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Differential effect of sorbitol and polyethylene glycol on antioxidant enzymes in rice leaves

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Abstract

Polyethylene glycol (PEG) and sorbitol (ST) have each been used in osmotically induced water stress studies in plants, however, these osmotica may not have equivalent effects in plants. The present study was designed to examine whether antioxidant enzyme responses in rice leaves are different for PEG and ST of osmotic potential −1.5 MPa. As judged by relative water content, PEG treatment resulted in a higher degree of water stress in rice leaves than ST treatment. PEG treatment markedly increased lipid peroxidation, judged by malondialdehyde content, in rice leaves. However, ST treatment had no effect on lipid peroxidation. An increase in peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR) activities was observed in rice leaves treated with ST. PEG treatment had no effect on POX and APX activities and decreased GR activity in rice leaves. The decrease in superoxide dismutase activity induced by PEG was more pronounced than by ST. Cycloheximide blocked the enhanced activities of POX, APX and GR by ST, indicating de novo synthesis of the enzymes. Results suggest that ST but not PEG treatment can up-regulate antioxidant system in rice leaves.

Abbreviations: APX – ascorbate peroxidase, CAT – catalase, GR – glutathionine reductase, MDA – malondialdehyde, PEG – polyethylene glycol, POX – peroxidase, RWC – relative water content, SOD – superoxide dismutase, ST – sorbitol

Introduction

Drought is a major stress that dramatically limits plant growth and productivity (Boyer 1982). Leaves are known to close their stomata under water stress (Yordanov et al. 2000). During water stress leaf stomatal closure limits water loss and the influx of CO_2. Lowered CO_2 influx leads to a decrease in carbon reduction by the Calvin cycle and to a decrease in oxidized $NADP^+$ to serve as an electron acceptor in photosynthesis. As a result, electrons flow to the alternative electron acceptor, O_2, producing superoxide radical and consequently other reactive oxygen species, the most damaging of which is the hydroxyl radical (Scandalios 1993). It has been shown that thylakoid membrane electron leakage to O_2 increased in sunflower (Sgherri et al. 1996) and in wheat (Biehler and Fock 1996) after drought. Reactive oxygen species can directly damage proteins, amino acids and nucleic acids and cause peroxidation of membrane lipids (Dat et al. 2000).

Plants have evolved specific protective mechanisms, involving enzymatic and non-enzymatic antioxidants in order to defend themselves against reactive oxygen species. Non-enzymatic antioxidants include glutathione and ascorbate (Alscher 1989; Dat et al. 2000), while enzymatic antioxidants include superoxide dismutase (SOD, EC 1. 15. 1. 1), catalase (CAT, EC 1. 11. 1. 6), peroxidase (POX, EC 1. 11.1. 7), ascorbate peroxidase (APX, EC 1. 11. 1. 11) and glutathione reductase (GR, EC 1. 6. 4. 2) (Bowler et al. 1992; Dat et al. 2000). It has been demonstrated that GR (Gamble and Burke 1984; Smirnoff and Colombé 1988; Baisak et al. 1994; Boo and Jung 1999),

APX (Smirnoff and Colombé 1988; Mittler and Zilinskas 1992; Baisak et al. 1994; Mittler and Zilinskas 1994), and POX (Zhang and Kirkham 1994) were all stimulated upon exposure to water stress. On the other hand, SOD (Quartacci and Navari-Izzo 1992; Baisak et al. 1994) and CAT (Mukherjee and Choudhuri (1983, 1985); Quartacci and Navari-Izzo 1992; Zhang and Kirkham 1994) activities which can catalytically scavenge superoxide radical and H_2O_2, respectively, declined in water stressed plants. Activities of cytosolic and chloroplastic Cu/Zn SOD increased during drought of pea plants (Mittler and Zilinskas (1992, 1994)) and osmotic stress enhanced Mn SOD transcript abundance in maize (Zhu and Scandalios 1994). It appears that different components of the reactive oxygen scavenging system are modulated differently under water stress conditions.

