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Exogenous Hydrogen Peroxide Changes Antioxidant Enzyme Activity and Protects Ultrastructure in Leaves of Two Cucumber Ecotypes Under Osmotic Stress

Zhong-Jing Liu · Yan-Kui Guo · Ji-Gang Bai

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Abstract Cucumber (*Cucumis sativus* L.) varieties cv. Jinchun no. 4 (a North China ecotype) and cv. Lvfeng no. 6 (a South China ecotype) were cultivated to explore the effects of osmotic stress on the ultrastructure of chloroplasts and mitochondria, as well as to assess the possible protective effect of exogenous hydrogen peroxide (H_2O_2). Under osmotic stress induced by 10% polyethylene glycol 6000, 84.3% of the chloroplasts in Jinchun no. 4 were abnormal, whereas 88.6% were abnormal in Lvfeng no. 6. Abnormal mitochondria occurred in these two strains at rates of 78.5 and 87.1%, respectively. The stress condition disintegrated the membranes of most chloroplasts and mitochondria in the leaf cells of both cucumber ecotypes, and it also increased the malondialdehyde (MDA) content. We subjected the two cultivars to a combined treatment with H_2O_2 and osmotic stress and made the following observations: (1) Abnormal chloroplasts occurred at rates of 25.7 and 28.6%, and abnormal mitochondria were observed at rates of 22.9 and 32.8%, respectively. (2) Most of the investigated membranes were well organized in leaves of Jinchun no. 4 and Lvfeng no. 6, and the levels of endogenous H_2O_2, superoxide anion, and MDA were lower. Osmotic stress and exogenous H_2O_2 both increased the activities of antioxidative enzymes such as manganese superoxide dismutase, glutathione peroxidase, catalase, guaiacol peroxidase, ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase, and the antioxidants ascorbate and reduced glutathione. The combined effect of osmotic stress and exogenous H_2O_2 resulted in the highest antioxidant activities in both cucumber ecotypes. We propose that exogenous H_2O_2 increases antioxidant activity in cucumber leaves and thereby decreases lipid peroxidation to some extent, thus protecting the ultrastructure of most chloroplasts and mitochondria under osmotic stress.

Keywords Antioxidant · Cucumber · Hydrogen peroxide · Polyethylene glycol · Ultrastructure

Introduction

In many regions of the world, drought occurs every year (Ludlow and Muchow 1990). Some crops such as cucumber (*Cucumis sativus* L.) have shallow root systems and large leaf areas and therefore are sensitive to inadequate water supply. Water stress not only reduces the yield and quality of such crops (Chaves and others 2003), but it also induces the production of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$) (Smirnoff 1993). The accumulation of ROS damages the ultrastructure of plants (Xu and others 2008) and leads to the death of cells (Molassiotis and others 2006). To scavenge the ROS, plants possess antioxidative enzymes, including superoxide dismutase, catalase (CAT), guaiacol peroxidase (GPX), glutathione peroxidase (GSH-Px), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase (MDHAR), and antioxidants such as reduced glutathione (GSH) and ascorbate (AsA) (Asada 1992).

Hydrogen peroxide (H_2O_2) not only is a harmful ROS but also has a role as a signaling molecule in pathways of stress signal transduction (Foyer and others 1997; Chamnongpol and others 1998). Pretreatment with low concentrations of

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the molecule increases the accumulation of GSH (Murphy and others 2002) and antioxidant enzyme activity (De Azevedo Neto and others 2005) in plants, thereby alleviating harm from ROS (Murphy and others 2002; Wahida and others 2007). Osmotic stress can result from water deficit (Morgan 1984; Kusaka and others 2005), and we hypothesized that H_2O_2 pretreatment would improve the tolerance of cucumber plants to osmotic stress by enhancing antioxidant activity and protecting ultrastructure. It has not been previously reported that H_2O_2 pretreatment alleviates the effects of osmotic stress on ultrastructure.

Polyethylene glycol (PEG) 6000 influences osmotic potential and is not absorbed by plants (Carpita and others 1979; van den Berg and Zeng 2006). To determine the effects of osmotic stress on ultrastructure, we used PEG 6000 to induce osmotic stress (Murillo-Amadaor and others 2002) in cucumber seedlings. Also, to explore a possible protective strategy, seedlings were pretreated with exogenous H_2O_2.

Materials and Methods

Plant Materials and Treatments

In China, *Cucumis sativus* is divided into North China and South China ecotypes, and Jinchun no. 4 and Lvfeng no. 6 belong to these two ecotypes, respectively. To study the effects of H_2O_2 pretreatment on both ecotypes of Chinese cucumber, seeds from the two cultivars were germinated on moist gauze at 25°C for 2 days and then were planted in 10-cm plastic pots filled with sand. Cucumber seedlings were grown at 25°C with 12-h light (600 μmol m^{-2} s^{-1})/12-h dark and were watered twice per day with Hoagland nutrient solution containing 5 mM KNO_3, 5 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 2 mM $MgSO_4$, 10 μM $MnSO_4$, 50 μM H_3BO_3, 0.7 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 0.01 μM $(NH_4)_6Mo_7O_{24}$, and 70 μM Fe-EDTA-Na_2. When the second leaves were fully expanded, seedlings were selected and were divided into four groups. The aboveground organs of two groups were sprayed with freshly prepared 1.5 mM exogenous H_2O_2, and the other two groups were sprayed with water. The H_2O_2-sprayed plants were kept at 25°C for an additional 8 h and then were watered with either the Hoagland nutrient solution only or Hoagland nutrient solution containing 10% (w/v) PEG 6000 and were designated as "H_2O_2 pretreatment" and "H_2O_2 + PEG treatment," respectively. The other two groups of seedlings were separately watered with either of the two types of nutrient solutions and were named "control" and "PEG treatment." After 2 days of treatment, samples of the second leaf were used for electron microscopy studies or were ground with liquid nitrogen for the subsequent experiments. Three different sets of plants grown at different times were separately tested with all four treatments.

Transmission Electron Microscopy

Leaf samples were treated with 3.5% glutaraldehyde for 24 h and then were postfixed with 1% osmic acid at 4°C for 4 h (Helliot and others 2003, as modified by Xu and others 2008). Leaf cells were dehydrated with ethanol and were embedded in Spurr resin at 60°C for 24 h, after which thin sections were cut with an LKB ultramicrotome and placed upon 250-mesh grids. Then leaf cells were poststained with uranyl acetate and lead citrate and were observed in a transmission electron microscope (JEM-1200EX; JEOL Ltd., Tokyo, Japan) at 80 kV. The magnifications were 10,000× and 25,000×. To count the number of starch granules, 70 chloroplasts per treatment were analyzed.

Determination of Malondialdehyde Content

The levels of malondialdehyde (MDA) were determined according to the method of Dhindsa and others (1981) with the modifications of Xu and others (2008), and the absorbance was measured at 450, 532, and 600 nm (Zhang and others 2005).

Determination of $O_2^{\bullet-}$ Formation Rate and H_2O_2 Content

The formation rate of $O_2^{\bullet-}$ was determined according to the method of Elstner and Heupel (1976). Leaves (0.2 g) were homogenized in 3 ml of 65 mM phosphate buffer (pH 7.8) and were then centrifuged at 4°C and 5000×*g* for 10 min. The supernatants (0.75 ml) were mixed with 0.675 ml of 65 mM phosphate buffer (pH 7.8) and 0.075 ml of 10 mM hydroxylamine chlorhydrate and then were incubated at 25°C. After 20 min, 0.375 ml of 17 mM sulfanilamide and 0.375 ml of 7 mM α-naphthylamine were added, and the mixtures were incubated at 25°C for another 20 min before they were mixed with 2.25 ml of ether. The absorbance was measured at 530 nm and the formation rate of $O_2^{\bullet-}$ was calculated from a standard curve of $NaNO_2$.

