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Expression of ascorbate peroxidase and glutathione reductase in roots of rice seedlings in response to NaCl and H_2O_2

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Summary

The accumulation of H_2O_2 by NaCl was observed in the roots of rice seedlings. Treatment with NaCl caused an increase in the activities of ascorbate peroxidase (APX) and glutathione reductase (GR) and the expression of *OsAPX* and *OsGR* in rice roots. Exogenously applied H_2O_2 also enhanced the activities of APX and GR and the expression of *OsAPX* and *OsGR* in rice roots. The accumulation of H_2O_2 in rice roots in response to NaCl was inhibited by the NADPH oxidase inhibitors, diphenyleneiodonium chloride (DPI) and imidazole (IMD). However, DPI, IMD, and dimethylthiourea, a H_2O_2 trap, did not reduce NaCl-enhanced activities of APX and GR and expression of *OsAPX* and *OsGR*. It appears that H_2O_2 is not involved in the regulation of NaCl-induced APX and GR activities and *OsAPX* and *OsGR* expression in rice roots.

Introduction

Soil salinity, particularly due to NaCl, can be considered as the single most widespread soil toxicity problem that global rice production faces at present. Roots play a number of important roles during plant growth and development and typically are the first part of the plant to encounter soil salinity. When growing in saline soils, roots have to cope with two types of stresses, osmotic and ionic. These stresses in turn cause a reduction in water uptake and inhibition of root growth (Munns, 1993).

The increase in active oxygen species (AOS) seems to occur as a response to most, if not all,

Abbreviations: AOS, active oxygen species; APX, ascorbate peroxidase; DMTU, dimethylthiourea; DPI, diphenyleneiodonium chloride; DW, dry weight; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; IMD, imidazole; PVP, polyvinyl-pyrrolidone

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abiotic stresses including drought (Smirnoff, 1993) and salinity (e.g. Dionisio-Sese and Tobita, 1998; Lin and Kao, 2000; Hernández et al., 2001; Lee et al., 2001; Sudhakar et al., 2001; Hernández and Almansa, 2002). These AOS can damage DNA, protein, chlorophyll, and membrane functions. To mitigate and repair damage initiated by AOS, plants have developed a complex antioxidant system (Van Breusegem et al., 2001; del Rio et al., 2002).

Ascorbate peroxidase (APX, EC 1.11.1.1) plays a crucial role in the detoxification of cellular H_2O_2, the toxic product of superoxide dismutation. Glutathione reductase (GR, EC 1.6.4.2) is a flavoenzyme and has been found in all organisms examined. This enzyme, which catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) with the accompanying oxidation of NAD(P)H, is thought to be an important reaction of the system for the detoxification of AOS in plants. Therefore, APX and GR have been suggested to be regulated in response to various environmental stresses and to contribute to stress tolerance in APX- and GR-overexpressing plants (Aono et al., 1991; Broadbent et al., 1995; Kubo et al., 1995; Sato et al., 2001). Lee et al. (2001) showed that NaCl stress resulted in a higher activity of APX in rice leaves but not in rice roots. In shoot cultures of rice, activity of APX was similar whether the shoots were grown in the presence or absence of NaCl (Fadzilla et al., 1997). In contrast, there was an early increase in GR activity in NaCl-exposed shoot cultures of rice (Fadzilla et al., 1997). Expression of *APX* and *GR* has been reported to be enhanced in plants by NaCl treatment (Kaminaka et al., 1998; Savoure et al., 1999; Kawasaki et al., 2001). However, Lopez et al. (1996) demonstrated that APX activity, not the mRNA level, was enhanced in NaCl-stressed *Raphanus sativus* plants.

Recently, many researchers have focused on functional aspects of H_2O_2 generation. H_2O_2 is a constituent of oxidative metabolism. Because H_2O_2 is relatively stable and diffusible through membrane, H_2O_2 is thought to constitute a general signal molecule inducing cellular stress (Foyer et al., 1997; Neill et al., 2002). It has been shown that H_2O_2 stimulated APX and GR activities in maize leaves (Pastori and Trippi, 1992, 1993). H_2O_2 induced the expression of gene encoding APX in germinating rice embryos (Morita et al., 1999) and in *Arabidopsis* leaves (Karpinski et al., 1999). However, the failure of induction of *APX* and *GR* genes by H_2O_2 has also been reported (Babiychuk et al., 1995; Vansuyt et al., 1997; Xiang and Oliver, 1998). It has been suggested that cytosolic APX transcripts can be upregulated by increased levels of H_2O_2 in tobacco chloroplasts as a result of Cu-Zn-superoxide dismutase overexpression (Gupta et al., 1993). However, Karpinski et al. (1997) reported that a photooxidative burst of H_2O_2 was not involved in the regulation of *APX1* or *APX2* gene expression in *Arabidopsis*. de Agazio and Zacchini (2001) demonstrated that dimethylthiourea (DMTU), a H_2O_2 trap, partially prevented the increase of *APX* gene expression in spermidine-treated maize roots. They concluded that induction of *APX* gene expression in spermidine-treated maize roots is mediated through H_2O_2, a spermidine catabolic product.

