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ORIGINAL PAPER

Paraquat pre-treatment increases activities of antioxidant enzymes and reduces lipid peroxidation in salt-stressed cucumber leaves

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Abstract To investigate whether paraquat (PQ) is involved in regulation of antioxidant enzymes and lipid peroxidation under short-term salt stress, and to elucidate the physiological mechanism of salt stress mitigated by PQ, a cucumber cultivar (cv. Chunguang no. 2) was exposed to 100 mM NaCl for 48 h after pre-treatment with 10 μM PQ for 1 h. When compared to the control, salt stress increased the levels of malonaldehyde (MDA), superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) and the activities of antioxidant enzymes, such as superoxide dismutase (EC 1.15.1.1), ascorbate peroxidase (EC 1.11.1.11) and glutathione reductase (EC 1.6.4.2) in the cucumber leaves. Under salt conditions, PQ pre-treatment prevented oxidative stress as observed by the decreases in MDA, H_2O_2 and $O_2^{\cdot-}$ that correlated with the increase in antioxidant defenses. We propose that, at low concentrations, the PQ pre-treatment can reduce the salt-induced oxidative damage by increasing the antioxidative mechanisms in cucumber plants.

Keywords Antioxidant · Cucumber · Malonaldehyde · Paraquat · Salt stress

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Introduction

In some regions of the world, salinity is one of the most important environmental hazards (Pitman and Läuchli 2002), which not only affects the photosynthesis process in plants (Parida and Das 2005), but also induces the generation of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (Hernández et al. 2000). The accumulation of ROS damages lipids, proteins, nucleic acids and carbohydrates (Rodriguez and Redman 2005) and will trigger apoptosis (Fath et al. 2001). To alleviate the effects of ROS, plants have evolved an antioxidant system, which can be generally categorized into enzymatic and non-enzymatic types (Xie et al. 2008). Enzymatic antioxidants include superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (GPX, EC 1.11.1.7) (Foyer et al. 1994), glutathione peroxidase (GSH-Px, EC 1.11.1.9) (Xue et al. 2001), ascorbate peroxidase (APX, EC 1.11.1.11), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2) (Asada 1992). The commonly known non-enzymatic antioxidants are the reduced glutathione (GSH) and ascorbate (AsA) (Kumar and Knowles 1993).

Paraquat (PQ) is a type of bipyridinium herbicide. It can be photoreduced by photosystem I and then is re-oxidized by transferring electrons to O_2 under light, resulting in the generation of $O_2^{\cdot-}$ (Asada and Takahashi 1987; Foyer et al. 1994). Concentrations of PQ above 2 mM cause significant damage to the photosystems (Chagas et al. 2008). In PQ treated plants, there are significant decreases in water contents and protein levels (Iturbe-Ormataexe et al. 1998). However, PQ usually induces antioxidant enzymes in leaves (Donahue et al. 1997; Casano et al. 1999; Ekmekci

and Terzioglu 2005). By enhancing the antioxidant activities, pre-treatment with PQ was shown to increase the tolerance of cucumber leaves to polyethylene glycol (PEG)-induced drought stress (Liu et al. 2009). Drought and salinity both induce osmotic stress in plants (Soria et al. 2006). However, seedlings do not absorb the PEG molecules (Carpita et al. 1979), while salinity should have an ion-specific effect (Chen et al. 2002). Therefore, salinity is a different stress in comparison to PEG-induced drought. To date, no effort has been undertaken to describe the impact of PQ pre-treatment on the antioxidant system, which may reveal the free-radical mechanism of PQ action on plant cells under salt stress.

Cucumber is an important horticultural crop and is highly sensitive to salinity during the seedling stage. In this study, its seedlings were pre-treated with PQ and then exposed to short-term salt stress. Our aim was to investigate whether PQ is involved in regulation of antioxidant enzymes and lipid peroxidation under short-time salt stress and, thereby to elucidate the physiological mechanism of salt stress mitigated by PQ in cucumber plants.

Materials and methods

Plant material and treatments

Cucumber seeds (*Cucumis sativus* cv. Chunguang no. 2) were germinated on moist gauze at 25°C for 2 days and then were planted into 14-cm plastic pots filled with sand. The cucumber seedlings were grown at 25°C with the photoperiod of 12 h light (600 μmol m^{-2} s^{-1})/12 h darkness and were watered once per day with the 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. At the two-leaf stage, cucumber seedlings were selected for treatments. In preliminary experiments, the cucumbers were pre-treated with three concentrations (5, 10 and 15 μM) of PQ in light (100 μmol m^{-2} s^{-1}) for three different pre-treatment times (0.5, 1 and 2 h), and then, they were rinsed 12 times with the Hoagland nutrient solution and were kept in darkness for 24 h. After that the plants were transferred into the Hoagland nutrient solution containing 100 mM NaCl for 48 h. We found that pre-treatment with 10 μM PQ for 1 h resulted in the lower levels of MDA, $O_2^{\cdot-}$ and H_2O_2 under salt stress, and thereby was closer to the optimum combination to acclimate the seedlings. Thus, two groups of cucumber plants were pre-treated with 10 μM PQ or H_2O for 1 h in light intensity of 100 μmol m^{-2} s^{-1}. All plants were successively eluted 12 times with the Hoagland nutrient solution and were put into darkness for 24 h. To study the short-term salt effects, the cucumbers pre-treated with 10 μM PQ were then separately watered with the Hoagland nutrient solution (PQ pre-treatment) or Hoagland nutrient solution containing 100 mM NaCl (PQ + salt treatment) for 48 h. Other seedlings, which were pre-treated with H_2O, were watered for 48 h with either of the two types of nutrient solutions as indicated above, and were separately, designated as the control and salt treatment groups. Every plant was maintained at 25°C with 12 h light (600 μmol m^{-2} s^{-1})/12 h dark cycles and was watered twice per day. At 0 h before PQ pre-treatment and 25 and 73 h after PQ pre-treatment, samples of the second leaf were harvested at the end of dark period. At 1 h after PQ pre-treatment, the leaves were collected at the end of light period. Then, leaf samples were ground with liquid nitrogen for the determination of antioxidant parameters. Three different sets of plants grown at different times were used for the experiment.