The H_2O_2 content was measured according to the modified method of Bernt and Bergmeyer (1974). Samples of the second leaf were cut from cucumber seedlings and ground with liquid nitrogen at once. Then 0.1 g of the ground leaves was homogenized in 5 ml of cold (−20°C) acetone and centrifuged at 3000×*g* and 4°C for 10 min. One milliliter of the supernatant was mixed with 0.2 ml of ammonia and 0.1 ml of 95% (v/v) hydrochloric acid containing 20% (v/v) titanium tetrachloride. After being centrifuged at 10,000×*g* and 4°C for 10 min, the sediment was repeatedly washed with cold (−20°C) acetone and

centrifuged at 14,000×*g* and 4°C, and then it was dissolved in 3 ml of 1 M H_2SO_4. The absorbance of the resulting solution was measured at 410 nm and the content of H_2O_2 in the leaves was calculated with an H_2O_2 solution-derived standard curve.

Extraction of Antioxidative Enzymes

After being ground with liquid nitrogen, 0.3 g of leaves were suspended in 3 ml of ice-cold HEPES buffer (25 mM, pH 7.8) containing 0.2 mM EDTA and 2% (w/v) PVP. The homogenate was centrifuged at 4°C and 12,000×*g* for 20 min, and the resulting supernatants were used for determination of manganese superoxide dismutase (Mn-SOD, EC 1.15.1.1), CAT (EC 1.11.1.6), GPX (EC 1.11.1.7), DHAR (EC 1.8.5.1), MDHAR (EC 1.6.5.4), and GR (EC 1.6.4.2) (Ramiro and others 2006). To extract GSH-Px (EC 1.11.1.9), 0.3 g of ground leaves was suspended in 0.3 ml of HEPES buffer (25 mM, pH 7.8) containing 0.2 mM EDTA and 2% (w/v) PVP. The enzyme APX (EC 1.11.1.11) was extracted with HEPES buffer (25 mM, pH 7.8) containing 0.2 mM EDTA, 2% (w/v) PVP, and 2 mM AsA (Zhu and others 2004).

Determination of Antioxidant Enzyme Activities

The activity of Mn-SOD was measured in the presence of 3 mM H_2O_2 using the method of Hwang and others (1999). Fifteen microliters of the enzyme extract was mixed with 10 µl of 300 mM H_2O_2, 2.54 ml of 100 mM phosphate buffer (pH 7.8), 75 µl of 55 mM methionine, 300 µl of 0.75 mM nitroblue tetrazolium (NBT), and 60 µl of 0.1 mM riboflavin in a test tube. The test tubes containing the reaction solution were irradiated under fluorescent light at 40 µmol m^{-2} s^{-1} for 10 min. The absorbance of the irradiated and nonirradiated solutions was determined at 560 nm.

The CAT activity was calculated by the disappearance of H_2O_2 ($\varepsilon = 40$ M^{-1} cm^{-1}) in 1 min at 240 nm (Pereira and others 2002, as modified by Xu and others 2008), whereas the GPX activity was determined by following the change of absorbance due to guaiacol oxidation ($\varepsilon = 6.39$ mM^{-1} cm^{-1}) at 470 nm (Ramiro and others 2006, as modified by Xu and others 2008). In the GR enzyme reaction, NADPH oxidation ($\varepsilon = 6.2$ mM^{-1} cm^{-1}) was recorded as the decrease in the absorbance at 340 nm in 1 min (Lee and Lee 2000, as modified by Xu and others 2008).

The activity of GSH-Px was measured by using H_2O_2 as the substrate (Xue and others 2001). Enzyme extract (40 µl) was mixed with 40 µl of 1 mM GSH for the enzyme reaction. For the nonenzyme reaction, 40 µl of boiled enzyme extract was mixed with 40 µl of 1 mM GSH. The mixtures were incubated at 37°C for 5 min, and then 20 µl of 1.5 mM H_2O_2 was added to initiate the reaction. The reaction was allowed to run for 3 min and was stopped by adding 1.67% (w/v) metaphosphoric acid [containing 0.05% (w/v) EDTA and 28% (w/v) NaCl]. The mixtures were centrifuged at 2000×*g* for 10 min, and then 400 µl of the supernatant was mixed with 500 µl of 0.32 M Na_2HPO_4 and 100 µl of 1 mM 5,5-dithio-bis(2-nitrobenzoic acid) for 5 min. The absorbance was measured at 412 nm, and the enzyme activity was calculated from the decrease in GSH within the enzyme reaction as compared to the nonenzyme reaction.

To determine the activity of APX, 50 µl of enzyme extract was mixed with 850 µl of 25 mM phosphate buffer (pH 7.0, containing 0.1 mM EDTA), 50 µl of 5 mM AsA, and 50 µl of 20 mM H_2O_2 (Chen and Asada 1989), and the H_2O_2-dependent oxidation of AsA ($\varepsilon = 2.8$ mM^{-1} cm^{-1}) was followed by the decrease in absorbance at 290 nm. The activity of MDHAR was assayed by monitoring the change of absorbance at 340 nm due to NADPH oxidation ($\varepsilon = 6.2$ mM^{-1} cm^{-1}) for 1 min. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADH, 2.5 mM AsA, 0.15 U ascorbate oxidase, and 50 µl of enzyme extract (Hossain and others 1984). To measure the activity of DHAR, 50 µl of enzyme extract was mixed with 850 µl of 25 mM phosphate buffer (pH 7.0, containing 0.1 mM EDTA), 50 µl of 70 mM GSH, and 50 µl of 8 mM dehydroascorbate, and the enzyme activity was determined by the formation of AsA ($\varepsilon = 14$ mM^{-1} cm^{-1}) in 1 min at 265 nm (Doulis and others 1997).

Determination of Nonenzymatic Antioxidant Contents

Ground leaves (0.6 g) were suspended in 6 ml of 1% (w/v) oxalic acid and were centrifuged at 4°C and 8000×*g* for 10 min. The supernatant was used for determining the levels of AsA and total ascorbate.

The AsA content was assayed according to the methods of Klein and Perry (1982) and Raghu and others (2007) with some modifications. The AsA extract (0.5 ml) was mixed with 250 µl of 0.1% (w/v) 2,6-dichlorophenol and 625 µl of xylene and was incubated for 20 s. The absorbance was measured at 500 nm within 1.5 min, and the content of AsA was calculated from a standard curve of ascorbate.

The content of total ascorbate was determined according to the method of Mukherjee and Choudhuri (1983) with slight modifications. Charcoal (0.2 g) was activated with 15 ml of 1 M HCl and then was suspended in 2.5 ml of ascorbate extract for 1 min. After filtration, 200 µl of filtrate was mixed with 200 µl of 2% (w/v) thiourea and 100 µl of 2% (w/v) 2,4-dinitrophenylhydrazine. After being incubated at 37°C for 3 h, the mixture was cooled at room temperature and mixed with 0.5 ml of 85% (v/v) H_2SO_4 in an ice bath. The absorbance was recorded at

500 nm and the contents of AsA and total ascorbate were calculated from a standard curve of ascorbate. Oxidized ascorbate was estimated from the difference of total ascorbate and AsA.