We have previously shown that NaCl increases H_2O_2 level in roots of rice seedlings (Lin and Kao, 2001). In this study, we first examined the effect of NaCl and H_2O_2 on the expression of APX and GR and then the possible interactions between H_2O_2- and NaCl-induced expression of APX and GR in roots of rice seedlings.

Materials and methods

Plant material

Rice (*Oryza sativa* L., cv. Taichung Native 1) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. In order to get more uniformly germinated seeds, rice seeds in Petri dish (20 cm) containing distilled water were pre-treated at 37 °C for 1 day under dark condition. Uniformly germinated seeds were then selected and transferred to a Petri dish (9.0 cm) containing two sheets of Whatman No. 1 filter paper moistened with 10 mL of distilled water for 2 days. Two-day-old seedlings were then treated with distilled water, NaCl, or H_2O_2 at the desired concentration as specified in the individual experiments. Root growth of rice seedlings grown in distilled water is similar to that grown in medium containing inorganic salts, thus seedlings grown in distilled water were used as the controls. For the experiments to examine the role of H_2O_2 in regulating NaCl-induced activities of APX and GR, and expression of *OsAPX* and *OsGR* in roots, diphenyleneiodonium chloride (DPI) and imidazole (IMD), which are known to inhibit NADPH oxidase, and DMTU, a chemical trap for H_2O_2, were used. Each Petri dish contained 20 germinated seeds. Each treatment was replicated four times. The germinated seeds were allowed to grow at 27 °C in darkness.

H_2O_2 determination

The H_2O_2 level was colorimetrically measured as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing with phosphate buffer (50 mmol L^{-1}, pH 6.8) including 1 mmol L^{-1} hydroxylamine. The homogenate was centrifuged at 6000g_n for 25 min. To determine H_2O_2 levels, extracted solution was mixed with 0.1% titanium chloride (Aldrich) in 20% (v/v) H_2SO_4 and the mixture was then centrifuged at 6000g_n for 15 min. The intensity of yellow color of supernatant was measured at 410 nm. H_2O_2 level was calculated using the extinction coefficient 0.25 $\mu mol^{-1}\,cm^{-1}$. H_2O_2 level was expressed on the basis of dry weight (DW).

Ascorbate peroxidase and glutathione reductase assays

For extraction of enzymes, root tissues were homogenized with 0.1 mol L^{-1} sodium phosphate buffer (pH 6.8) containing 0.1 mmol L^{-1} EDTA, 1% (v/v) polyvinyl-pyrrolidone (PVP), and 0.5% (v/v) Triton X-100 in a chilled mortar. For analysis of APX activity, 2 mmol L^{-1} ascorbic acid was added to the extraction buffer. The homogenate was centrifuged at 12,000g_n for 20 min and the resulting supernatant was used for determination of enzyme activity. The whole extraction procedure was carried out at 4 °C. APX was determined according to Nakano and Asada (1981). The decrease in ascorbate concentration was followed as a decline in optical density at 290 nm and activity was calculated using the extinction coefficient [2.8 (mmol L^{-1})$^{-1}$ cm^{-1} at 290 nm] for ascorbate. One unit of APX was defined as the amount of enzyme that breaks down μmol of ascorbate per minute. GR was determined by the method of Foster and Hess (1980). One unit of GR was defined as the amount of enzyme that decreases 1 A_{340} min^{-1}. Activities of all enzymes were expressed on the basis of DW.

