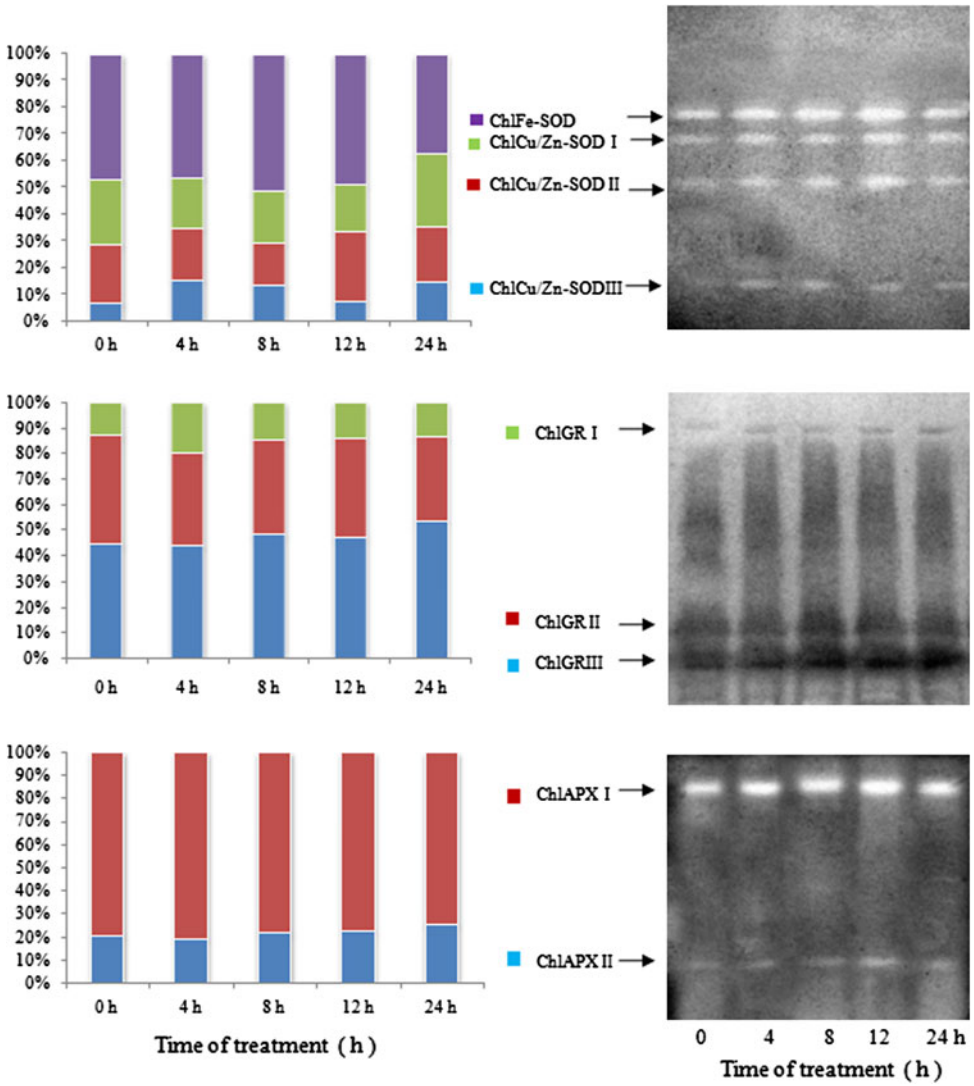

Fig. 2 Effects of water stress on the activities of antioxidant isoenzymes in chloroplasts. Native PAGE of SOD, GR, and APX isoenzymes in chloroplastic fractions from leaves of detached maize plants exposed to –0.7 MPa osmotic stress for various times. All experiments were repeated at least three times with similar results

The activities of the detected mitochondrial antioxidant isoenzymes exhibited concomitant changes with total enzyme activity, increasing from 4 to 12 h, then almost down to the basal level (Figs. 3, 6), showing the same change trend observed in chloroplasts (Figs. 1, 2). Among those SOD isoenzymes resolved in mitochondria, one was Cu/Zn–SOD, one was Fe–SOD, and the remaining two were Mn–SODs (Fig. 6). Though it has been reported that Fe–SOD is located almost entirely in chloroplasts (Mittler and others 2004), Fe–SOD was also detected in mitochondria and its activity accounted for 20% of the total SOD activity (Fig. 6). However, the stacked activities of two Mn–SOD isoenzymes accounted for more than 60% of the total SOD activity, and the contribution of Mn–SODI was higher than that of Mn–SODII (Fig. 6). Moreover, four kinds of GR isoenzymes and only one APX isoenzyme were detected (Fig. 6).

Effects of Water Stress on the Activities of Antioxidant (Iso)enzymes in Peroxisomes

Under water stress, the activities of SOD, GR, APX, and CAT isoenzymes in peroxisomes also increased from 4 to 12 h and then declined (Fig. 7), the same tendency as found in chloroplasts (Fig. 2) and mitochondria (Fig. 6). PEG treatment for 12 h enhanced the activities of SOD, CAT, GR, and APX by 61.4, 28.8, 123.7, and 153.7% in peroxisomes, respectively, compared with the control values (Fig. 4A). PEG treatment for 12 h also enhanced the contents of ASC and GSH by 270.1 and 214% in peroxisomes, respectively (Fig. 4B).

In peroxisomes, there were three kinds of SOD isoenzymes, including one Cu/Zn–SOD and two Mn–SODs, two kinds of GR isoenzymes, one kind of APX isoenzyme, and two kinds of CAT isoenzymes (Fig. 7). Among the SOD isoenzymes, two Mn–SODs' stacked activities accounted