The content of GSH was determined at 412 nm as described by Guri (1983) with the modifications of Xu and others (2008) and was calculated from a standard curve of reduced glutathione. The level of oxidized glutathione (GSSG) was measured using the GSH and GSSG assay kit (Beyotime Institute of Biotechnology, China).

Data Analysis

The total enzyme activities of certain enzyme types were assayed with three replicates. Data were expressed as mean ± standard error. Differences were analyzed with analysis of variance (ANOVA) and least significant difference (LSD) in SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). *P* values less than 0.05 were considered to be significant.

Results

Under the osmotic stress induced by PEG, the edges of the second leaves were withered in 83.3 ± 4.2% of Jinchun no. 4 seedlings and in 87.5 ± 7.2% of Lvfeng no. 6 seedlings (Fig. 1). When H_2O_2 pretreatment was combined with PEG, most of the second leaves were normal, and only 25.0 ± 7.2% of Jinchun no. 4 seedlings and 29.2 ± 4.2% of Lvfeng no. 6 seedlings had withered leaves. To understand how H_2O_2 induced tolerance to osmotic stress, we studied the ultrastructure and antioxidant system of leaf cells.

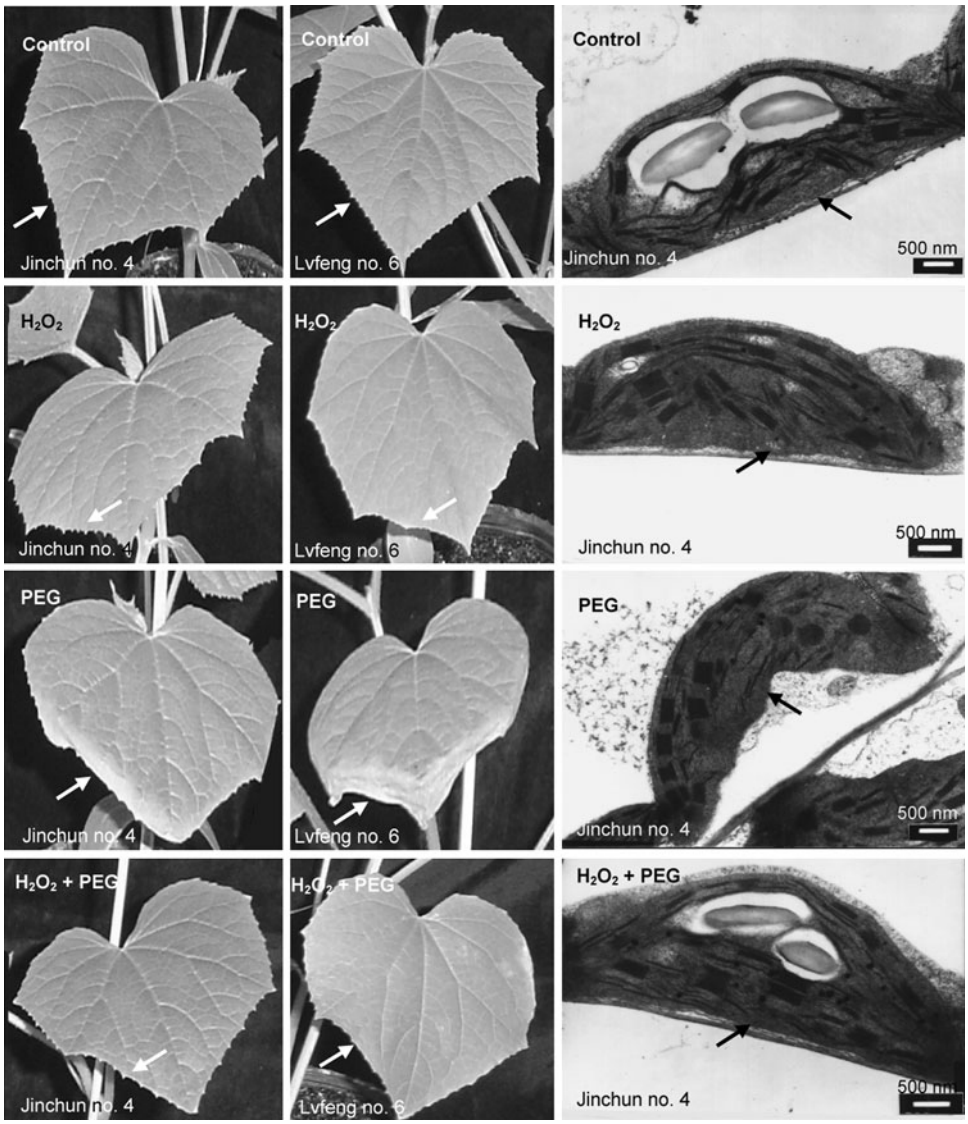

Fig. 1 Leaves of Jinchun no. 4 and Lvfeng no. 6 and chloroplasts in the first-layer palisade parenchyma of Jinchun no. 4 at 56 h after H_2O_2 pretreatment. Control, untreated; H_2O_2, pretreated with 1.5 mM H_2O_2; PEG, watered with 10% PEG; H_2O_2 + PEG, pretreated with 1.5 mM H_2O_2 and watered with 10% PEG. The differences among treatments are marked with *white arrows* in leaves and *black arrows* in chloroplasts. Eight seedlings per treatment were used in three independent experiments and 70 chloroplasts per treatment were analyzed. The magnification of the chloroplasts is 10,000×

Changes in Cell Ultrastructure

After applying exogenous H_2O_2 and PEG, the shapes of chloroplasts were the same in both cucumber varieties. Therefore, the organelles of just one cucumber ecotype are shown in Fig. 1. The chloroplasts were similar and normal in the H_2O_2 pretreatment and control groups; all exhibited long ellipsoidal shapes. PEG treatment caused 84.3 ± 3.9% of 70 chloroplasts from Jinchun no. 4 and 88.6 ± 3.5% of 70 chloroplasts from Lvfeng no. 6 to curve inward and were therefore abnormal in three independent experiments. However, treatment with H_2O_2 + PEG reduced the percentage of abnormal chloroplasts to 25.7 ± 4.0% in Jinchun no. 4 and 28.6 ± 5.0% in Lvfeng no. 6, and most chloroplasts were long and ellipsoidal. In the chloroplasts of Jinchun no. 4, the starch granules were smaller in the H_2O_2 pretreatment group than they were in either the H_2O_2 + PEG group or the control group, and the average number of granules in each chloroplast was 2.5 ± 0.5, 1.5 ± 0.5, and 0.5 ± 0.5, respectively. In Lvfeng no. 6 and the PEG treatment group of Jinchun no. 4, there were almost no starch particles to be found. Under higher magnification (Fig. 2), the chloroplast membranes and thylakoids in the two cucumber ecotypes were well