Isolation of *OsAPX* and *OsGR* cDNA

Rice cDNA clones encoding *OsAPX* and *OsGR* were isolated through RT-PCR approach using root RNA as template. Total RNA was isolated from hydroponically cultivated rice roots using the TRIZOL reagent and following manufacture's instructions (Invitrogen Life Technologies, Carlsbad, CA). Using 1 μg of template RNA, *OsAPX* (0.8 kb) and *OsGR* (1.6 kb) cDNA were amplified by Super Script™ one-step RT-PCR with Platinum® Taq (Invitrogen®), respectively. The gene-specific primers were designed according to the cDNA sequences published in NCBI GeneBank (accession numbers: *OsAPX*, AY254495; *OsGR*, D78136). The primer pairs used for the RT-PCR were: *OsAPX*, 5′-TTCGAATTCTCCACCCGCAGC-CAT GGCGA-3′ (*Eco*RI site underlined) and 5′-ACTGTCTAGAACGG GCAATGTACT AGGCAGT-3′ (*Xba*I site underlined); *OsGR*, 5′-AGCGAATTCTT-GAGGATCCAT GGC-3′ (*Eco*RI site underlined) and 5′-TCATCTAGACCAGAGTACACTTTGCC-3′ (*Xba*I site underlined). The PCR products were subcloned into the *Eco*RI and *Xba*I sites of pBluescript (Strategene) to generate pBS/APX and pBS/GR, respectively.

RNA gel blot Analysis

RNA gel blot analysis was employed to study *OsAPX* and *OsGR* transcription levels in roots following different treatments. Sample of 7 μg of total RNA was size fractionated on 1% agarose gel containing 10 mmol L^{-1} sodium phosphate buffer (pH 6.5), transferred to a nylon filter, and hybridized with ^{32}P random primer labeled *OsAPX* DNA (0.8 kb) or *OsGR* DNA (1.6 kb) probe (Thomas, 1983). All hybridization reagents were prepared as described in Sambrook et al. (1989). The hybridization signals were scanned (Microtek ScanMaker 8700 scanner, software Quantity One, Bio-Rad), and the ratio of *OsAPX* and *OsGR* mRNA to 18S rRNA was determined. The results are expressed as the degree of increase over mRNA levels for the untreated roots.

Statistical analysis

The results presented were the means of four replicates. Means were compared by either Student's *t*-test or Duncan's multiple range test.

Results

The changes in APX and GR activities in roots of rice seedlings after treatment of 150 mmol L^{-1} are presented in Figs. 1B and C. NaCl treatment had higher activities of APX and GR than the controls. NaCl treatment caused an increase in H_2O_2 content in rice roots (Fig. 1A). The increase in H_2O_2 was evident at 8 h after treatment of NaCl. These results suggest that H_2O_2 may play an important role in regulating the increase of APX and GR activities in rice roots treated with NaCl.

To test whether H_2O_2 is involved in NaCl-induced APX and GR activities in roots of rice seedlings, DMTU, a chemical trap for H_2O_2 (de Agazio and Zacchini, 2001) was used. Roots of rice seedlings were pre-treated with or without 5 mmol L^{-1} DMTU for 12 h. As indicated in Figs. 2B and C, when rice