Determination of malonaldehyde (MDA) content

The content of MDA was measured according to the method of Dhindsa et al. (1981). Leaf samples (0.2 g) were homogenized in 4 mL of 10% trichloroacetic acid and then centrifuged at 10,000×*g* for 15 min. The supernatant (1 mL) was mixed with 1 mL of 0.6% thiobarbituric acid and then heated at 95°C for 30 min. The absorbances were determined at 450, 532 and 600 nm, and the MDA content was estimated by the formula C (μM) = 6.45 $(A_{532} - A_{600}) - 0.56\, A_{450}$ (Tang et al. 2010).

Assay of the formation rate of $O_2^{\cdot-}$

The formation rate of $O_2^{\cdot-}$ was determined according to the method of Elstner and Heupel (1976). Leaves (0.3 g) were ground in liquid nitrogen, extracted in 3 mL of 65 mM phosphate buffer (pH 7.8) and then centrifuged at 4°C and 5,000×*g* for 10 min. The supernatants (0.5 mL) were added to 0.4 mL of 65 mM phosphate buffer (pH 7.8) and 0.1 mL of 10 mM hydroxylammoniumchloride. The mixture was incubated at 25°C for 20 min. Then, the reaction mixture was added to 1 mL of 58 mM sulfanilamide and 1 mL of 7 mM α-naphthylamine. After 20 min, 2 mL of chloroform was added, and the mixture was centrifuged at 10,000×*g* and 4°C for 3 min. The absorbance of the supernatant was measured at 530 nm and the $O_2^{\cdot-}$ concentration was calculated from a standard curve of $NaNO_2$ in the concentration range of 0–16 μM.

Determination of H_2O_2 content

After being ground in liquid nitrogen, the leaves (0.3 g) were homogenized with 1 mL of 100 mM sodium

phosphate buffer (pH 6.8) and then centrifuged at 4°C and 18,000×*g* for 20 min. Then, 0.5 mL of H_2O_2 extraction was mixed with 2.5 mL of peroxide reagent [consisting of 83 mM sodium phosphate (pH 7.0), 0.005% *o*-dianisidine and 1 mM peroxidase] and was incubated at 30°C for 10 min. The reaction was subsequently stopped by adding 0.5 mL of 1 M perchloric acid (Bernt and Bergmeyer 1974). The absorbance was measured at 436 nm, and the H_2O_2 contents of the leaves were calculated from a standard curve of H_2O_2 reagent in the concentration range of 0–500 μM.

Determination of antioxidant enzyme activities

The ground leaves (0.5 g) were suspended in 3 mL of ice-cold HEPES buffer (25 mM, pH 7.8) which contained 0.2 mM EDTA and 2% PVP. The homogenate was centrifuged at 4°C and 12,000×*g* for 20 min, and the resultant supernatants were used for the determination of SOD, CAT, GPX, MDHAR, DHAR and GR (Ramiro et al. 2006). To extract GSH-Px, the leaves (0.3 g) were ground with liquid nitrogen and was suspended in 0.3 mL of HEPES buffer (25 mM, pH 7.8) containing 0.2 mM EDTA and 2% PVP, while the HEPES buffer (25 mM, pH 7.8) containing 0.2 mM EDTA, 2% PVP and 2 mM AsA was used for APX extraction.

SOD activity was assayed based on the reduction in nitroblue tetrazolium (NBT) (Hwang et al. 1999). The reaction mixture (3 mL) was composed of 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 1.5 μM riboflavin and 20 μL of enzyme extract. Test tubes containing the reaction solution were irradiated with light of 40 μmol m^{-2} s^{-1} for 10 min. The absorbance of the irradiated and non-irradiated solutions was determined at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to cause a 50% inhibition of NBT reduction.

The activity of CAT was calculated by the disappearance of H_2O_2 ($\varepsilon = 40$ M^{-1} cm^{-1}) at 240 nm (Pereira et al. 2002). The reaction mixture contained 100 mM phosphate buffer (pH 7.8), 10 mM H_2O_2 and 50 μL of enzyme extract.

GPX activity was determined at 470 nm by following the change in absorption due to guaiacol oxidation ($\varepsilon = 6.39$ mM^{-1} cm^{-1}) (Ramiro et al. 2006). The reaction solution contained 100 mM phosphate buffer (pH 7.0), 0.05% guaiacol, 15 mM H_2O_2 and 50 μL of enzyme extract.