Sorbitol (ST) and polyethylene glycol (PEG) have each been used to induce water stress in plant tissue. In experiments where osmotic stress is imposed on plant tissues by ST, it is difficult to separate purely osmotic effects from effects due to water stress generated during such treatments. It is known that PEG does not enter the cell wall space (Rubinstein 1982) and ST does (Flores and Galston 1984). PEG molecules with a molecular weight greater than 3000 are apparently not absorbed at all (Tarkow et al. 1966). Thus, PEG and ST may not have equivalent effects. This study was therefore designed to investigate whether antioxidant enzyme responses in rice leaves are different for ST and PEG.

Materials and methods

Plant material and treatments

Rice (*Oryza sativa* c.v. Taichung Native 1) was cultured in a stainless net floating on half-strength Johnson's modified nutrient solution (pH 4.2) in a 500-ml beaker (Lin et al. 1999). The nutrient solution was replaced every three days. Rice plants were grown for 12 days in a greenhouse, under natural light and the day/night temperature of 30/25 °C. The apical 3 cm of the third leaf of 12-day-old seedling was used for the experiment. A group of 10 segments floated in a Petri dish containing 10 ml of distilled water served as controls. For induction of water stress, leaf segments were exposed to PEG-6000 or ST solution of osmotic potential −1.5 MPa. All samples were kept at temperature at 27 °C and irradiance of 40 mmol m^{-2} s^{-1} for 4, 8 and 12 h.

RWC, chlorophyll and protein measurements

RWC, defined as water content of leaf tissue as a percentage of that of the fully turgid tissue, was determined by the method of Weatherley (1950). Chlorophyll was determined according to Wintermans and De Mots (1965) after extraction in 96% (v/v) ethanol. For protein extraction, leaf segments were homogenised in 50 mM sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,600 *g* for 20 min, and the supernatants were used for determination of protein by the method of Bradford (1976) and for enzyme assays.

Determinations of lipid peroxidation and H_2O_2

Malondialdehyde (MDA), routinely used as an indicator of lipid peroxidation, was extracted with 5% (w/v) trichloroacetic acid and determined according to Heath and Packer (1968). The H_2O_2 content was colorimetrically measured, as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenising 50 mg leaf tissue with 3 ml of phosphate buffer (50 mM, pH 6.5). The homogenate was centrifuged at 6 000 *g* for 25 min. To determine H_2O_2 contents, 3 ml of extracted solution was mixed with 1 ml of 0.1% titanium chloride in 20% (v/v) H_2SO_4. The mixture was then centrifuged at 6,000 *g* for 15 min. The intensity of the yellow colour of the supernatant was measured at 410 nm. H_2O_2 level was calculated using the extinction coefficient 0.28 $mmol^{-1}$ cm^{-1}.

Determinations of antioxidant enzymes

POX activity was measured using modification of the procedure of MacAdam et al. (1992). Activity was calculated using the extinction coefficient (26.6 mM^{-1} cm^{-1} at 470 nm) for tetraguaiacol. CAT activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Kato and Shimizu 1987). The decrease in H_2O_2 was followed as the decline in optical density at 240 nm, and activity was calculated using the extinction coefficient (40 mM^{-1} cm^{-1} at 240 nm) for H_2O_2 (Somashekaraiah et al. 1992). SOD was determined according to Paoletti et al. (1986). APX was determined according to Nakano and Asada (1981). The decrease in ascorbate concentration was followed as the decline in optical density at 290 nm

and activity was calculated using the extinction coefficient (2.8 mM^{-1} cm^{-1} at 290 nm) for ascorbate. GR was determined by the method of Foster and Hess (1980). One unit of activity for CAT, POX, SOD, APX, and GR were defined as the amount of enzyme which broke down 1 mmol of H_2O_2 per min, caused the formation of 1 mmol tetraguaiacol per min, inhibited 50% the rate of NADH oxidation observed in control, broke down 1 mmol of ascorbate per min, and decreased 1 A_{340} per min, respectively.

Experimental design

Chlorophyll, protein, H_2O_2 and MDA contents were expressed per initial g fresh weight. Enzyme activities were expressed as unit per mg protein. In the present investigation, rice seedlings were grown for 12 days in a greenhouse, where natural light was provided. The growth of rice seedlings is sensitive to light and varies with different light intensities. Experiments were carried out at the different time of the year. Thus, absolute levels of each measurement varied among experiments because of seasonal effects. However, the patterns of response to PEG or ST were reproducible. For biochemical analysis, four independent extractions were performed for each treatment. All experiments described here were repeated at least three times. Similar results and identical trends were obtained each time. The data reported here are from a single experiment.