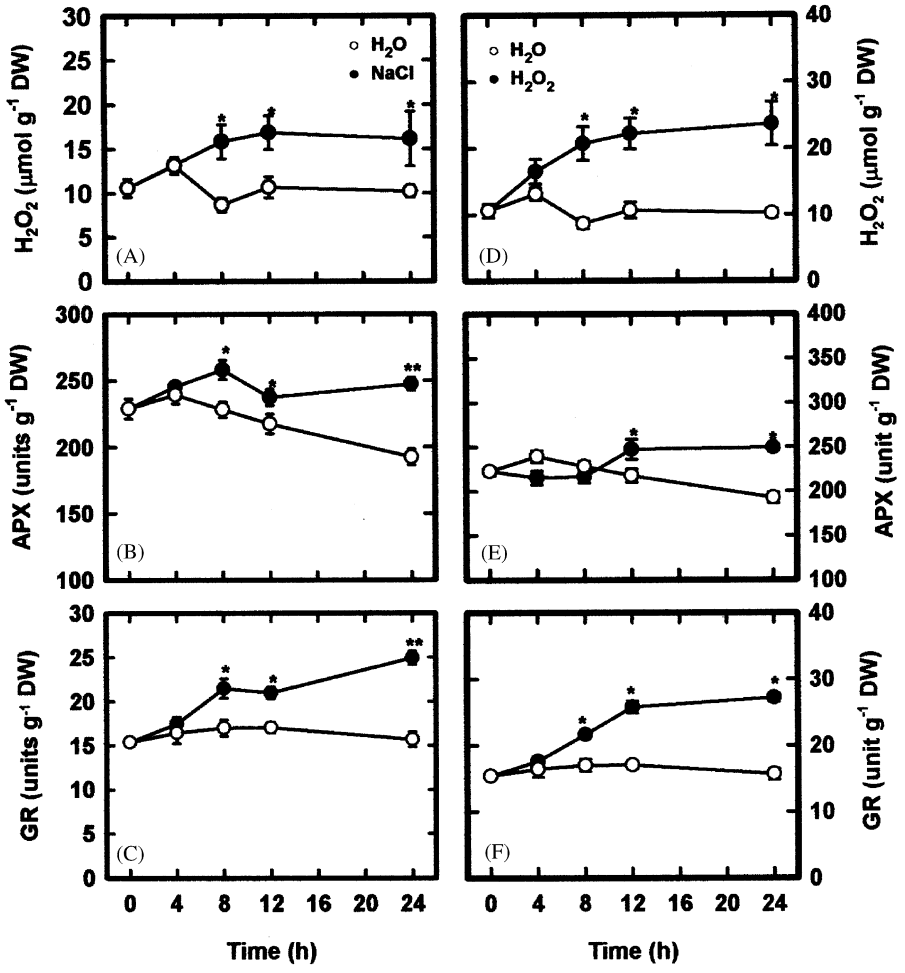

Figure 1. Changes in the levels of H_2O_2 (A, D) and the activities of APX (B, E) and GR (C, F) in H_2O- , NaCl-, and H_2O_2-treated roots of rice seedlings. Two-day-old rice seedlings were treated with either distilled water or NaCl (150 mmol L^{-1}) or H_2O_2 (10 mmol L^{-1}). * and ** indicate significance at $P<0.05$ and $P<0.01$ ($n=4$), respectively, by Student's *t*-test when compared to water control.

roots were pre-treated with DMTU, NaCl-induced APX and GR activities in rice roots were not reduced. AOS originating from the plasma-membrane NADPH oxidase, which transfers electrons of cytoplasmic NADPH to O_2 to form O_2^-, followed by dismutation of O_2^- to H_2O_2, has been a recent focus in AOS signaling. In several model systems investigated in plants, the oxidative burst and accumulation of H_2O_2 appear to be mediated by the activities of plasma-membrane NADPH oxidase complex (Pei et al., 2000; Orozco-Cárdenas et al., 2001; Jiang and Zhang, 2002, 2003). When rice roots were pre-treated with DPI or IMD, which is known to inhibit NADPH oxidase (Pei et al., 2000; Cross, 1990; Orozco-Cárdenas et al., 2001; Jiang and Zhang, 2002, 2003), it was observed that NaCl-induced accumulation of H_2O_2 was significantly reduced (Fig. 2A). However, DPI and IMD pre-treatments had no effect on NaCl-induced APX and GR activities in rice roots (Figs. 2B and C).

The effect of 150 mmol L^{-1} NaCl on the expression of genes of APX and GR is shown in Figs. 3A and B. The steady-state transcript levels of *OsAPX* and *OsGR* increased after NaCl treatment. However, NaCl-induced expression of *OsAPX* and *OsGR* in rice roots was not reduced by pre-treatment of DPI and DMTU (Figs. 3A and B).

Exogenous application of H_2O_2 resulted in an increase in the content of H_2O_2 (Fig. 1D), the activities of APX and GR (Figs. 1E and F) and the transcript levels of *OsAPX* and *OsGR* in rice roots (Figs. 4A and B).

Discussion

Environmental stresses are known to cause oxidative stress within plant cells. The accumulation of H_2O_2 has been observed in response to chilling