APX activity was determined according to the method of Zhu et al. (2004) and the H_2O_2-dependent oxidation of AsA ($\varepsilon = 2.8$ mM^{-1} cm^{-1}) was followed by a decrease in the absorbance at 290 nm. The reaction mixture contained 25 mM phosphate buffer (pH 7.0), 1 mM H_2O_2, 0.1 mM EDTA, 0.25 mM ascorbate and 50 μL of enzyme extract.

DHAR activity was measured at 265 nm by the formation of AsA ($\varepsilon = 14$ mM^{-1} cm^{-1}) in 1 min (Doulis et al. 1997). The reaction mixture contained 25 mM phosphate buffer (pH 7.0), 3.5 mM GSH, 0.1 mM EDTA, 0.4 mM dehydroascorbate and 50 μL of enzyme extract and the reactive blank was composed of 25 mM phosphate buffer (pH 7.0), 3.5 mM GSH, 0.1 mM EDTA and 0.4 mM dehydroascorbate.

MDHAR activity was assayed by monitoring the change in the absorbance at 340 nm due to NADPH oxidation ($\varepsilon = 6.2$ mM^{-1} cm^{-1}) for 1 min (Hoque et al. 2007). 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.

GR activity was assayed by following the decrease in absorbance at 340 nm due to NADPH oxidation ($\varepsilon = 6.2$ mM^{-1} cm^{-1}) for 1 min (Foyer and Halliwell 1976). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.5 mM oxidized glutathione (GSSG), 0.15 mM NADPH, 5 mM $MgCl_2$ and 50 μL of enzyme extract.

GSH-Px activity was measured according to the method of Xue et al. (2001). The mixture of 40 μL of enzyme extract and 40 μL of 1 mM GSH was used for the enzymatic reaction. For the non-enzymatic reaction, 40 μL of enzyme extract was boiled for 25 min and was then mixed with 40 μL of 1 mM GSH. The two mixtures were heated at 37°C for 5 min. Then, 20 μL of 1.5 mM H_2O_2 was added to initiate the reaction. The reaction was run for 3 min and was terminated by the addition of 400 μL of 1.67% metaphosphoric acid (containing 0.05% EDTA and 28% NaCl). After centrifugation at 2,000×*g* for 10 min, 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 and then measured at 412 nm. The GSH-Px activity was calculated as the decrease in GSH level in the enzymatic reaction when compared with the non-enzymatic reaction.

Determination of protein content

To define the activities of antioxidant enzymes, the protein contents of the enzyme extracts were determined using the Bradford method (1976).

Determination of non-enzymatic antioxidant contents

After being ground with liquid nitrogen, the leaves (0.4 g) were suspended in 2 mL of 6% trichloroacetic acid and centrifuged at 4°C and 15,000×*g* for 20 min. The supernatant was used for the determination of AsA and total

ascorbate (T-AsA). The contents of AsA and T-AsA were measured at 525 nm according to the methods of Kampfenkel et al. (1995) and calculated from a standard curve of ascorbate. Oxidized ascorbate was estimated from the difference between T-AsA and AsA.

The levels of total glutathione and oxidized glutathione (GSSG) were measured using the GSH and GSSG assay kit (Beyotime Institute of Biotechnology, China), and GSH content was estimated from the difference between total glutathione and GSSG.

Statistics

The levels of antioxidants were assayed with three replicates, and data were expressed as mean ± standard errors. Differences were analyzed using one-way ANOVA and Tukey's test (SSPS 13.0 for Windows). $P < 0.05$ were considered to be statistically significant.

Results and discussion

MDA contents

Malonaldehyde is produced during the peroxidation of membrane lipids and is often used as an indicator of stress damage (Ohkawa et al. 1979). In the current experiment, cucumber seedlings were cultured in flexible pots and selected carefully before treatment. Because the selection process might have influenced the plants, the MDA level at 0 h was the highest during treatment (Fig. 1). PQ treatment causes a significant increase in MDA content of wild wheat (Ekmekci and Terzioglu 2005). In our study, half of the cucumber seedlings were pre-treated with PQ for 1 h, and

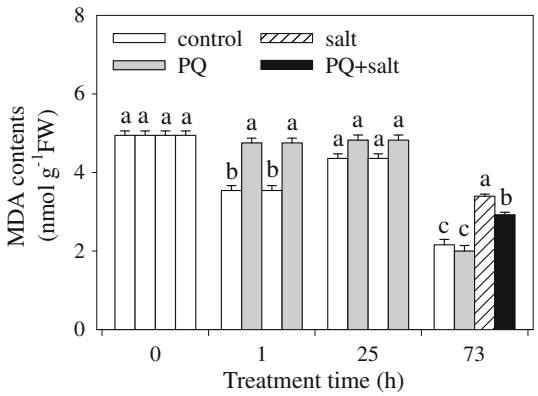

Fig. 1 Effects of PQ and salt on MDA contents in cucumber leaves. Control, untreated; PQ, pre-treated with 10 μM PQ; salt, watered with 100 mM salt; PQ + salt, pre-treated with 10 μM PQ and watered with 100 mM salt. *Bars* represent standard errors. *Different small letters* denote significant differences among the treatment groups at a treatment time by Tukey's test at $P < 0.05$