Results

RWC of detached rice leaves exposed to ST or PEG solution of −1.5 MPa, with light exposure, decreased during 12-h of incubation, suggesting that both ST and PEG treatments in our study did indeed cause water stress in the leaves. The decrease in RWC in PEG-treated leaves was greater than that in ST-treated leaves, indicating that water stress induced by PEG is more severe than ST (Figure 1).

The obvious character of leaf senescence is yellowing. Chlorophyll loss has been the principal criterion of senescence for the largest number of workers. The protein break down during leaf senescence has been realized from earliest studies. In the present paper, ST- or PEG-induced senescence of the leaves was assessed by the decrease in chlorophyll and protein contents. It is clear from Figure 1, both ST and PEG decreased chlorophyll and protein contents.

The time courses of lipid peroxidation and H_2O_2 content in detached rice leaves treated with ST and PEG are given in Figure 1. MDA was used as an indicator of lipid peroxidation. MDA contents remained almost unchanged in control leaves during 12-h incubation (Figure 1). However, there was a significant increase in MDA content in PEG-treated leaves (Figure 1). It is clear that PEG treatment resulted in an increase in lipid peroxidation in detached rice leaves. In contrast, ST treatment had no effect on lipid peroxidation (Figure 1). Figure 1 also demonstrated that there was no significant difference in the content of H_2O_2 between control and ST- or PEG-treated leaves.

There was no difference in the activity of CAT, the enzyme responsible for eleminating H_2O_2, between control and ST-treated leaves (Figure 2). CAT activity in PEG-treated leaves was lower than water-treated leaves at 4 h after treatment (Figure 2). ST treatment resulted in a higher activity of POX as compared with the control leaves (Figure 2). In contrast, no difference in POX activity was observed between PEG- and H_2O-treated leaves (Figure 2). Figure 2 also demonstrated that ST treatment resulted in higher activities in SOD, APX and GR than PEG treatment.

Since ST treatment caused an increase in the activities of POX, APX and GR, the effect of cycloheximide, an inhibitor of protein synthesis, was studied in order to know if these enhancements were due to new protein synthesis. Cycloheximide treatment prevented any ST-induced increase in POX, APX and GR activities (Table 1) indicating that the increases in POX, APX and GR activities in leaves incubated in ST solution are probably due to de novo protein synthesis.

Table 2 shows that rice leaves pretreated with ST resulted in a reduction of toxicity of paraquat, judged by the changes in protein contents.

Discussion

In the present investigation, ST and PEG were used to induce water stress. As judged by RWC, PEG treatment resulted in a higher degree of water stress in rice leaves than ST treatment (Figure 1). ST molecules can be absorbed by plant cells (Flores and Galston 1984). Thus, a lower degree of water stress induced by ST seems to result from a certain amount of osmotic adjustment, due to the accumulation of ST. Baisak et al. (1994) reported the enhancement of lipid peroxidation in the leaves subjected to higher degree