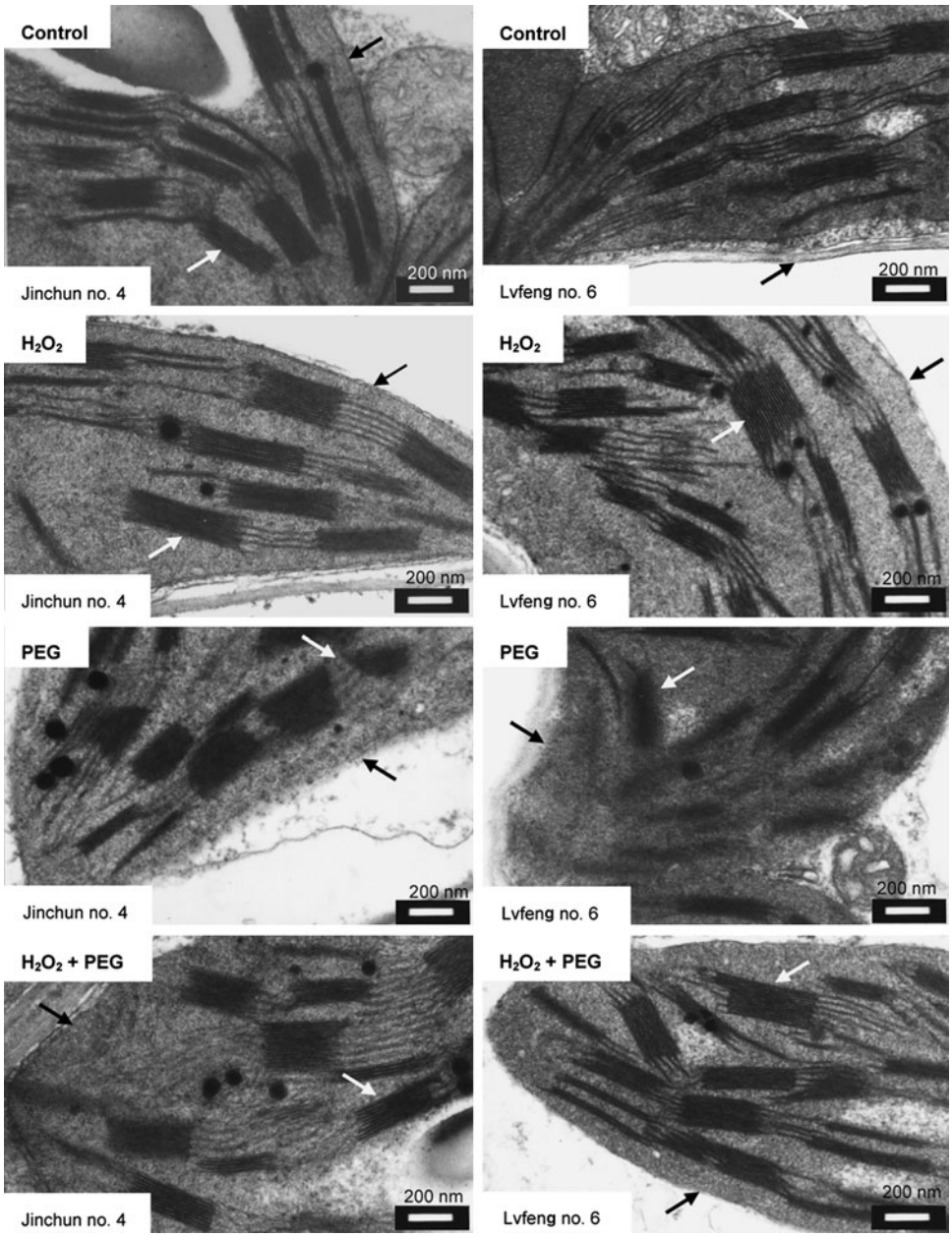

Fig. 2 Chloroplast thylakoids (marked with *white arrows*) and chloroplast membrane (marked with *black arrows*) in cucumber leaves at 56 h after H_2O_2 pretreatment. Control, untreated; H_2O_2, pretreated with 1.5 mM H_2O_2; PEG, watered with 10% PEG; H_2O_2 + PEG, pretreated with 1.5 mM H_2O_2 and watered with 10% PEG. The magnification is 25,000×

organized in the H_2O_2 pretreatment, control, and H_2O_2 + PEG treatment groups. However, in the PEG treatment group, the lamellae were disorganized and no longer parallel, and the envelopes of the abnormal chloroplasts were disintegrated.

In Jinchun no. 4 and Lvfeng no. 6, the mitochondrial membranes were relatively continuous in the H_2O_2 pretreatment and control groups (not shown). In the PEG treatment group, 78.5 ± 2.3% of the mitochondria from Jinchun no. 4 and 87.1 ± 3.1% of the mitochondria from Lvfeng no. 6 were abnormal, with membranes that appeared indistinct and cristae beginning to disintegrate. In the H_2O_2 + PEG treatment group, only 22.9 ± 1.0% of the mitochondria were abnormal in Jinchun no. 4 and 32.8 ± 2.8% were abnormal in Lvfeng no. 6.

MDA Content

Compared to the control group, H_2O_2 pretreatment significantly ($P < 0.05$) decreased the MDA content in leaves of Jinchun no. 4 and Lvfeng no. 6, whereas PEG treatment increased the MDA content ($P < 0.01$) (Fig. 3). Moreover, treatment with H_2O_2 + PEG elevated ($P < 0.01$) the MDA content in Jinchun no. 4 and did not change the MDA content in Lvfeng no. 6 ($P > 0.05$). The MDA content in H_2O_2 + PEG-treated plants of both ecotypes was higher ($P < 0.01$) than that in H_2O_2 pretreated plants. However, compared with PEG treatment, treatment with H_2O_2 + PEG lowered ($P < 0.05$) the MDA content in leaves of both the cucumber ecotypes. The osmotic stress induced by PEG significantly ($P < 0.01$) increased the MDA content of cucumber leaves, whereas exogenous H_2O_2 decreased it ($P < 0.01$).

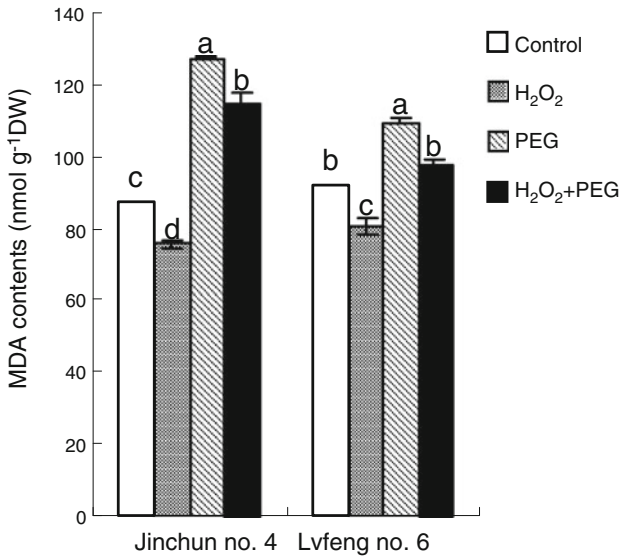

Fig. 3 Effects of exogenous H_2O_2 and PEG on MDA content in cucumber leaves. Control, untreated; H_2O_2, pretreated with 1.5 mM H_2O_2; PEG, watered with 10% PEG; H_2O_2 + PEG, pretreated with 1.5 mM H_2O_2 and watered with 10% PEG. Samples represent values at 56 h after H_2O_2 pretreatment. *Bars* represent the standard error of three independent experiments. Values with the same letter are not significantly different at $P < 0.05$ according to the least significant difference (LSD) test