the MDA content in leaves of the PQ pre-treatment group was higher ($P < 0.01$) than that in the control group. At 25 h after the start of the experiment, the PQ had been rinsed-off the plants for 24 h, so the MDA levels of the PQ pre-treatment and the control groups were not significantly different ($P > 0.05$). The treatment with salt has been shown to enhance the MDA contents in tomato leaves (Mittova et al. 2004). Meanwhile, pre-treatment with proline and glycine betaine (Banu et al. 2009) lowers the MDA levels and improves the tolerance of plants to NaCl stress. At 73 h, the PQ-pretreated seedlings were transferred to salt conditions for 48 h, and we found that the salt treatment resulted in the highest contents of MDA ($P < 0.05$). In the PQ + salt treatment group, the MDA content was decreased by 13.9% ($P < 0.05$) than that in the salt treatment group and was still higher ($P < 0.01$) than those in the control and PQ pre-treatment groups. Cucumber seedlings, which were cultured in flexible pots, would be influenced during selection process; however, the selecting equally affected the plants in different treatment groups at the same treatment time and would not interfere with the difference trends of MDA content among the four treatment groups at 73 h. At 0, 1, 25 and 73 h, plants in the control groups were subjected to different experimental conditions and were in dissimilar physiological states, so their MDA contents varied greatly; nevertheless, when PQ-pretreated or PQ-untreated cucumber seedlings were watered with salt or not, the leaf samples of four treatment groups at 73 h were all harvested at the end of dark period, and all plants in the four groups were kept under the same experimental conditions except for the watered solutions. Therefore, the differences of MDA contents among the four treatment groups at 73 h should be the affection of salt stress and PQ treatment. According to the ANOVA results, salt significantly ($P < 0.01$) increased the MDA content in cucumber leaves, while PQ decreased ($P < 0.05$) the MDA level. Liu et al. (2009) transferred cucumber seedlings into PEG conditions after PQ pre-treatment and found similar changes in MDA contents. The results of our study indicated that salt induced oxidative stress, but PQ pre-treatment decreased the lipid peroxidation to some extent under short-term salt stress.

Formation rate of $O_2^{\cdot-}$ and contents of H_2O_2

Stress conditions induce the overproduction of ROS that can damage lipid membranes and increase MDA levels (Smirnoff 1993). In the study, the careful selection of cucumber seedlings before treatment perhaps influenced the plants and caused the levels of $O_2^{\cdot-}$ (Fig. 2a) and H_2O_2 (Fig. 2b) at 0 h to be higher during treatment. Under light, PQ generates $O_2^{\cdot-}$ and gives plants a stress (Ananieva et al. 2004). On the other hand, since the SOD activity in the PQ

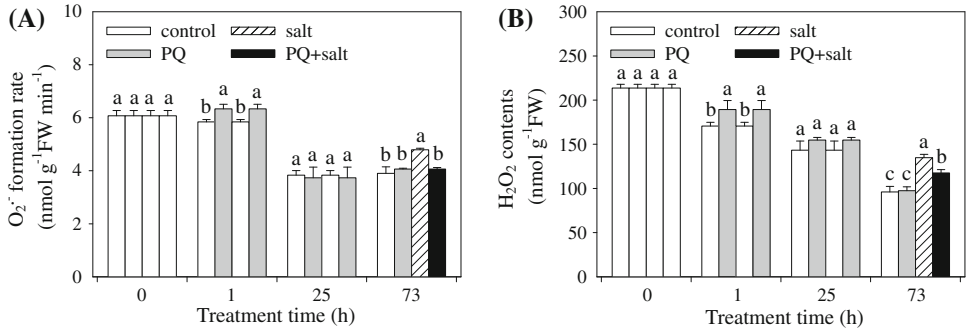

Fig. 2 Effects of PQ and salt on $O_2^{\cdot-}$ formation rate (**a**) and H_2O_2 contents (**b**) in cucumber leaves. Control, untreated; PQ, pre-treated with 10 μM PQ; salt, watered with 100 mM salt; PQ + salt, pre-treated with 10 μM PQ and watered with 100 mM salt. *Bars* represent standard errors. *Different small letters* denote significant differences among the treatment groups at a treatment time by the Tukey's test at $P < 0.05$

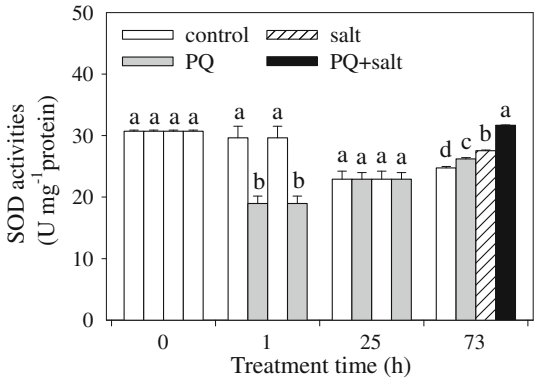

Fig. 3 Effects of PQ and salt on SOD activities in cucumber leaves. Control, untreated; PQ, pre-treated with 10 μM PQ; salt, watered with 100 mM salt; PQ + salt, pre-treated with 10 μM PQ and watered with 100 mM salt. *Bars* represent standard errors. *Different small letters* denote significant differences among the treatment groups at a treatment time by the Tukey's test at $P < 0.05$