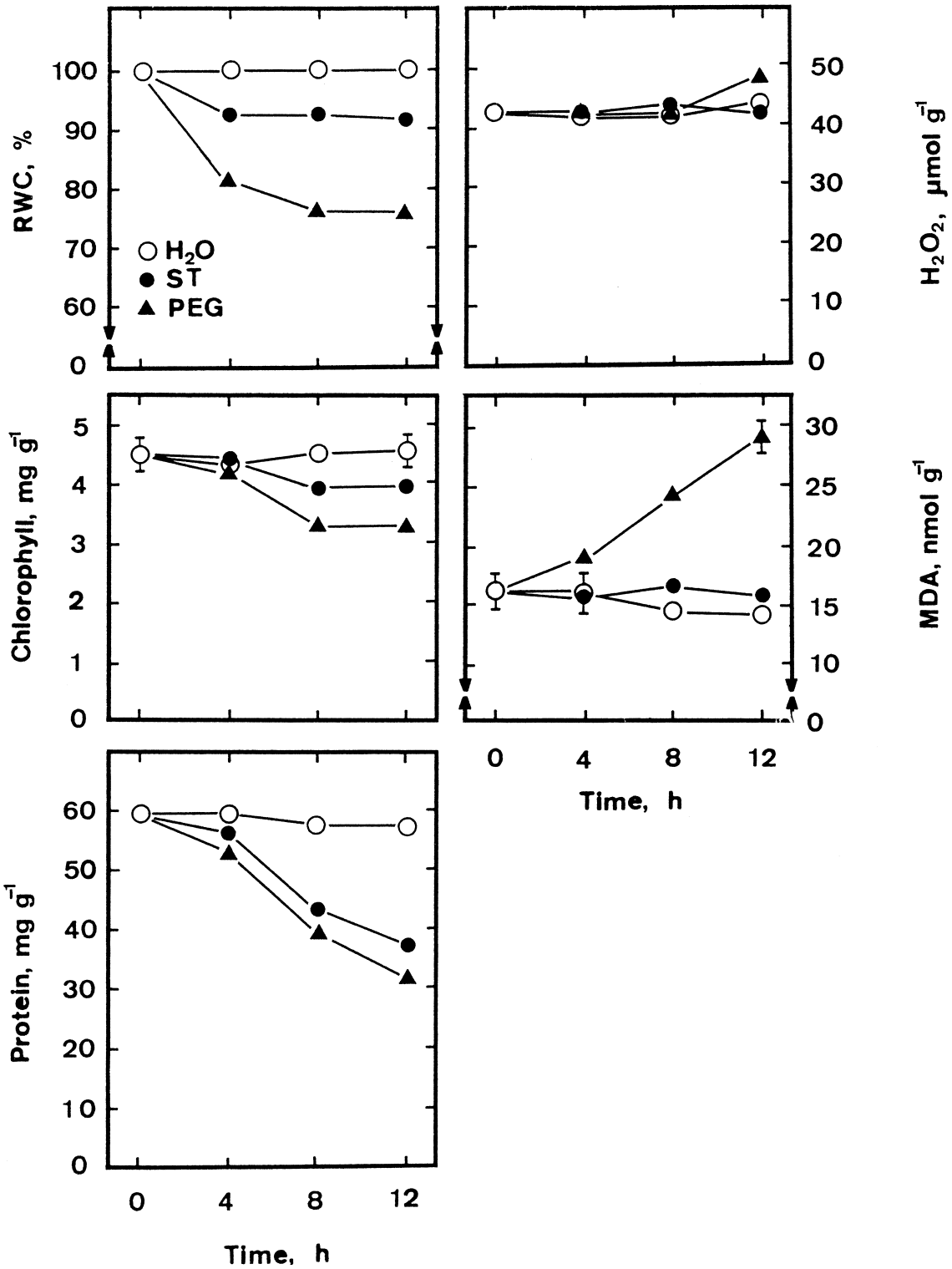

Figure 1. Changes in RWC, chlorophyll, protein, H_2O_2 and MDA contents of detached rice leaves floated on water, ST (−1.5 MPa) or PEG (−1.5 MPa) solutions in the light. Means ± SE, n = 4.