$O_2^{\bullet-}$ Formation Rates and H_2O_2 Content

The endogenous H_2O_2 content of cucumber leaves was determined before pretreatment with H_2O_2 (0 h) and at 8 and 56 h after pretreatment. Compared to the level at 0 h, endogenous H_2O_2 at 8 h went up 143 ± 5.0% ($P < 0.05$) in Jinchun no. 4 and 137 ± 5.0% ($P < 0.05$) in Lvfeng no. 6. However, at 56 h, the endogenous H_2O_2 levels in the H_2O_2 pretreatment group had decreased. In Fig. 4, the ROS levels of the four treatments were compared at 56 h after pretreatment. Compared to the control group, H_2O_2 pretreatment did not significantly change the endogenous H_2O_2 level ($P > 0.05$) in Jinchun no. 4, and it reduced the level of endogenous H_2O_2 in Lvfeng no. 6. It also decreased ($P < 0.05$) the formation rates of $O_2^{\bullet-}$ in leaves of both cucumber ecotypes. Furthermore, PEG treatment significantly ($P < 0.01$) increased the levels of the two molecules in each ecotype. Compared to PEG treatment, treatment with H_2O_2 + PEG reduced ($P < 0.05$) the levels of endogenous H_2O_2 and the formation rate of $O_2^{\bullet-}$ in leaves of both cucumber ecotypes. Therefore, the osmotic stress induced by PEG significantly ($P < 0.01$) increased the levels of endogenous H_2O_2 and $O_2^{\bullet-}$ in cucumber leaves, whereas exogenous H_2O_2 decreased ($P < 0.01$) them.

Antioxidant Enzyme Activities

When comparing the H_2O_2 pretreatment group to the control group, the activities of antioxidant enzymes such as Mn-SOD, GSH-Px, CAT, GPX, DHAR, and GR were all enhanced ($P < 0.05$) in leaves from both cucumber ecotypes, whereas the MDHAR activity increased ($P < 0.05$) in Jinchun no. 4 but did not change significantly ($P > 0.05$) in Lvfeng no. 6. However, H_2O_2 pretreatment did not change the APX activity ($P > 0.05$) in either ecotype (Figs. 5 and 6). In leaves from both cucumber ecotypes, treatment with PEG elevated the activity of all the investigated enzymes ($P < 0.05$) relative to the controls; moreover, treatment with H_2O_2 + PEG caused the enzymes to increase more ($P < 0.05$) than PEG treatment alone. In leaves from Jinchun no. 4 and Lvfeng no. 6, exogenous H_2O_2 and the osmotic stress induced by PEG significantly ($P < 0.05$) increased the activity of antioxidant enzymes such as Mn-SOD, GSH-Px, GPX, CAT, APX, DHAR, MDHAR, and GR, and the combination of H_2O_2 pretreatment and osmotic stress resulted in the highest antioxidant enzyme activity.

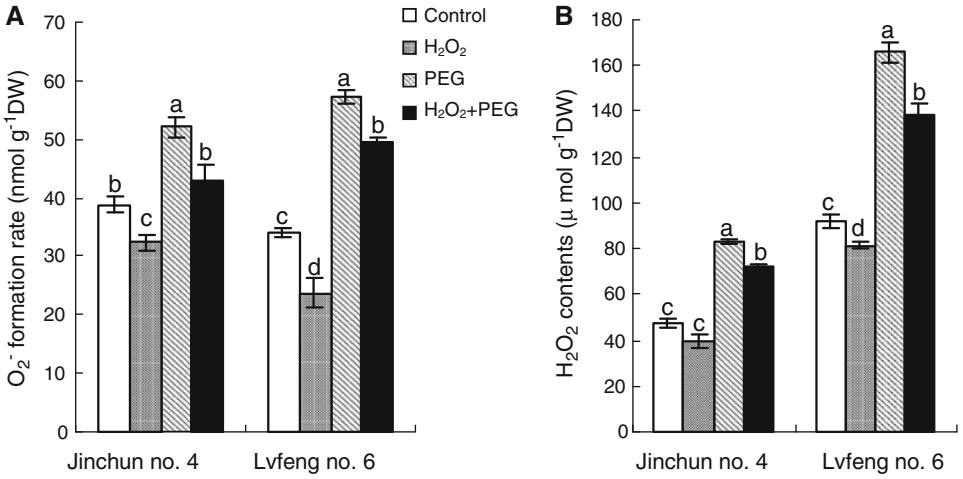

Fig. 4 Effects of exogenous H_2O_2 and PEG on the $O_2^{\bullet-}$ formation rate (**a**) and endogenous H_2O_2 levels (**b**) in cucumber leaves. Control, untreated; H_2O_2, pretreated with 1.5 mM H_2O_2; PEG, watered with 10% PEG; H_2O_2 + PEG, pretreated with 1.5 mM H_2O_2 and watered with 10% PEG. Samples represent values at 56 h after H_2O_2 pretreatment. *Bars* represent the standard error of three independent experiments. Values with the same letter are not significantly different at $P < 0.05$ according to the least significant difference (LSD) test

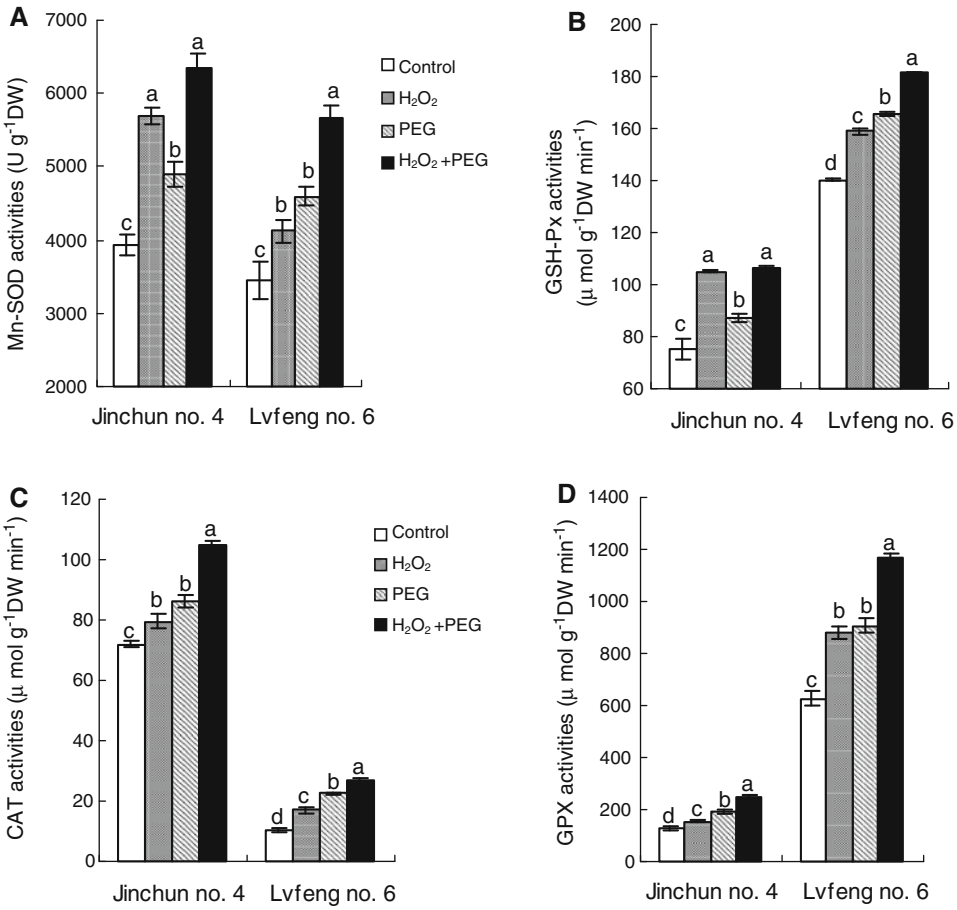

Fig. 5 Effects of exogenous H_2O_2 and PEG on the activities of Mn-SOD (**a**), GSH-Px (**b**), CAT (**c**), and GPX (**d**) in cucumber leaves. Control, untreated; H_2O_2, pretreated with 1.5 mM H_2O_2; PEG, watered with 10% PEG; H_2O_2 + PEG, pretreated with 1.5 mM H_2O_2 and watered with 10% PEG. Samples represent values at 56 h after H_2O_2 pretreatment. *Bars* represent the standard error of three independent experiments. Values with the same letter are not significantly different at $P < 0.05$ according to the least significant difference (LSD) test