pre-treatment at 1 h was lower ($P < 0.01$) than the control (Fig. 3), the enzyme could not dismutate $O_2^{\cdot-}$ into more H_2O_2. However, stress will enhance H_2O_2 generation via many other sources such as plasma-membrane-localized NADPH oxidases and cell wall peroxidases (Neill et al. 2002). Therefore, at 1 h in our study, not only the level of $O_2^{\cdot-}$ but also the content of H_2O_2 were higher ($P < 0.05$) in the seedlings pre-treated with PQ than those in the control. At 25 h after the start of the experiment, which was 24 h after PQ has been rinsed-off the plants, the levels of the two ROS in the PQ pre-treatment group were not significantly different ($P > 0.05$) from those in the control. It was previously shown that salt increases the rate of $O_2^{\cdot-}$ production and the level of H_2O_2 (Møller 2001), and the application of exogenous sodium nitroprusside reduces the accumulation of H_2O_2 in cucumber roots under NaCl conditions (Shi et al. 2007). As shown in Fig. 2, after the PQ pre-treated seedlings were transferred into salt conditions for 48 h, not only the formation rate of $O_2^{\cdot-}$ but also the content of H_2O_2 was the highest ($P < 0.05$) in the salt treatment group at 73 h. When compared with salt treatment, the levels of $O_2^{\cdot-}$ and H_2O_2 were separately decreased by 15.1 and 13.7% ($P < 0.05$) in the PQ + salt treatment group. Cucumber seedlings were cultured in flexible pots and would be influenced during selection process; however, the selecting equally affected the plants in different treatment groups at the same treatment time and would not interfere with the difference trends of $O_2^{\cdot-}$ and H_2O_2 levels among four treatment groups at 73 h. At treatment time of 0, 1, 25 and 73 h, plants in the control groups were subjected to different experimental conditions and were in dissimilar physiological states, so their levels of $O_2^{\cdot-}$ and H_2O_2 varied greatly; nevertheless, when PQ-pretreated or PQ-untreated cucumber seedlings were watered with salt or not, the leaf samples of four treatment groups at 73 h were all harvested at the end of dark period, and all plants in the four groups were kept under the same experimental conditions except for the watered solutions. Therefore, the differences in $O_2^{\cdot-}$ and H_2O_2 levels among the four treatment groups at 73 h should be the affection of salt stress and PQ treatment. The results of $O_2^{\cdot-}$ and H_2O_2 were consistent with the change in MDA contents at 73 h suggesting that pre-treatment with PQ decreased the lipid peroxidation by reducing the levels of ROS under short-term salt stress.

Antioxidative metabolism

The tolerance of plants to ROS requires the adaptation of many complex and multifaceted processes. For example, ROS-scavenging enzymes and antioxidant molecules in plants prevent or alleviate the damage from $O_2^{\cdot-}$ and H_2O_2 (Taşgin et al. 2006). Liu et al. (2009) cultured cucumber seedlings in hard pots, but we used flexible pots. When we selected cucumber seedlings for treatments, the selection process likely influenced the plants. Thus, at 0 h, not only

the ROS levels, but also the antioxidant activities changed significantly in the study in comparison to the controls at 25 and 73 h. The enzyme SOD catalyzes the conversion of $O_2^{\cdot-}$ to H_2O_2 and O_2 in the chloroplast, mitochondrion, cytoplasm and peroxisome (Vyas and Kumar 2005). Salt tolerance is directly related to the increase in SOD activity (Hernández et al. 2000). At 73 h, salt treatment significantly induced the SOD activity to higher levels than in the controls ($P < 0.001$) suggesting that this may be a general adaptive defense of plants to stress environments (Liang et al. 2003). However, the level of $O_2^{\cdot-}$ in plants with salt treatment was the highest among the four treatment groups at 73 h, indicating that the generation of $O_2^{\cdot-}$ exceeded the capacity of SOD to eliminates $O_2^{\cdot-}$. PQ treatment preferentially induces the activity of SOD in young expanding leaves (Casano et al. 1999). Ekmekci and Terzioglu (2005) also found that PQ causes a significant activation of SOD. Here, leaves of plants pre-treated with PQ displayed lower SOD activity at 1 h ($P < 0.01$) than did those in the control group (Fig. 3). This result was dissimilar to that of Liu et al. (2009). After the pre-treated plants were rinsed and put into darkness for 24 h, the SOD activity in leaves had no difference ($P > 0.05$) between the PQ pre-treatment group and the control. However, the activity of CAT in the PQ pre-treatment group was higher ($P < 0.01$) than that in the control (Fig. 4) indicating that pre-treatment with PQ influenced a part of enzymes in the antioxidant system at 25 h. The expression of an antioxidant enzyme gene regulates activities of other antioxidant enzymes in plants (Yun et al. 1998; Wang et al. 2004), so the enzymes in the antioxidant system are interactional. At 73 h in the study, the SOD activity in leaves of the PQ pre-treatment group was higher ($P < 0.01$) than that in the control. The reason may be that PQ pre-treatment induced CAT and thereby activated the activity of SOD. Exogenous silicon (Liang et al. 2003) increases the SOD activity in salt-stressed plants. When PQ pre-treated plants were transferred to salt conditions, the SOD activity in the PQ + salt treatment group was the highest ($P < 0.001$) at 73 h indicating that the SOD activity was further altered by salt stress after it was influenced by PQ pre-treatment, that is, there was an inverse relationship between SOD activity and the formation rate of $O_2^{\cdot-}$ in PQ pre-treated stressed cucumber leaves, and PQ pre-treatment increased the ability of cucumber leaves to dismutate $O_2^{\cdot-}$ via SOD under short-term salt stress.