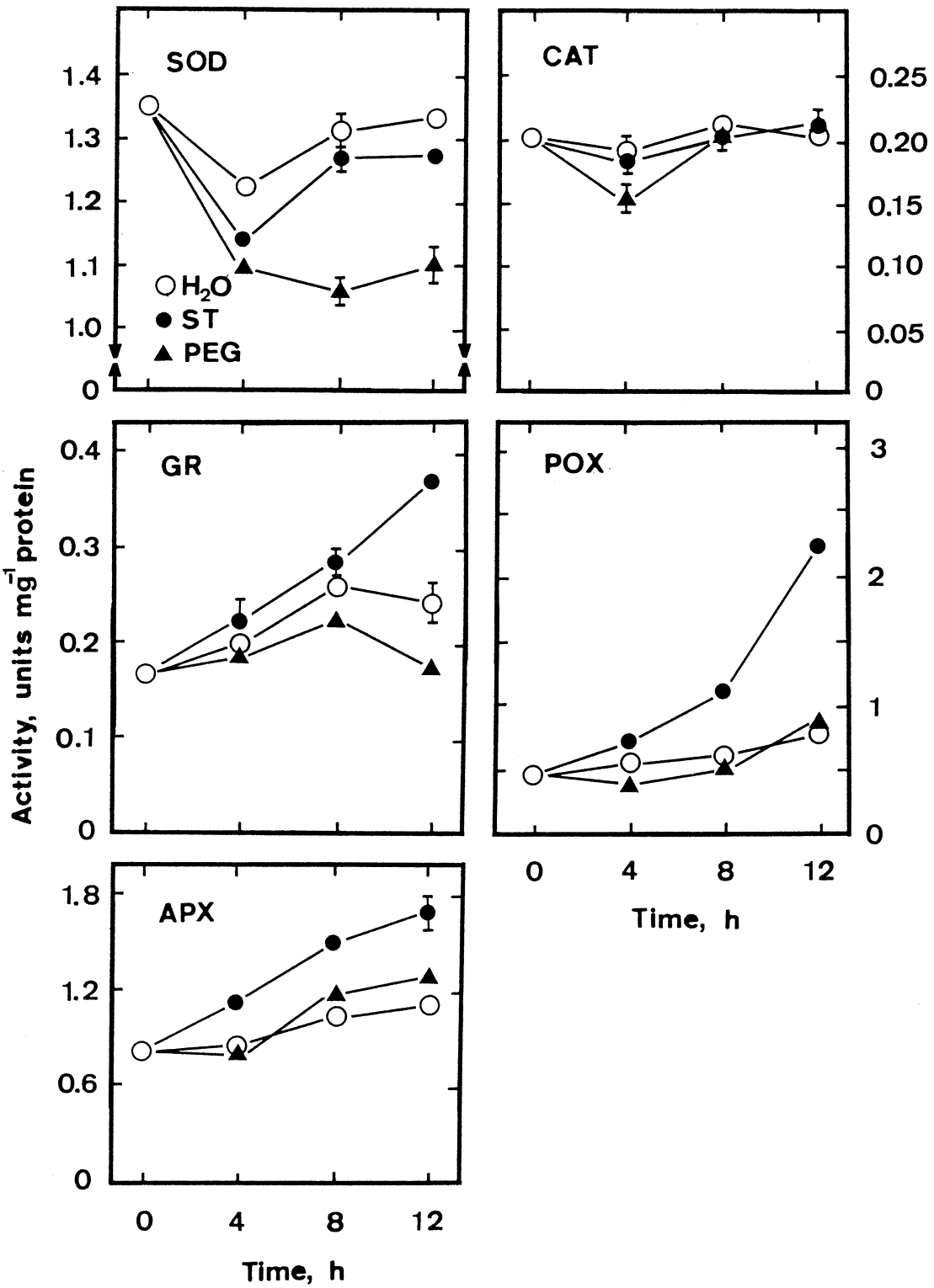

Figure 2. Changes in the activities of antioxidative enzymes in detached rice leaves floated on water, ST (−1.5 MPa) or PEG (−1.5 MPa) solution in the light. Means ± SE, n = 4.

Table 1. Effect of cycloheximide on the induction of POX, APX and GR activities by ST (−1.5 MPa).

Treatment	Enzyme activity, units mg^{-1} protein		
	POX	APX	GR
H_2O	0.62 ± 0.02	1.29 ± 0.03	0.31 ± 0.03
CHI	0.29 ± 0.03	1.38 ± 0.15	0.36 ± 0.03
ST	2.01 ± 0.09	1.80 ± 0.14	0.60 ± 0.06
ST + CHI	0.32 ± 0.02	1.55 ± 0.06	0.38 ± 0.04

The concentration of cycloheximide was 10 μM. Enzyme activities were determined 12 h after treatment in the light. Means ± SE, n = 4.

Table 2. Effect of ST pretreatment on contents of protein in detached rice leaves treated with paraquat.