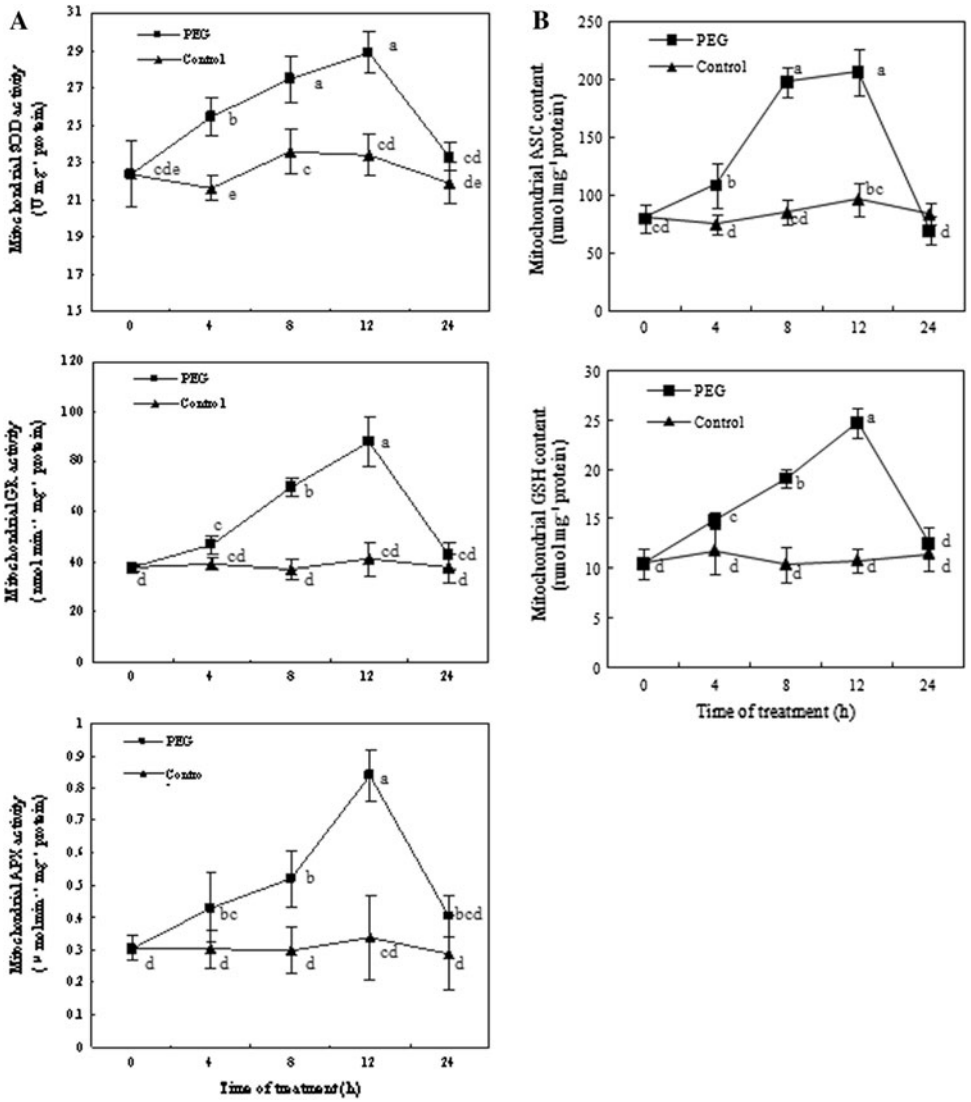

Fig. 3 Effects of water stress on the activities of antioxidant enzymes and the contents of ASC and GSH in mitochondria. Time course of changes in the activities of SOD, GR, and APX (**A**) and the ASC and GSH contents (**B**) in mitochondrial fractions from leaves of detached maize plants exposed to –0.7 MPa osmotic stress. The values are given as mean ± SE ($n = 6$) of six different experiments. Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Ducan's multiple-range tests

for around 63% of the total SOD activity in the peroxisomal fraction (Fig. 7). Regarding the two kinds of CAT isoenzymes resolved, the relative contribution of CATI was higher than that of CATII, and the priority of CATI increased with time under water stress (Fig. 7).

Effects of Water Stress on the Activities of Antioxidant (Iso)enzymes in Cytosol

In cytosol, PEG treatment for 12 h enhanced the activities of SOD, GR, and APX by 12.8, 44.5, and 45.6%, respectively, compared with the control values (Fig. 5A). PEG treatment for 12 h also enhanced ASC and GSH content by 57.5 and 49.2% in cytosol, respectively (Fig. 5B).

Under water stress, five kinds of SOD isoenzymes (including four Cu/Zn–SODs and one Fe–SOD), two kinds of GR isoenzymes, and four kinds of APX isoenzymes were detected in the cytosolic fraction (Fig. 8). Under water stress, the activities of Cu/Zn–SODIV, GRI, GRII, and APXI–IV, concomitant with the total enzyme activity for each, increased from 4 to 12 h, maximized at 12 h, and then declined (Fig. 8). However, the activities of the other four SOD isoenzymes kept increasing during the 24 h of water stress (Fig. 8). Thus, the relative contribution of Cu/Zn–SODIV declined from 41.3 to 27.6%, whereas that of Fe-SOD kept increasing from 12 to 15%, and the relative contribution of the other three Cu/Zn–SOD isoforms also increased from 46.7 to 57.5% during the whole 24 h after the initiation of water stress (Fig. 8).

Effects of Water Stress on the Activities of Antioxidant (Iso)enzymes and the Content of Antioxidants in Apoplasts

In apoplasts, the activity of SOD significantly increased within 4 h, steadily increased to 8 h, and was maximized at 12 h of PEG treatment, whereas the activities of CAT, APX, and GR increased markedly from 8 to 12 h and maximized at 12 h after PEG treatment (Fig. 9A). PEG treatment for 12 h