Abiotic and biotic stresses enhance the production of H_2O_2 (Neill et al. 2002), which is toxic and must be eliminated (Foyer et al. 1997). In plants, enzymes, such as CAT, GPX, GSH-Px and APX are important for regulation of intracellular H_2O_2 (Noctor and Foyer 1998). CAT acts in the peroxisomes of cells, and the enzyme GPX plays a role in the apoplast, chloroplast and cytosol (Shigeoka et al. 2002). It has been shown that chloroplasts contain GSH-Px, which is important for elimination of H_2O_2 and lipid peroxidation products (Djanaguiraman et al. 2005). In the chloroplasts, cytosol (Foyer et al. 1994), mitochondria and peroxisomes (Jiménez et al. 1998), there is an ascorbate–glutathione cycle in which APX plays an important role in removing H_2O_2 when catalyzing the oxidization of AsA.

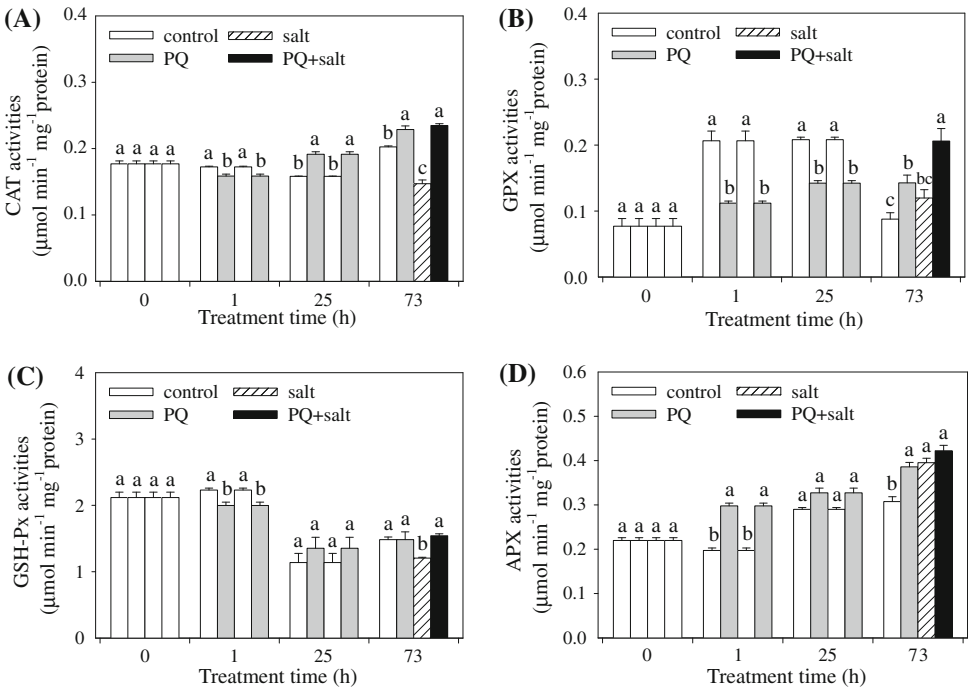

Fig. 4 Effects of PQ and salt on activities of CAT (**a**), GPX (**b**), GSH-Px (**c**) and APX (**d**) in cucumber leaves. Control, untreated; PQ, pre-treated with 10 μM PQ; salt, watered with 100 mM salt; PQ + salt, pre-treated with 10 μM PQ and watered with 100 mM salt. *Bars* represent standard errors. *Different small letters* denote significant differences among the treatment groups at a treatment time by the Tukey's test at $P < 0.05$