Nonenzymatic Antioxidant Levels

In leaves from the H_2O_2 pretreatment group, some antioxidant indices such as the GSH content in both cucumber ecotypes and the ratio of GSH/GSH + GSSG in Jinchun no. 4 and AsA/AsA + dehydroascorbate in Lvfeng no. 6 were not elevated ($P > 0.05$) relative to the controls, whereas other indices, including the AsA content,

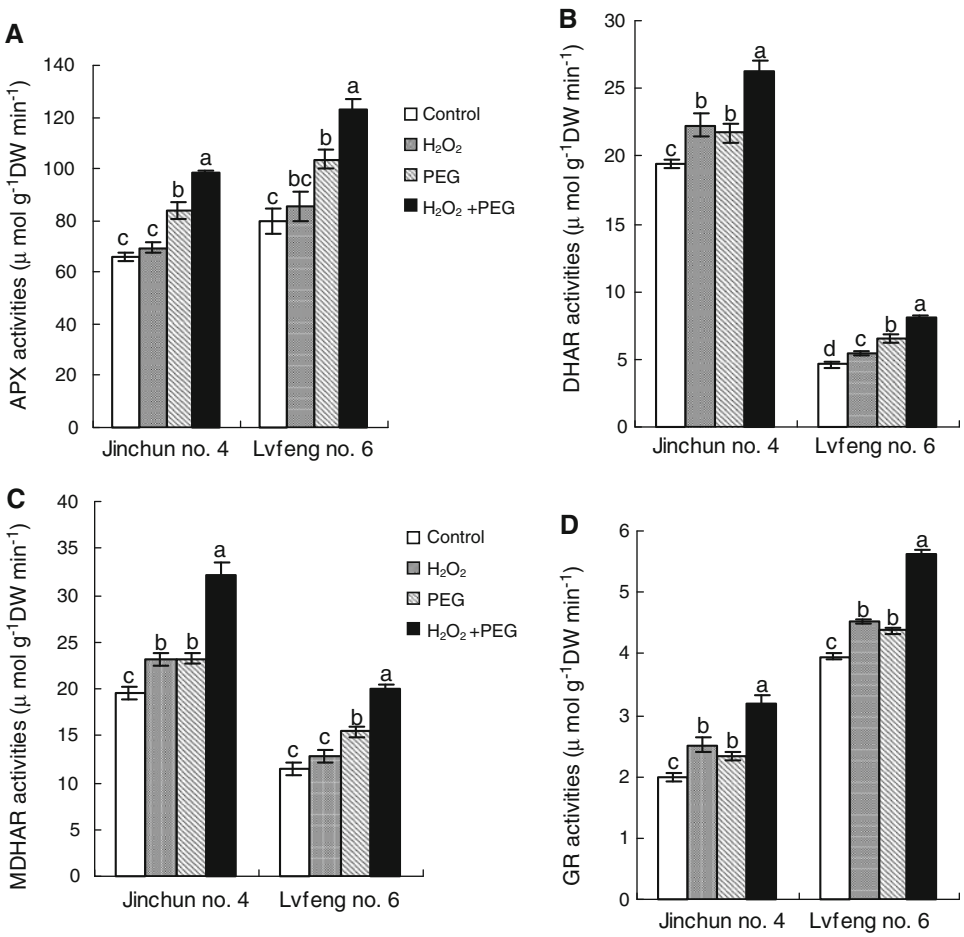

Fig. 6 Effects of exogenous H_2O_2 and PEG on the activities of APX (**a**), DHAR (**b**), MDHAR (**c**), and GR (**d**) in cucumber leaves. Control, untreated; H_2O_2, pretreated with 1.5 mM H_2O_2; PEG, watered with 10% PEG; H_2O_2 + PEG, pretreated with 1.5 mM H_2O_2 and watered with 10% PEG. Samples represent values at 56 h after H_2O_2 pretreatment. *Bars* represent the standard error of three independent experiments. Values with the same letter are not significantly different at $P < 0.05$ according to the least significant difference (LSD) test

GSH/GSH + GSSG in Lvfeng no. 6, and AsA/AsA + dehydroascorbate in Jinchun no. 4 were significantly ($P < 0.05$) enhanced (Fig. 7). When comparing PEG-treated plants to the controls, the levels of GSH and AsA and the ratios of GSH/GSH + GSSG and AsA/AsA + dehydroascorbate were enhanced ($P < 0.05$) in both cucumber ecotypes. Compared to PEG treatment alone, treatment with H_2O_2 + PEG significantly enhanced the antioxidant indices. Therefore, exogenous H_2O_2 and the osmotic stress induced by PEG significantly ($P < 0.05$) increased the levels of GSH and AsA and the ratios of GSH/GSH + GSSG and AsA/AsA + dehydroascorbate in leaves of Jinchun no. 4 and Lvfeng no. 6. However, the combined effect of osmotic stress and exogenous H_2O_2 resulted in the highest antioxidant indices.

Discussion

Cucumber ecotypes of North China and South China grow in different habitats, but they are both sensitive to water stress. In this study, the second leaves from 83.3% of Jinchun no. 4 seedlings and 87.5% of Lvfeng no. 6 seedlings were withered under osmotic stress.

Changes in Cell Ultrastructure

Chloroplasts and mitochondria are major ROS-generating sites under stress conditions (Salin 1991; Xu and others 2006). It has been demonstrated that the chloroplast membrane ruptures under saline or osmotic stress (García-Valenzuela and others 2005) and that the thylakoidal structure of the chloroplast is disrupted under conditions of salt stress (Hernández and others 1995) and drought (Palomäki and others 1994). In response to the PEG treatment used in this study, the shapes of some chloroplasts changed, their membranes and thylakoids were deliquescent, and the percentage of abnormal chloroplasts was 84.3% in Jinchun no. 4 and 88.6% in Lvfeng no. 6. Sucrose and trehalose help prevent membrane fusion in cells during stress (Crowe and others 1998). Pretreatment with exogenous H_2O_2 increased the percentage of normal chloroplasts and thylakoids in the PEG-stressed leaves of both cucumber ecotypes. This indicates that exogenous H_2O_2 protects the chloroplast ultrastructure under osmotic stress induced by PEG. Mäkelä and others (2000) have reported that the starch granules in tomato plants are smaller under drought stress but are larger with an application of exogenous glycinebetaine. In Jinchun no. 4, there were almost no