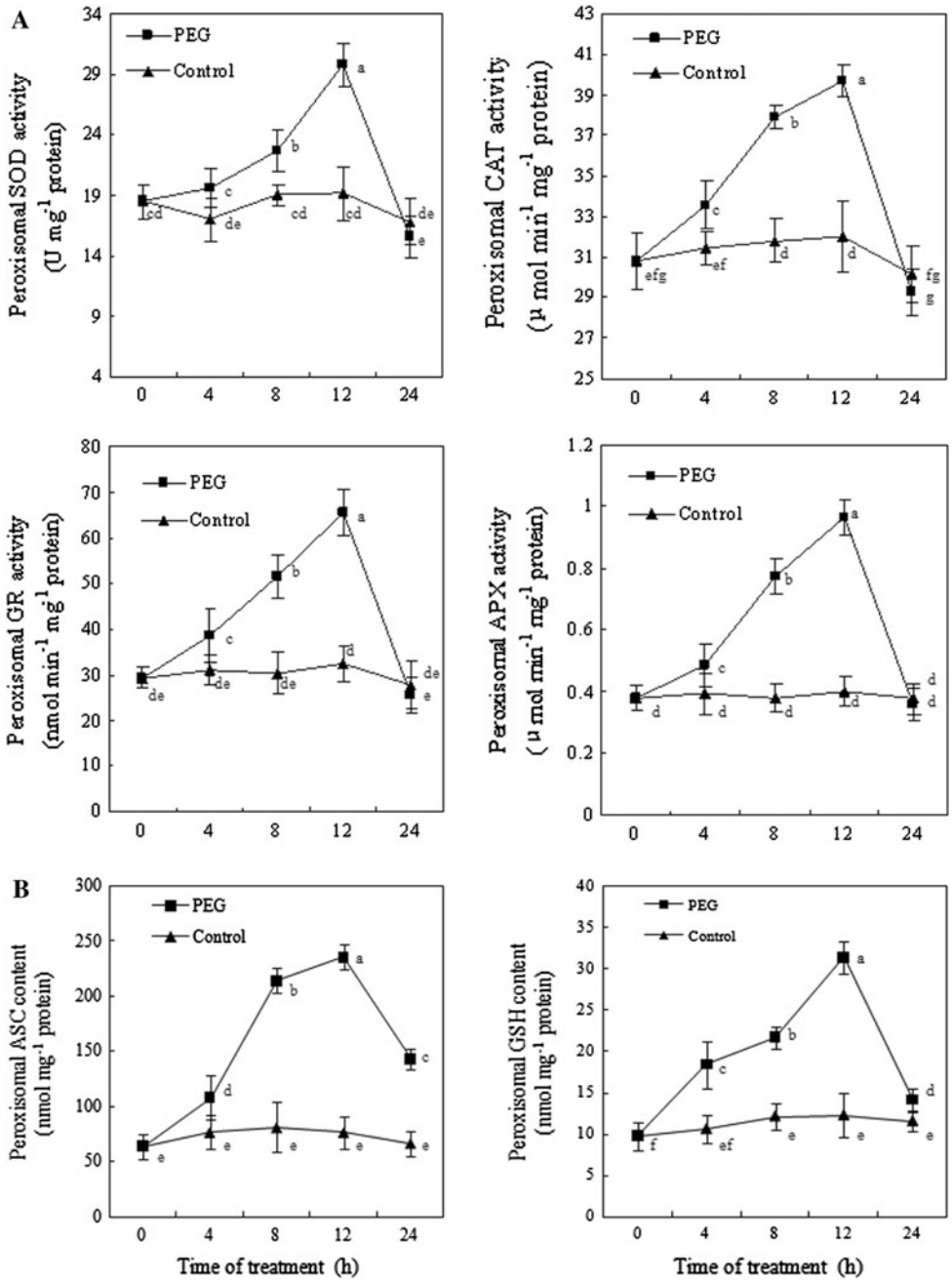

Fig. 4 Effects of water stress on the activities of antioxidant enzymes and the contents of ASC and GSH in peroxisomes. Time course of changes in the activities of SOD, CAT, GR, and APX (**A**) and the ASC and GSH contents (**B**) in peroxisomal fractions from leaves of detached maize plants exposed to –0.7 MPa osmotic stress. The values are given as mean ± SE ($n = 6$) of six different experiments. Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Ducan's multiple-range tests

enhanced the activities of SOD, CAT, GR, and APX by 81.3, 130, 53.5, and 20.5% in apoplasts, respectively, compared with the control values (Fig. 9A). PEG treatment for 12 h also enhanced the contents of ASC and GSH by 50.1 and 162.2% in apoplasts, respectively (Fig. 9B).

Under water stress, six kinds of SOD isoenzymes (including four Cu/Zn–SODs, one Fe–SOD, and one Mn–SOD), one CAT isoenzyme, four kinds of GR isoenzymes, and two kinds of APX isoenzymes were detected in apoplasts (Fig. 10). However, the activities of all detected isoenzymes of apoplastic antioxidant enzymes showed more complicated changes than those that occurred in intracellular compartments. Regarding SOD isoenzymes resolved, the activities of Mn–SOD and Fe–SOD decreased, those of Cu/Zn–SODII–IV increased continuously under water stress (Fig. 10), and the activity of Cu/Zn–SODI (Fig. 10) changed in parallel to the total SOD activities (Fig. 9A). However, the activities of isoforms of other antioxidant enzymes such as CAT, GR, and APX increased gradually from 4 to 12 h and then declined (Fig. 10), showing the same tendency as their total enzyme activity, respectively (Fig. 9). The relative contribution of Fe–SOD and Mn–SOD declined continuously from 80 to 43%, whereas the contribution of the other SOD isoenzymes kept increasing during water stress. For the two