The enzyme APX has also been localized in the apoplastic space (Diaz-Vivancos et al. 2006). PQ treatment causes a significant activation of APX and GPX (Murgia et al. 2004; Ekmekci and Terzioglu 2005) and CAT (Liu et al. 2009). In our case, after cucumber seedlings were pre-treated with PQ for 1 h, the activities of CAT, GPX and GSH-Px were significantly lower ($P < 0.05$), while the APX activity was higher ($P < 0.001$) in the PQ pre-treated leaves than those of the control (Fig. 4). At 25 h, when PQ had been rinsed-off the plants for 24 h, the CAT activity in the PQ pre-treatment group was higher ($P < 0.01$) than that in the control, while the GPX activity was decreased ($P < 0.001$). Meantime, the activities of GSH-Px and APX had no difference ($P > 0.05$) between the PQ pre-treatment and the control groups. These results indicated that PQ pre-treatment influenced the antioxidant system. Drought stress increases CAT activity (Liu et al. 2009); but salt, a different kind of osmotic stress, reduces the enzyme activity in maize (Azevedo Neto et al. 2006). Application of glycine betaine (Raza et al. 2007) enhances CAT activity under salt stress. Meanwhile, salt treatment causes an increase in GPX activity (Sudhakar et al. 2001). Exogenous silicon has been shown to significantly enhance the enzyme activity in salt-stressed cucumber leaves (Zhu et al. 2004). Moreover, salt stress increases GSH-Px activity in plants (Ben-Hayyim et al. 1993). Zinc supplements also increase the GSH-Px activity under cadmium-induced oxidative stress (Aravind and Prasad 2005). However, decreased GSH-Px activity has been detected in plants under acid rain stress (Gabara et al. 2003). The APX activity is enhanced in salt-stressed leaves (Lee et al. 2001), especially in the presence of exogenous proline (Hoque et al. 2007). In our study, salt treatment decreased the activities of CAT ($P < 0.001$) and GSH-Px ($P < 0.01$) when compared with the control at 73 h. The cucumber plants had a general adaptive defense to salt treatment, which increased the activities of GPX ($P > 0.05$) and APX ($P < 0.01$). Meantime, the endogenous H_2O_2 level was the highest with salt treatment indicating that the generation of endogenous H_2O_2 exceeded the capacity of the cellular antioxidant defense system to eliminate H_2O_2. From the ANOVA results, PQ significantly ($P < 0.05$) increased the activities of CAT, GPX, GSH-Px and APX in leaves at 73 h. The activities of CAT, GPX and GSH-Px were all higher ($P < 0.05$) in the PQ + salt treatment group than those in the salt treatment group, suggesting that some enzymes of the antioxidant system in leaves was further stimulated by salt stress after the system was influenced by PQ pre-treatment. The higher activities of CAT, GPX, GSH-Px and APX in salt-stressed cucumber leaves coincided with a decrease in the levels of endogenous H_2O_2, suggesting that PQ pre-treatment increased the ability of cucumber to scavenge endogenous H_2O_2 via CAT, GPX, GSH-Px and APX under short-term salt stress.

To remove H_2O_2, APX needs the substrate AsA, whose regeneration relies on GSH-dependent DHAR and GR (a key enzyme in GSH regeneration cycle) or NADH-dependent MDHAR (Luster and Donaldson 1987). Drought stress was shown to increase the activities of GR, MDHAR and DHAR (Liu et al. 2009). At 73 h in our study, salt treatment did not change the activities of DHAR and MDHAR significantly ($P > 0.05$), but it did increase ($P < 0.01$) the GR activity when compared with the control (Fig. 5). This was similar to the report that salt conditions enhances the GR activity and induces the decrease in MDHAR and DHAR activities (Chaparzadeh et al. 2004). In plants treated by PQ, the activities of DHAR and GR (Miyagawa et al. 2000) are activated. In the current experiment, the ANOVA results showed that PQ pre-treatment increased ($P < 0.01$) the activities of DHAR, MDHAR and GR in cucumber leaves at 73 h. Exogenous proline (Hoque et al. 2007) increases the activities of MDHAR, DHAR and GR under salt stress. Here, treatment with PQ + salt induced the higher ($P < 0.05$) activities of DHAR, MDHAR and GR in comparison to the salt treatment alone. As shown in Fig. 6, the contents of AsA and GSH and the ratios of AsA/oxidized ascorbate and GSH/GSSG in the PQ + salt treatment group were higher than those in the salt-only treatment group. Therefore, AsA and GSH could be regenerated well via DHAR, MDHAR and GR when PQ was combined with short-term salt stress.

AsA and GSH not only perform as substrates in the ascorbate–glutathione cycle, but also act non-enzymatically. The beneficial effects of AsA in mitigating drought stress may be the activation of certain enzymatic reactions, and AsA also directly scavenges ROS, thus protecting plant cellular membranes (Thomas et al. 1992). The main function of GSH is in the regulation of protein thiol-disulfide redox status (Alscher et al. 1997), so the antioxidant plays a protective role in salinity tolerance (Gossett et al. 1996). The levels of AsA and GSH and the ratios of AsA/oxidized ascorbate and GSH/GSSG are increased by drought stress (Liu et al. 2009), but we found that they were reduced by salt treatment in this study (Fig. 6). This might be attributed to the high cost of AsA and GSH in mitigating ROS against the effects of salt (Shi and Zhu 2008). The application of paclobutrazol elevates the GSH levels in *Catharanthus* plants under salt stress (Jaleel et al. 2007). In our study, compared with salt-only treatment, PQ + salt treatment resulted in higher ($P < 0.01$) levels of GSH, AsA, AsA/oxidized ascorbate and GSH/GSSG and lower MDA content. According to the ANOVA results, PQ significantly ($P < 0.01$) increased the levels of AsA and GSH and the ratios of AsA/oxidized ascorbate and GSH/GSSG. These results suggest that PQ induced the elevation of AsA and GSH in salt-stressed cucumber leaves, and the two