Pretreatment	Treatment	Protein content, mg m^{-1} FW
H_2O	H_2O	54.23 ± 1.60
	Paraquat	11.65 ± 0.33
ST	H_2O	40.36 ± 1.02
	Paraquat	19.59 ± 1.69

Detached rice leaves were pretreated with water or ST (−1.5 MPa) for 12 h and then transferred to either water or paraquat (10 μM) for 12 h in the light. Means ± SE, n = 4.

of water stress but not subjected to mild stress. Parallel to these observations, we also noticed an increase in lipid peroxidation in detached rice leaves treated with PEG, on the other hand, detached rice leaves did not exhibit an increase in lipid peroxidation with exposure to ST (Figure 1). The occurrence of lipid peroxidation is an indicator of the prevalence of free radical reactions and a change in the balance between $O_2^{\cdot-}/H_2O_2$ in leaves. However, we were not able to provide any evidence for the increased formation of $O_2^{\cdot-}$. Increase in the contents of H_2O_2 in *Vigna catjang* seedlings subjected to water stress had been reported (Mukherjee and Choudhuri (1983, 1985)). In contrast, Boo and Jung (1999) demonstrated that H_2O_2 content decreased in rice seedlings in response to water stress. However, neither PEG nor ST had any effect on H_2O_2 content in detached rice leaves (Figure 1).

In the present investigation, we were able to show that ST can up-regulate its antioxidant system by increasing the activities of POX, APX and GR (Figure 2). We also demonstrated that SOD activity in ST-treated leaves were higher than that in PEG-treated leaves (Figure 2). All these results would explain why ST treatment resulted in no increase in lipid peroxidation.

It has been demonstrated that transgenic plants overexpressing POX, APX and GR had increased resistance to paraquat-mediated oxidative stress (Aono et al. (1991, 1993); Sen Gupta et al. 1993; Yun et al. 2000). Thus, it would be interesting to know whether ST-treated detached rice leaves are resistant to paraquat. To test this, detached rice leaves were pretreated with either ST or water for 12 h and then transferred to either water or paraquat (10 μM) for 12 h in the light. It is indeed that ST pretreatment reduced paraquat toxicity, judged by the decrease in protein content (Table 2). It seems that ST induction of POX, APX and GR activities could be a possible cause of reduced paraquat-mediated oxidative stress in rice leaves.

Water stress usually enhances the senescence of leaves (Thomas and Stoddart 1980). We also demonstrated that ST and PEG enhanced senescence of detached rice leaves (Figure 1). Since ST had no effect on lipid peroxidation (Figure 2), it is unlikely that ST-enhanced senescence of detached rice leaves is linked to reactive oxygen-mediated lipid peroxidation.

Treatment of detached rice leaves with cycloheximide, an inhibitor of protein synthesis, prevented ST induced increase in the activities of POX, APX and GR (Table 1) indicating that ST stimulates the de novo synthesis of POX, APX and GR proteins probably as an adaptive mechanism to protect the leaves against oxygen radical damage. It is not known as to how ST lead to the stimulation of POX, APX and GR activities at a time when soluble protein content was declining. It has been shown that $O_2^{\cdot-}$ and H_2O_2 generated in the leaves during water stress might be responsible for the induction of GR (Pastori and Trippi 1992; Baisak et al. 1994). Since paraquat treatment, which generates superoxide radical internal, resulted in a decrease in POX activity and H_2O_2 treatment had no effect on POX activity in detached rice leave (Fang and Kao 2000), it seems that ST-induced POX may not be mediated through reactive oxygen species.

In conclusion, the results presented here suggest (a) that PEG treatment results in higher degree of water stress in detached rice leaves than ST treatment, (b) that PEG but not ST treatment results in a significant increase in lipid peroxidation, and (c) that ST but not PEG treatment can up-regulate antioxidant system. It should be noted that the conclusion obtained from the present work is simply based on the studies using detached rice leaves. Since cutting increased POX activity and prevented the normal de-

crease in CAT activity in tobacco leaves (Parish (1968a, 1968b)), one may argue that the changes in antioxidant enzymes are probably a wound response. When detached leaves were used as an experimental system, wounding is always a problem. However, in the present work, each long and narrow rice leaf was cut once transversely, the aera of wounding was very small. Since no changes in RWC and proline content were observed in control rice leaves during 12-h incubation in the light (Figure 1, Cheng et al. (2002) (in press)), the results from the present investigation are unlikely to be associated with the changes in water-channel function or stomatal movement. Nevertheless, detached leaf system lacks the natural features of transport in the whole plant. Thus, the results from experiments using detached rice leaves are not necessarily similar to those using whole plant system.

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