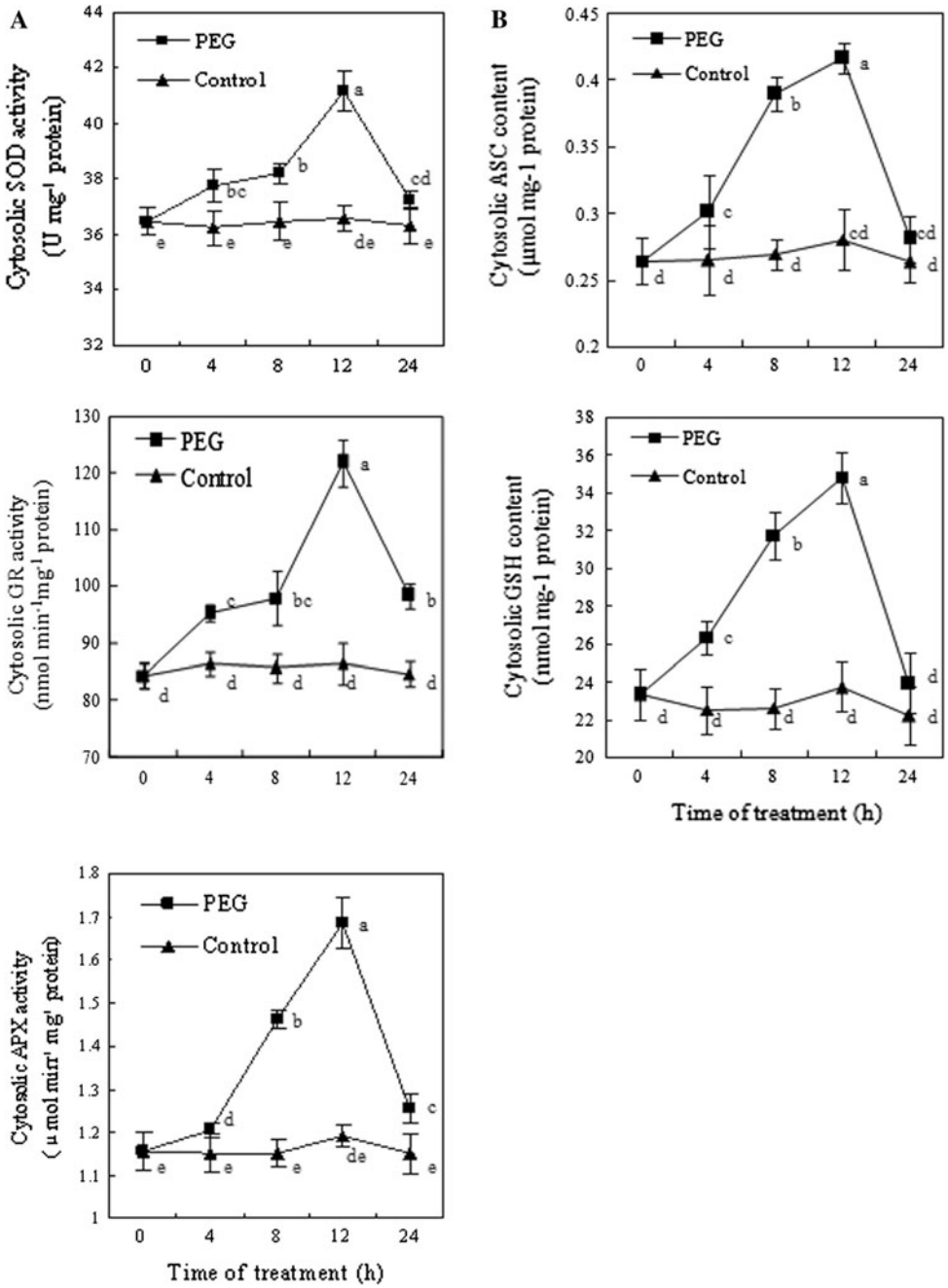

Fig. 5 Effects of water stress on the activities of antioxidant enzymes and the contents of ASC and GSH in cytosol. **A** Time course of changes in the activities of SOD, GR, and APX (**A**) and the ASC and GSH contents (**B**) in cytosolic fractions from leaves of detached maize plants exposed to –0.7 MPa osmotic stress. The values are given as mean ± SE ($n = 6$) of six different experiments. Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Ducan's multiple-range tests

kinds APX isoenzymes resolved, the relative contribution of APXI was about twofold higher than APXII, and the priority of APXI increased with the duration of water stress (Fig. 10).

Discussion

Broad and Special Distribution of Antioxidant Enzyme Isoforms

Previous studies showed that in plants, antioxidant enzymes act as isoenzymes in subcellular compartments and respond to various stimuli (Mittler and others 2004; Wang and others 2008a; Pérez-López and others 2009; Seckin and others 2009). In this study, our results showed that at least one isoform for the detected antioxidant enzymes was resolved in each subcellular compartment (Figs. 2, 6, 7, 8, 10).

Although all three SOD isoenzymes are nuclear encoded, the SOD enzyme is present in all subcellular compartments (Blokhina and others 2003; Wang and others 2008a). Similarly in this study, SOD isoenzymes were also resolved in all subcellular fractions (Figs. 2, 6, 7, 8, 10). Moreover, Fe–SOD coupled with Mn–SOD was well

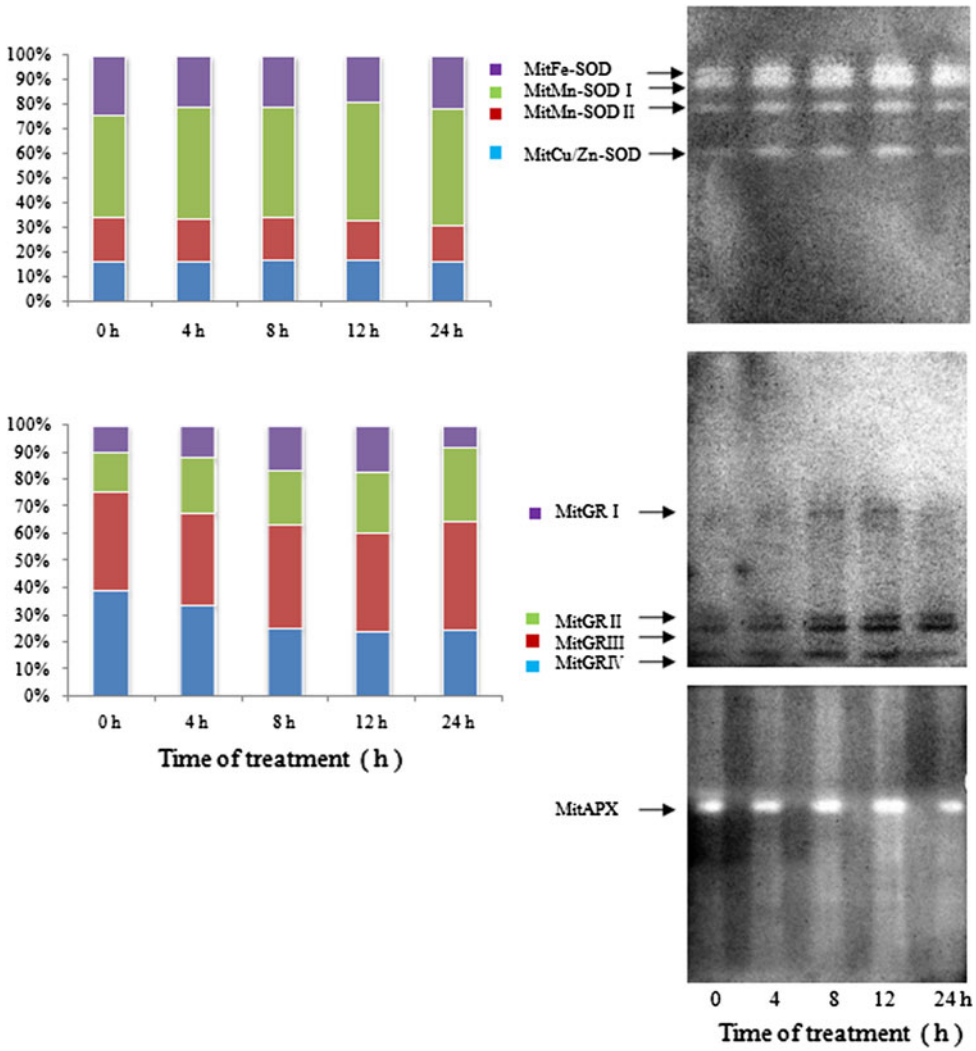

Fig. 6 Effects of water stress on the activities of antioxidant isoenzymes in mitochondria. Native PAGE of SOD, GR, and APX isoenzymes in mitochondrial fractions from leaves of detached maize plants exposed to –0.7 MPa osmotic stress for various times. All experiments were repeated at least three times with similar results

resolved in apoplasts (Fig. 10). Though it has been reported that Mn–SOD is located predominantly in plant mitochondria and peroxisomes (Wang and others 2008a), it has also been demonstrated in apoplasts (Yamahara and others 1999; Hernández and others 2001; Sgherri and others 2007). In the present study, Mn–SOD was resolved not only in mitochondria and peroxisomes (Figs. 6 and 7), but also in apoplasts (Figs. 2, 6, 7, 8, 10). CAT is often considered a peroxisomal marker enzyme because its presence is suggested to be limited to these organelles; however, it has also been localized in other cellular compartments (Wang and others 2008a; Locato and others 2009). Through systematic investigation, we found that besides two CAT isoenzymes in peroxisomes (Fig. 7), one CAT isoenzyme was detected in the apoplasts from maize under water stress (Fig. 10). APX seems to be the most versatile enzyme. At least one APX isoenzyme was detected in the five subcellular compartments focused on in this investigation, and even in cytoplasm, four isoenzymes of APX were resolved (Fig. 8). Moreover, APX has a much higher affinity for H_2O_2 than CAT, which makes APX a very versatile enzyme for a fine modulation of the levels of H_2O_2 in cells and/or within specific cellular compartments (Locato and others 2009). GR is always concomitant with APX, and at least two isoenzymes of GR were detected in all subcellular compartments investigated in this study (Figs. 2, 6, 7, 8, 10). Therefore, GR coupled with APX, which presented in all five subcellular compartments tested here, thus indicated, on one hand, the key role of the ASC–GSH cycle in ROS removal under water stress, and on the other hand, their orchestrated effects in scavenging H_2O_2.