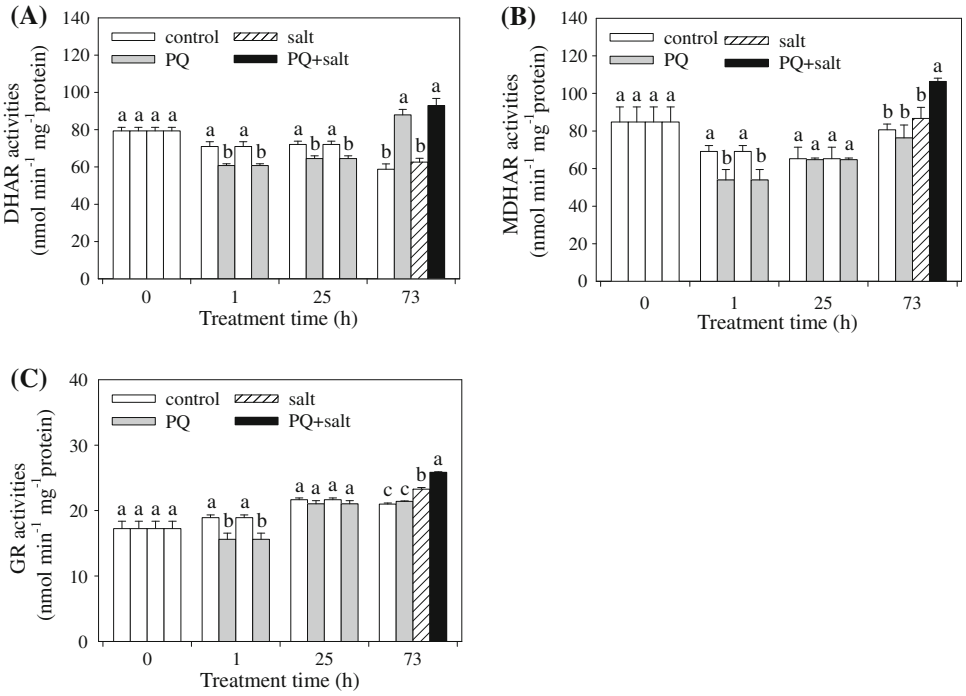

Fig. 5 Effects of PQ and salt on activities of DHAR (**a**), MDHAR (**b**) and GR (**c**) in cucumber leaves. Control, untreated; PQ, pre-treated with 10 μM PQ; salt, watered with 100 mM salt; PQ + salt, pre-treated with 10 μM PQ and watered with 100 mM salt. *Bars* represent standard errors. *Different small letters* denote significant differences among the treatment groups at a treatment time by the Tukey's test at $P < 0.05$

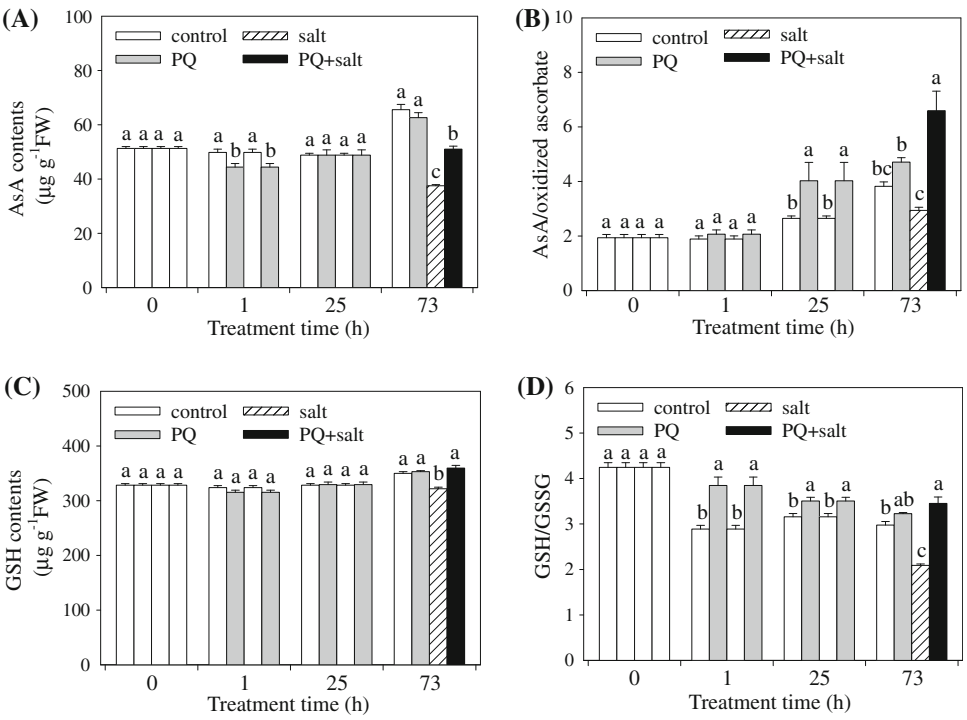

Fig. 6 Effects of PQ and salt on contents of AsA (**a**) and GSH (**c**) and ratios of AsA/oxidized ascorbate (**b**) and GSH/GSSG (**d**) in cucumber leaves. Control, untreated; PQ, pre-treated with 10 μM PQ; salt, watered with 100 mM salt; PQ + salt, pre-treated with 10 μM PQ and watered with 100 mM salt. *Bars* represent standard errors. *Different small letters* denote significant differences among the treatment groups at a treatment time by the Tukey's test at $P < 0.05$

antioxidants might play roles in increasing the cucumber tolerance to short-term salt stress.

In conclusion, salt stress increased the levels of $O_2^{\cdot-}$, H_2O_2 and MDA and modulated the antioxidative responses in cucumber leaves. When cucumber seedlings were pre-treated with low concentrations of PQ and then transferred in salt conditions, the activities of antioxidant enzymes, such as SOD, CAT, GPX, APX, DHAR, MDHAR and GR, as well as the contents of GSH and AsA increased; meanwhile, the levels of $O_2^{\cdot-}$, H_2O_2 and MDA decreased. PQ induced antioxidant activities and therefore decreases lipid peroxidation in salt-stressed cucumber leaves.

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