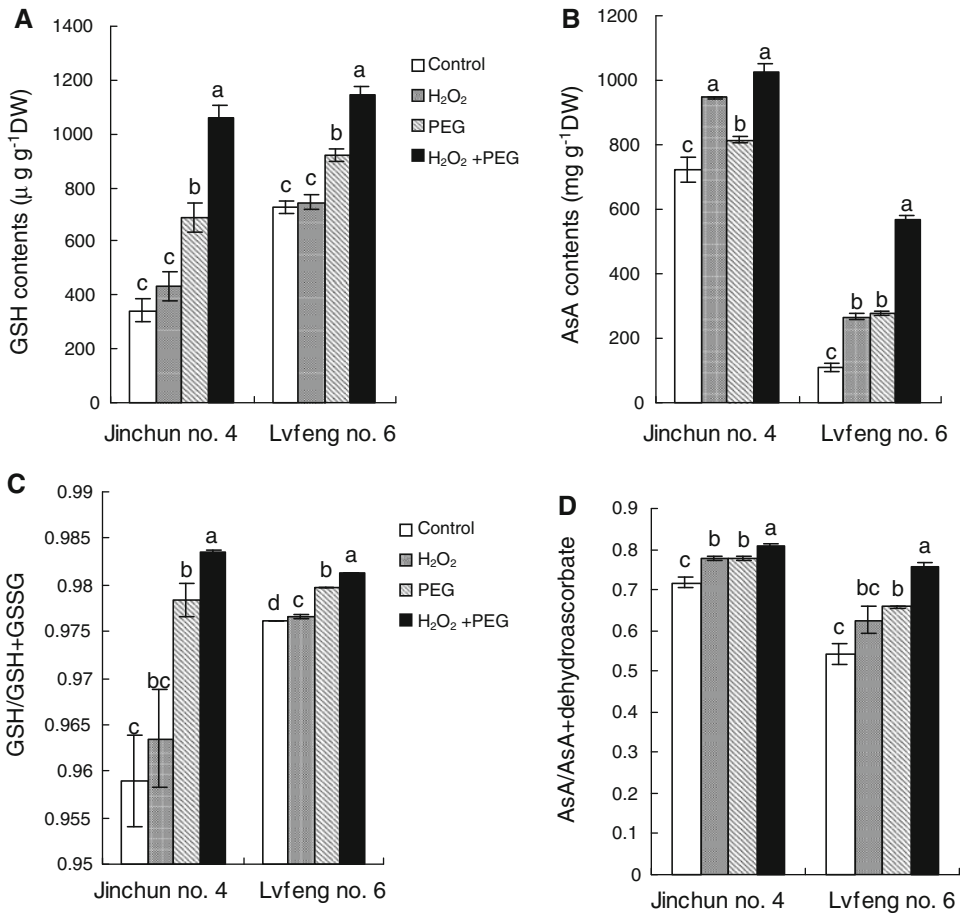

Fig. 7 Effects of exogenous H_2O_2 and PEG on GSH (**a**) and AsA (**b**) levels and on the GSH/GSH+GSSG (**c**) and AsA/AsA + dehydroascorbate ratios (**d**) in cucumber leaves. Control, untreated; H_2O_2, pretreated with 1.5 mM H_2O_2; PEG, watered with 10% PEG; H_2O_2 + PEG, pretreated with 1.5 mM H_2O_2 and watered with 10% PEG. Samples represent values at 56 h after H_2O_2 pretreatment. *Bars* represent the standard error of three independent experiments. Values with the same letter are not significantly different at $P < 0.05$ according to the least significant difference (LSD) test

starch particles in the PEG treatment group, whereas exogenous H_2O_2 made the particles appear. However, no starch particles were found in any of the Lvfeng no. 6 treatment groups. These results demonstrate the different photosynthetic activities of the two cucumber ecotypes. The integrity of the mitochondrial membrane is improved by exogenous selenium under salt stress (Kong and others 2005). In our study, the membranes of 78.5% of mitochondria from Jinchun no. 4 and 87.1% of mitochondria from Lvfeng no. 6 appeared to be disintegrated by PEG treatment, whereas pretreatment with exogenous H_2O_2 improved the membranes. H_2O_2 seems to protect the mitochondrial ultrastructure. Therefore, even though the starch granules are different between Jinchun no. 4 and Lvfeng no. 6, osmotic stress damages the membranes of chloroplasts and mitochondria in both cucumber ecotypes, whereas exogenous H_2O_2 may have a protective effect.

MDA and ROS Levels

Membrane destabilization is generally attributed to lipid peroxidation (Elstner 1982), which ultimately gives rise to the compound MDA (Wang and others 2004). Therefore, MDA levels are related to the extent of damage to the ultrastructure (Sairam and others 2002). It has been demonstrated that PEG results in elevated MDA levels (Chang and Kao 1997). Exogenous uniconazole (Zhang and others 2007) lowers the MDA content and separately improves the tolerance of plants to drought and NaCl stress. Applying brassinolide reduces MDA content under drought stress (Zhang and others 2008). In our study, PEG increased the MDA levels in Jinchun no. 4 and Lvfeng no. 6. In plants treated with H_2O_2 + PEG the MDA content was lower than in plants treated with PEG alone but was still higher than in the controls or after H_2O_2 pretreatment. These results are consistent with the difference in ultrastructure of the two ecotypes and indicate that pretreatment with exogenous H_2O_2 does not entirely eliminate the lipid peroxidation induced by PEG; however, it alleviates the damage to the ultrastructure of membranes under osmotic stress to some extent.

Environmental stresses induce the overproduction of ROS such as $O_2^{\bullet -}$ and H_2O_2, and this can increase the MDA content (Smirnoff 1993). Pretreatment with 2-aminoethanol results in a correlation between low levels of destroyed membranes and the accumulation of low concentrations of ROS, and it improves the tolerance of barley to drought (Mascher and others 2005). In our study, the osmotic stress

induced by PEG increased the formation rate of $O_2^{\bullet-}$ and the level of endogenous H_2O_2, whereas exogenous H_2O_2 decreased the levels of these two molecules. These results coincide with the changes in MDA levels, suggesting that exogenous H_2O_2 decreases the accumulation of $O_2^{\bullet-}$ and H_2O_2 in cucumber leaves, thereby reducing lipid peroxidation and protecting ultrastructure.

Antioxidant System

To control the concentration of ROS and protect the ultrastructure of membranes, cells express a network of antioxidants and antioxidative enzymes (Foyer and Noctor 2000). The enzyme SOD dismutates $O_2^{\bullet-}$ into H_2O_2 (Fridovich 1975). SODs are classified into manganese SOD (Mn-SOD), iron SOD (Fe-SOD), and copper/zinc SOD (CuZn-SOD), and the last two of these are inhibited by H_2O_2 (Vyas and Kumar 2005). According to our preliminary study, the activities of Fe-SOD and CuZn-SOD were not enhanced by H_2O_2 pretreatment. Therefore, we investigated Mn-SOD to understand the inductive effects of exogenous H_2O_2. Mn-SOD is located mainly in the mitochondria (Babitha and others 2002) and NaCl induces its activity (Hernández and others 1993). In our study, PEG treatment increased Mn-SOD activity compared to control; this may be a general adaptive defense of plants to stress environments (Liang and others 2003). However, the level of $O_2^{\bullet-}$ in PEG treatment was higher than that in the other three treatments, indicating that the generation of $O_2^{\bullet-}$ exceeds the capacity of SOD to eliminate $O_2^{\bullet-}$. Compared to the control, the Mn-SOD activity was increased in H_2O_2-pretreatment leaves. When H_2O_2 pretreatment was combined with PEG, Mn-SOD in the two cucumber ecotypes could be induced by H_2O_2 and PEG, so the enzyme activity was highest. There is an inverse relationship between Mn-SOD activity in cucumber leaves and the formation rate of $O_2^{\bullet-}$. Therefore, pretreatment with H_2O_2 increases the ability of cucumber leaves to dismutate $O_2^{\bullet-}$ via Mn-SOD under osmotic stress.