The broad distribution of the investigated antioxidant isoenzymes in different subcellular compartments suggests their cooperation in defense against mild water stress (–0.7 MPa induced by PEG, relative water content lowered by 14.2% during 24 h of PEG treatment) (Jiang and Zhang 2002; Hu and others 2006). Furthermore, the localization of antioxidant isoenzymes to specific subcellular compartments suggests a capacity to efficiently detoxify ROS

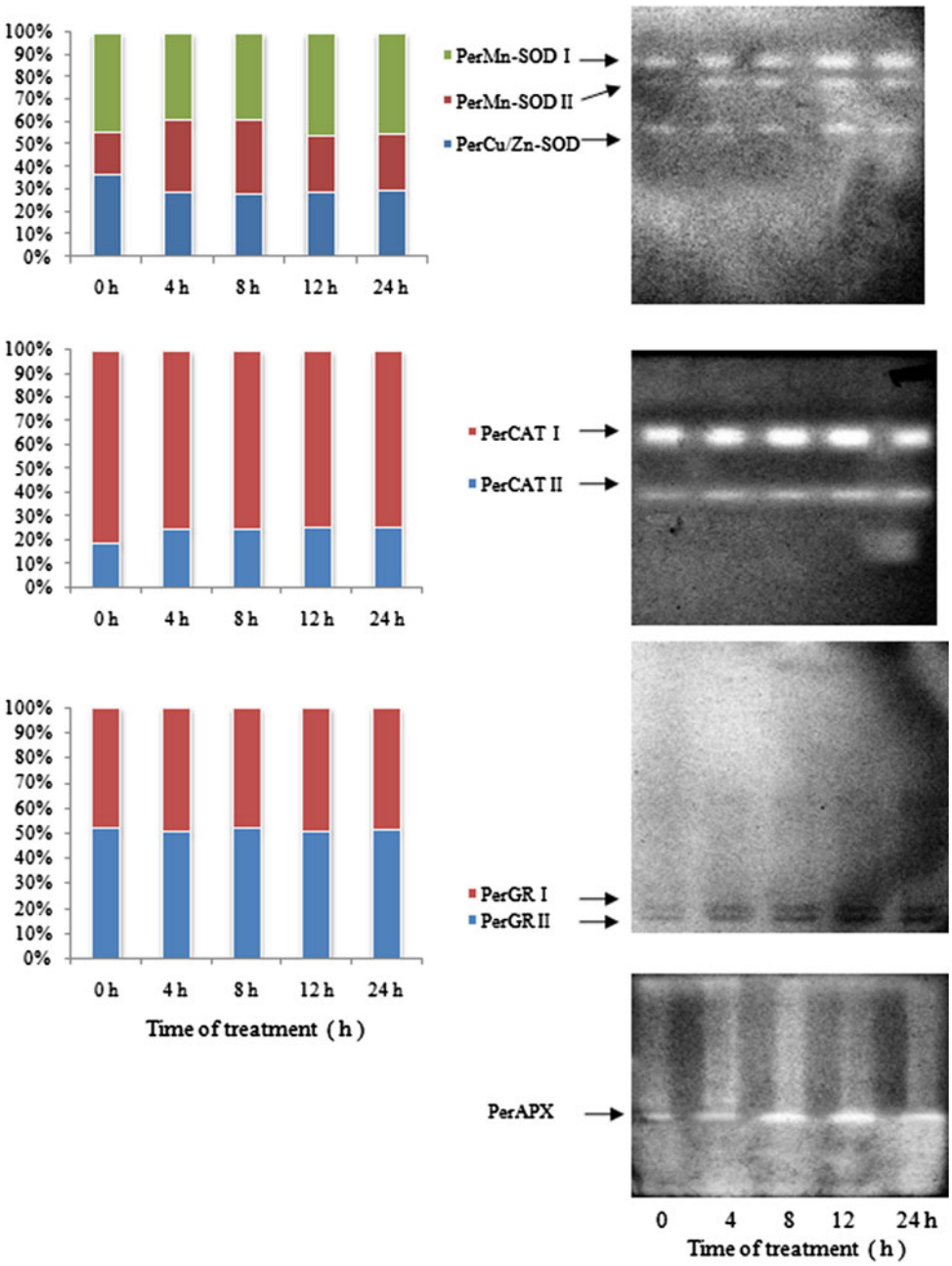

Fig. 7 Effects of water stress on the activities of antioxidant isoenzymes in peroxisomes. Native PAGE of SOD, CAT, GR, and APX isoenzymes in peroxisomal fractions from leaves of detached maize plants exposed to –0.7 MPa osmotic stress for various times. All experiments were repeated at least three times with similar results

at their production site, as proposed by D'Arcy-Lameta and others (2006).

Mitochondria and Apoplasts Might Be the Targets for Improving Plant Performance under Water Stress

The identification of subcellular sites that are sensitive to oxidative damage may contribute to the design of transgenic plants with improved performance under a variety of abiotic stresses (Bartoli and others 2004).

Although plasma membranes and organelles such as mitochondria, peroxisomes, chloroplasts, and even nuclei have been shown to act as ROS generators (Ashtamker and others 2007; Wang and others 2008a; Jubany-Marí and others 2009), under stress conditions much attention is focused on chloroplasts, mitochondria, and apoplasts (Jubany-Marí and others 2009; Liu and others 2009). A previous study showed that among the subcellular fractions studied, the largest increases in protein oxidation brought about by drought occurred in mitochondria and peroxisomes (Bartoli and others 2004), and the increase in the production of ROS in mitochondria might be an important alarm signal upregulating antioxidant defense systems (Bartoli and others 2004). In plants, ROS were produced

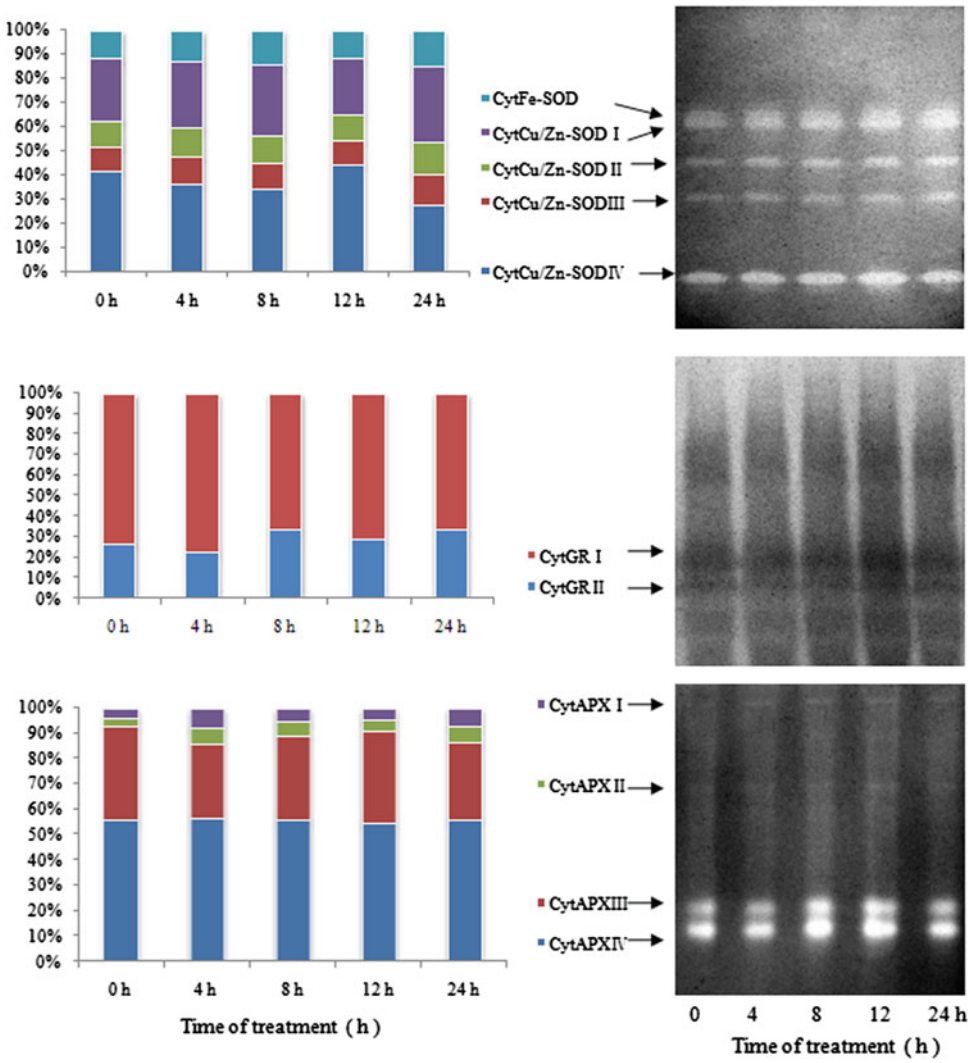

Fig. 8 Effects of water stress on the activities of antioxidant isoenzymes in cytosol. Native PAGE of SOD, GR, and APX isoenzymes in cytosolic fractions from leaves of detached maize plants exposed to –0.7 MPa osmotic stress for various times. All experiments were repeated at least three times with similar results

first in mitochondria and then in chloroplasts (Zhang and Xing 2008). Moreover, the changes in the activities of antioxidant (iso)enzymes investigated here (Figs. 1A, 3A) were proportional to changes in contents of H_2O_2 under water stress presented by our previous study (Jiang and Zhang 2002; Hu and others 2006). Thus, it is expected that mitochondria act more quickly and positively than chloroplasts in mobilizing the antioxidant system under water stress. This was the case in mitochondria where the significant increase of SOD activity occurred 4 h after PEG treatment (Fig. 3A), whereas in chloroplasts, the significant increase of SOD activity occurred within 8 h of PEG treatment (Fig. 1A). These results suggest that plant mitochondria may sense cellular stress early and serve as first relay stations where the initial alteration in ROS homeostasis is triggered (Jones 2000; Pastore and others 2007; Gao and others 2008).

Moreover, besides mainly localized Mn–SODs taking up more than 60% of the total SOD activity, Fe–SOD was also resolved in mitochondria and accounted for 20% of the total SOD activity; also, at least four GR isoenzymes were detected in mitochondria (Fig. 6). Furthermore, PEG treatment for 12 h enhanced the activities of SOD, GR, and APX by 29.4, 132, and 174% (Fig. 3A), respectively, and the ASC and GSH contents by 156.5 and 136.9% (Fig. 3B), respectively, in mitochondria, exhibiting a greater increase than that in chloroplasts [the increment of SOD, GR, APX, and ASC by 12.9, 40.7, 116, and 60.3%, respectively (Fig. 1)] or in cytosol [the increment of SOD, GR, and APX by 12.8, 44.5, and 45.6%, and ASC and GSH by 57.5 and 49.2%, respectively (Fig. 5)]. These results suggest that plant mitochondria play a crucial role in orchestrating antioxidant enzymes in the process of drought tolerance, as was described earlier (Pastore and others 2007; Atkin and Macherel 2009).

Besides mitochondria, apoplasts are also sensitive to water stress. Apoplasts are the major source of ROS production under water stress, and the accumulated ROS in