Endogenous H_2O_2 can rapidly diffuse across membranes and is toxic (Foyer and others 1997). To scavenge this molecule, plants have evolved an antioxidant system that includes GSH-Px, CAT, GPX, and APX. Chloroplasts contain GSH-Px, which is important for eliminating H_2O_2 (Djanaguiraman and others 2005). Increased GSH-Px activity has been detected in plants under salt stress (Ben-Hayyim and others 1993). The enzyme CAT eliminates H_2O_2 in the mitochondrion and microbody (Shigeoka and others 2002). Drought stress increases its activity in sorghum plants (Zhang and Kirkham 1996). Increased CAT activity has also been reported in salt-stressed *Catharanthus roseus* (Jaleel and others 2007). Treatment with propiconazole induces a significant increase in CAT activity in stressed plants (Manivannan and others 2007). The enzyme GPX may act in the apoplast, chloroplast, and cytosol (Shigeoka and others 2002). A prominent increase in GPX activity is related to the ability of plantlets to tolerate drought stress (Moran and others 1994). GPX activity is elevated by PEG treatment (Turkan and others 2005) and is enhanced significantly by foliar spraying with Spd or Spm under $CdCl_2$ stress (Zhao and Yang 2008). APX, a primary enzyme of the ascorbate-glutathione cycle, suppresses the accumulation of H_2O_2 in the chloroplasts, cytosol, peroxisomes, mitochondria, and apoplastic space (Jiménez and others 1997; Yoshimura and others 2000; Diaz-Vivancos and others 2006) by converting AsA to dehydroascorbate. This enzyme's activity was enhanced by drought stress (Mittler and Zilinskas 1994; Liu and others 2009), and the expression of APX can be induced by H_2O_2 (Hernández and others 2004). After treatment with silicon, there is an increase in APX activity in salt-stressed cucumbers (Zhu and others 2004). Propiconazole increased APX activity in *Vigna* plants under drought stress (Manivannan and others 2007). Jinchun no. 4 and Lvfeng no.6 belong to different cucumber ecotypes so their GSH-Px, CAT, GPX, and APX activities are significantly different under osmotic stress. Also, H_2O_2 pretreatment did not increase the CAT activity of Jinchun no. 4 relative to controls. Compared to controls, cucumbers display a general adaptive defense against PEG treatment that involves the induction of the enzymes GSH-Px, CAT, GPX, and APX. Nevertheless, the level of endogenous H_2O_2 was the highest in PEG-treated plants, indicating that the generation of endogenous H_2O_2 exceeds the capacity of the cellular antioxidant defense system to eliminate H_2O_2. The results of the ANOVA showed that exogenous H_2O_2 increased the activities of CAT, GPX, GSH-Px, and APX in leaves from both cucumber ecotypes. The activities of the four enzymes after H_2O_2 + PEG treatment were induced by H_2O_2 and PEG and therefore were higher than after PEG treatment alone. In the H_2O_2-pretreated stressed plants, the higher activities of CAT, GPX, GSH-Px, and APX coincide with a decrease in the levels of endogenous H_2O_2, suggesting that pretreatment with exogenous H_2O_2 increases the ability of cucumber leaves to scavenge endogenous H_2O_2 via CAT, GPX, GSH-Px, and APX under osmotic stress.

The enzyme APX acts by utilizing AsA as the electron donor in the ascorbate-glutathione cycle. The regeneration of AsA relies on NADH-dependent MDHAR or GSH-dependent DHAR being coupled with GR (a key enzyme in the GSH regeneration cycle) (Luster and Donaldson 1987; Bowdith and Donaldson 1990). Under drought stress, the activities of DHAR, MDHAR, and GR are elevated in sorghum plants (Zhang and Kirkham 1996). PEG increases GR activity (Turkan and others 2005). In tobacco plants treated with paraquat, the activities of DHAR and GR are enhanced (Miyagawa and others 2000). In the current

experiment, Jinchun no. 4 and Lvfeng no. 6 have different growth habitats, and the activities of DHAR, MDHAR, and GR under PEG treatment in the two ecotypes are different. However, the combination of exogenous H_2O_2 and osmotic stress resulted in the highest DHAR, MDHAR, and GR activities in leaves of both cucumber ecotypes. This combination also led to the highest levels of AsA and GSH and the highest GSH/GSH + GSSG and AsA/AsA + dehydroascorbate ratios, indicating that AsA and GSH can be regenerated well when exogenous H_2O_2 is combined with osmotic stress.

AsA and GSH not only act as substrates in the ascorbate-glutathione cycle, they also detoxify ROS (Xiong and Zhu 2002; Kumar and others 2003; Wang and others 2003). The beneficial effects of AsA in mitigating drought stress may be the activation of certain enzymatic reactions (Kefeli 1981). AsA can directly scavenge reactive oxygen radicals, thus providing membrane protection (Thomas and others 1992). Fletcher and others (2000) reported that the protective effect of triazole against stress is mediated by an increase in AsA levels. GSH takes part in the control of H_2O_2 levels (Alscher and others 1997) and has been shown to regulate the expression of genes whose products are involved in redox regulation and enhancement of stress tolerance (Noctor and others 2002). An increase in GSH levels is induced by paclobutrazol in *Catharanthus* plants under salt stress (Jaleel and others 2007). As a general adaptive defense of plants to stress environments (Liang and others 2003), the levels of GSH and AsA in leaves of Jinchun no. 4 and Lvfeng no. 6 were higher in PEG treatment than those in controls. Meanwhile, the levels of $O_2^{\bullet-}$ and endogenous H_2O_2 were the highest in PEG treatment. Exogenous H_2O_2 increased the levels of GSH and AsA in our study. In plants treated with H_2O_2 + PEG, the levels of AsA and GSH were induced by H_2O_2 and PEG and thereby were the highest. In H_2O_2-pretreated stressed plants, the higher levels of AsA and GSH coincide with the decrease in levels of $O_2^{\bullet-}$, endogenous H_2O_2, and MDA, suggesting that exogenous H_2O_2 can increase the ability of cucumber leaves to eliminate ROS via the higher contents of GSH and AsA under osmotic stress.

In conclusion, the starch particles and the activities of antioxidants such as CAT, DHAR, and GR were different in Jinchun no. 4 compared with Lvfeng no. 6 when the H_2O_2 pretreatment group was compared to the control group. However, in both cucumber ecotypes, the shapes of most of the chloroplasts and their thylakoid structures were significantly affected under osmotic stress induced by PEG treatment, and the membranes of most of the chloroplasts and mitochondria were indistinct. When H_2O_2 pretreatment was combined with osmotic stress, most of the investigated ultrastructures were similar to those of the controls in leaves of both cucumber ecotypes, and the levels of ROS and MDA were reduced. PEG increased the activity of antioxidative enzymes such as Mn-SOD, GSH-Px, CAT, GPX, APX, MDHAR, DHAR, and GR and the antioxidants AsA and GSH. However, H_2O_2 + PEG treatment increased their activities even more. Exogenous H_2O_2 can induce tolerance to osmotic stress in cucumber leaves by increasing antioxidant activity and decreasing lipid peroxidation to some extent, and thereby protecting the ultrastructure of most membranes.

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