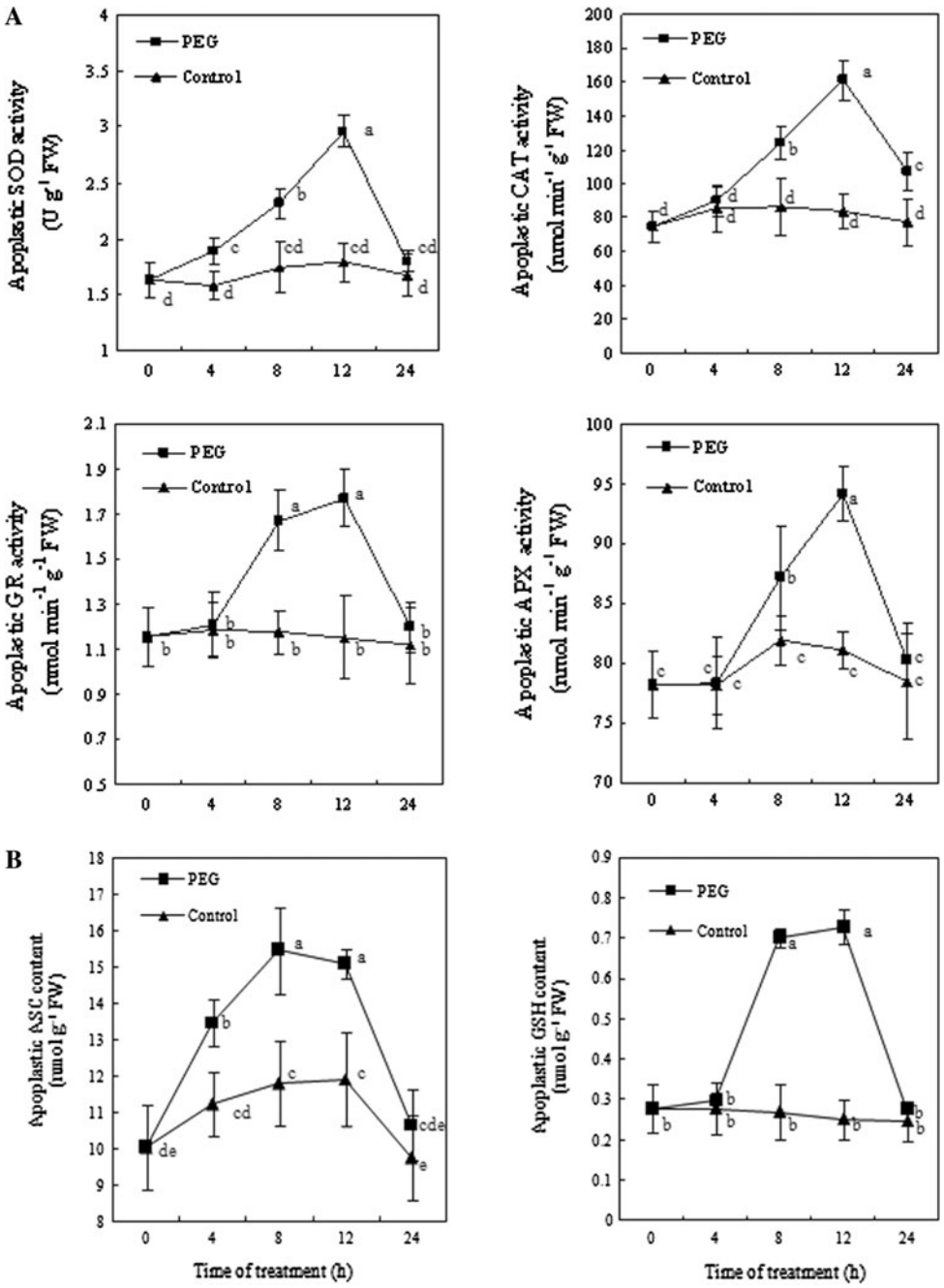

Fig. 9 Effects of water stress on the activities of antioxidant enzymes and the contents of ASC and GSH in apoplasts. Time course of changes in the activities of SOD, CAT, GR, and APX (**A**) and the ASC and GSH contents (**B**) in apoplastic fractions from leaves of detached maize plants exposed to –0.7 MPa osmotic stress. The values are given as mean ± SE ($n = 6$) of six different experiments. Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Ducan's multiple-range tests

apoplasts cannot cross the plasma membrane (Hu and others 2006), suggesting that apoplasts represent the first active line of defense against ROS under water stress and could have the potential to scavenge ROS in this compartment to avoid oxidative damage. Coincidentally, SOD, APX, GR, and CAT, the four antioxidant enzymes investigated in this study, were all located in apoplasts (Fig. 9A). Furthermore, PEG treatment for 12 h enhanced the activities of SOD and CAT by 81.3 and 130%, respectively, in apoplasts (Fig. 9A), which are remarkably higher than that in intracellular organelles [the increment of SOD by 12.9% in chloroplasts (Fig. 1A), 29.4% in mitochondria (Fig. 3A), 12.8% in cytosol (Fig. 5A), and the increment of SOD and CAT by 61.4 and 28.8%, respectively, in peroxisomes (Fig. 4A)]. These results suggest that apoplasts are most active among the investigated subcellular compartments in response to water stress.

In addition, the activities of all detected antioxidant isoenzymes showed more complicated changes in apoplasts than those occurring in intracellular compartments. In particular, the activities of apoplastic Mn–SOD and

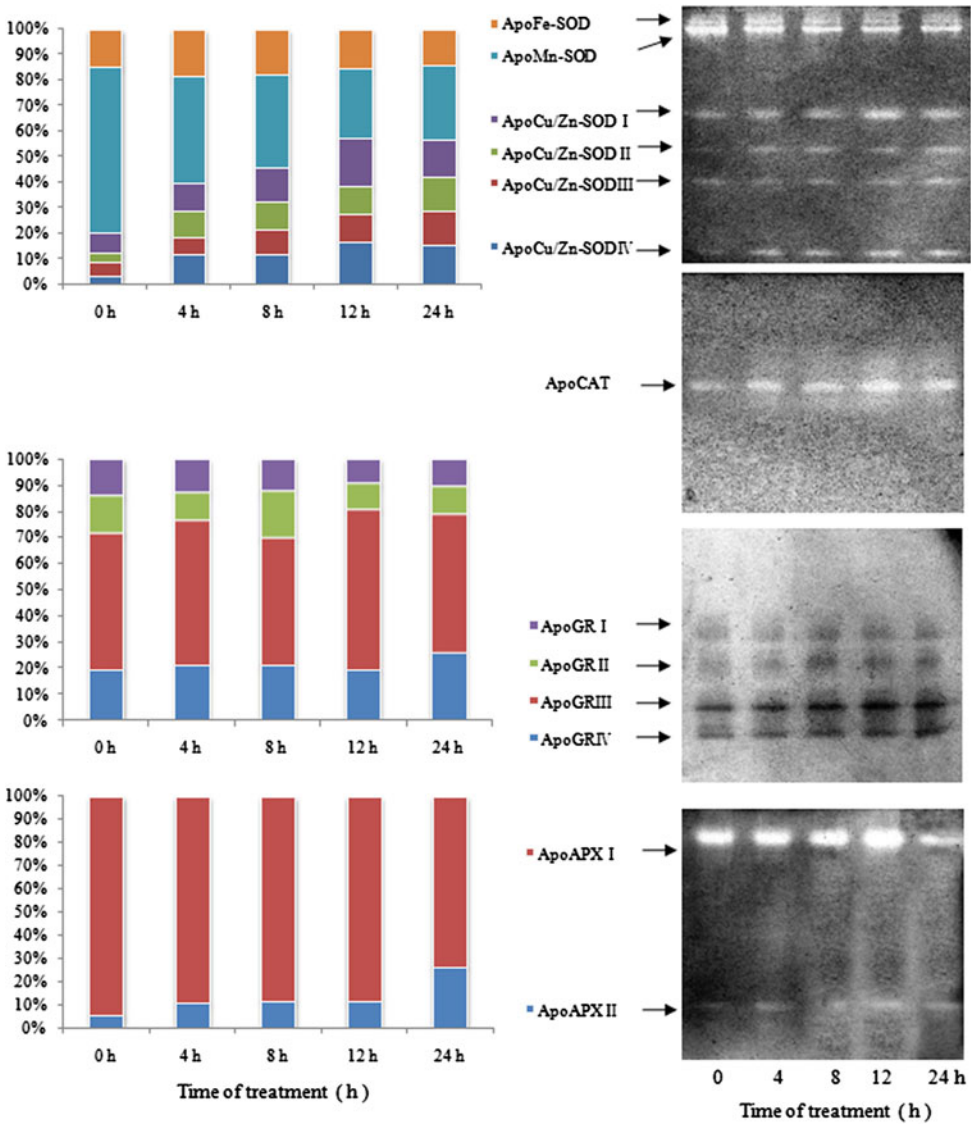

Fig. 10 Effects of water stress on the activities of antioxidant isoenzymes in apoplasts. Native PAGE of SOD, CAT, GR, and APX isoenzymes in apoplastic fractions from leaves of detached maize plants exposed to –0.7 MPa osmotic stress for various times. All experiments were repeated at least three times with similar results

Fe–SOD, the most active SOD isoenzymes in apoplasts, declined throughout the water stress (Fig. 10), whereas in chloroplasts and mitochondria/peroxisomes, activities increased gradually (Figs. 2, 6, 7). This was reminiscent of the hypothesis that subtle changes in the subcellular distribution of antioxidant enzymes and different sensitivities to ROS exhibited by antioxidant isoenzymes might be more important for protection than an overall increase in total enzyme activity (D'Arcy-Lameta and others 2006). Meanwhile, the amounts of ASC and GSH, powerful antioxidants, increased almost to the maximum in apoplasts during the 8 h after water stress (Fig. 9B), suggesting that plants utilize pre-existing and mobilize the newly developed antioxidant defense system in apoplasts to scavenge the accumulated H_2O_2 in situ, thus acclimating to the water stress. Here, the critical role of the apoplastic antioxidant system in water stress response emerged.

All the results discussed above indicate that protecting mitochondria against ROS and manipulating ROS accumulation in apoplasts might improve plant performance under water-stress conditions.

Antioxidant Defense System Was Mobilized Omnidirectionally in Cooperation in Response to Water Stress

The mode of coordination between different components of the ROS removal network of plants is complex (Mittler and others 2004). In this investigation, the cooperation included not only the antioxidant isoenzymes and antioxidants in different subcellular compartments, but several intracellular organelles.

Several ROS-scavenging enzymes such as GR (Chew and others 2003; Contour-Ansel and others 2006) and APX

(Chew and others 2003) have been found to be targeted to both the chloroplasts and mitochondria, the two most important organelles sensitive to oxidative stress (Jones 2000; Pesaresi and others 2007; Fernández and Strand 2008; Gao and others 2008; Zhang and Xing 2008), suggesting a high degree of coordination in defense responses between these different cellular compartments (Mittler and others 2004). Besides GR and APX located in both chloroplasts and mitochondria, Fe–SOD, located almost entirely in chloroplasts (Mittler and others 2004), was detected in mitochondria (Fig. 6). This was reminiscent of the phenomenon of dual targeting. From the subcellular prediction result of ProtComp-PL (www.softberry.com), WoLF PSORT (Horton and others 2007), and TargetP (Emanuelsson and others 2000), the protein sequence of maize Q5FB28 (chloroplastic Fe–SOD, from www.uniprot.org) and maize P41978 (mitochondrial Mn–SOD, from www.uniprot.org) contain the sequence expected to be the transit peptide for the dual targeting to chloroplasts and mitochondria (data not shown). Combined with the point of dual targeting, the same types of antioxidant enzymes (SOD, GR and APX) and their isoenzymes, together with their similar trends of activity changes in chloroplasts and mitochondria (Figs. 1A, 3A), might explain the coordination of chloroplasts and mitochondria in response to oxidative stress (Mittler and others 2004).

To survive under stress conditions, it is very important that the plant antioxidant system is able to work in harmony, thus providing better defense and regeneration of the active reduced forms (Blokhina and others 2003). In this investigation, the changes in the activities of antioxidant (iso)enzymes and the ASC and GSH contents, with the same tendency in different subcellular compartments during the time course of water stress treatment, were proportional to changes in H_2O_2 content under water stress presented by previous studies (Jiang and Zhang 2002; Hu and others 2006).

The simultaneous change in the activity of antioxidant enzymes GR, APX, and CAT (particularly in peroxisomes and apoplasts) exhibited basically the same trend of changes in the activity of SOD (Figs. 4A, 9A). Moreover, the contents of antioxidants such as ascorbic acid (ASC) and glutathione (GSH) displayed the same trend of change as antioxidant enzymes in each subcellular compartment (Figs. 1B, 3B, 4B, 5B, 9B). These results, on one hand, indicated their orchestrated effects in scavenging H_2O_2 as well as other free radicals. On the other hand, the antioxidant system, including antioxidant enzymes and antioxidant molecules, cooperated at the subcellular level, for most of the resolved isoenzymes displayed the same trend of changes, similar to the time-course curve of their respective antioxidant enzyme activity changes (Figs. 1, 3, 4, 5, 9). Thus, from a broad view of this investigation, all antioxidant systems, including antioxidant enzymes and antioxidants, located in different subcellular compartments were mobilized in coordination under water stress.

One may question whether the changes monitored in antioxidant enzymes reflected a general effect of water stress on any enzyme activity in the leaf. Thus, some enzymes not related to antioxidant action, such as chloroplastic glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mitochondrial succinate dehydrogenase (SDH), cytosolic glucose-6-phosphate-dehydrogenase (G6PDH), and peroxisomal NADH-preferring hydroxypyruvate reductase (HPR), were assayed for changes in activities during water stress (see Supplementary Material for detail). As was expected, the time course of changes in the activities of non-ROS-related enzymes, the specific marker enzyme for the specific subcellular compartments, is dissimilar to that of antioxidant enzymes in the specific subcellular location (Supplementary Fig. S2), which suggests that the pattern of change in antioxidant enzyme activities is not a general effect of water stress on any enzyme activity located in different subcellular compartments.

The Proposed Mechanism of Plant Acclimation to Water Stress

In summary, under mild water stress, all subcellular compartments were mobilized to scavenge ROS in coordination with their own characterization, and antioxidant (iso)-enzymes together with ASC and GSH in subcellular compartments, with the same tendency of changes in activity or contents, were orchestrated to remove ROS together and with subcellular specificity.

Furthermore, the broad distribution of the antioxidant isoenzymes suggested their cooperation in defense against stress, whereas antioxidant isoenzymes localized to subcellular compartments suggested a capacity to efficiently scavenge ROS in situ.

Among the subcellular compartments investigated, apoplasts and mitochondria were the most active. Plant mitochondria played a crucial role in orchestrating antioxidant enzymes in the process of drought tolerance, and the critical role of the apoplastic antioxidant system in water stress response was also elucidated. Therefore, mitochondria and apoplasts might be targets for improving plant performance under mild water stress.

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