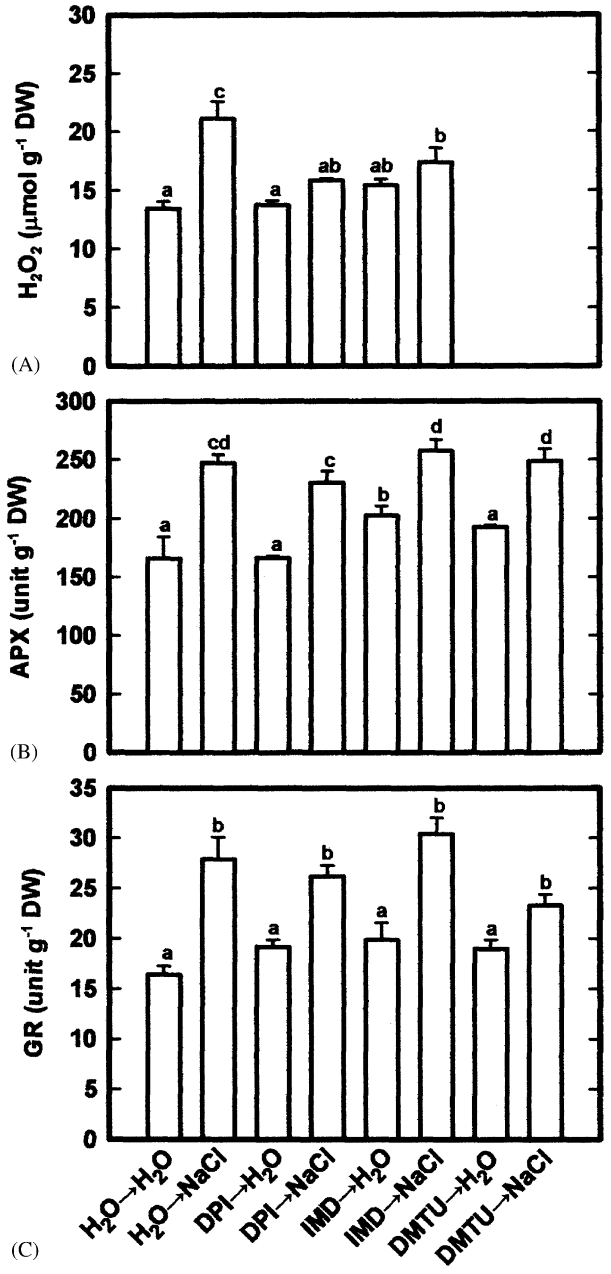

Figure 2. Effect of DPI and IMD pre-treatments on the levels of H_2O_2 (A) and DMTU, DPI, IMD on the activities of APX (B) and GR (C) in the roots of rice seedlings in the presence or absence of NaCl. Two-day-old rice seedlings were pre-treated with distilled water or DMTU (5 mmol L^{-1}), DPI (0.1 μmol L^{-1}) or IMD (10 mmol L^{-1}) for 12 h and then transferred to distilled water and NaCl (150 mmol L^{-1}) for 24 h, respectively. Means followed by the same letter do not differ significantly at $P<0.05$ ($n=4$) by Duncan's multiple range test.

(Okuda et al., 1991; Prasad et al., 1994; O'Kane et al., 1996; Fadzilla et al., 1996), heat (Dat et al., 1998), UV radiation (Murphy and Huerta, 1990), excess light (Karpinski et al., 1997), and anoxic stress (Blokhina et al., 2001). Lee et al. (2001) showed that NaCl treatment resulted in an accumulation of H_2O_2 in the leaves but not in the roots of rice plants. Here, we show that H_2O_2 levels accumulate in NaCl-treated roots of rice seedlings (Fig. 1A). NaCl-induced accumulation of H_2O_2 in rice leaves has been suggested to be due to NaCl-enhanced SOD and NaCl-deactivated CAT activities (Lee et al., 2001). This does not seem to be the case in the roots of rice seedlings, because NaCl had no effect on SOD and CAT activities (unpublished observation).

It has been shown that high concentration of DPI can affect other enzymes potentially involved in the generation of AOS, including cell wall peroxidase and nitric oxidase synthase (Bolwell et al., 1998; Frahry and Schopfer, 1998; Stuehr et al., 1991). The fact that NaCl-induced H_2O_2 accumulation in rice roots can be inhibited by low concentration DPI (0.1 μmol L^{-1}) and can be inhibited by IMD (10 mmol L^{-1}), another inhibitor of NADPH oxidase (Cross, 1990, Fig. 2A), strongly suggested that NaCl-induced accumulation of H_2O_2 was mediated through the activation of NADPH oxidase in rice roots. NADPH oxidase does not seem to be the only source H_2O_2 generation in NaCl-treated rice roots, because NaCl-induced cell wall bound NADH peroxidase and diamine oxidase activities, which devoted to H_2O_2 generation, have been detected in the roots of rice seedlings (Lin and Kao, 2001).

The role of APX and GR in the H_2O_2 scavenging in plant cells has been well established in ascorbate–glutathione cycle (Bowler et al., 1992). APX and GR activities have been shown to be enhanced by NaCl stress in rice leaves (Lee et al., 2001) and rice shoots cultured in the presence of NaCl (Fadzilla et al., 1997). There are other reports showing that NaCl had no effect on APX activity in rice roots (Lee et al., 2001). Here, we show that both APX and GR activities are enhanced by NaCl in roots of rice seedlings (Figs. 1B and C).

The results of the present study seem to be contradictory to those of Lee et al. (2001), who demonstrated that NaCl had no effect on H_2O_2 levels and APX activity in rice roots. This difference may be due to the different rice cultivars used.

Gene expression in response to environmental stress is usually studied at the level of steady-state mRNA abundance because this gives a more precise estimate of antioxidant gene activation than enzyme activity. Expression of *APX* and *GR* has been demonstrated to be enhanced in plants by NaCl treatment (Kaminaka et al., 1998; Savoure et al., 1999; Kawasaki et al., 2001). However, Lopez

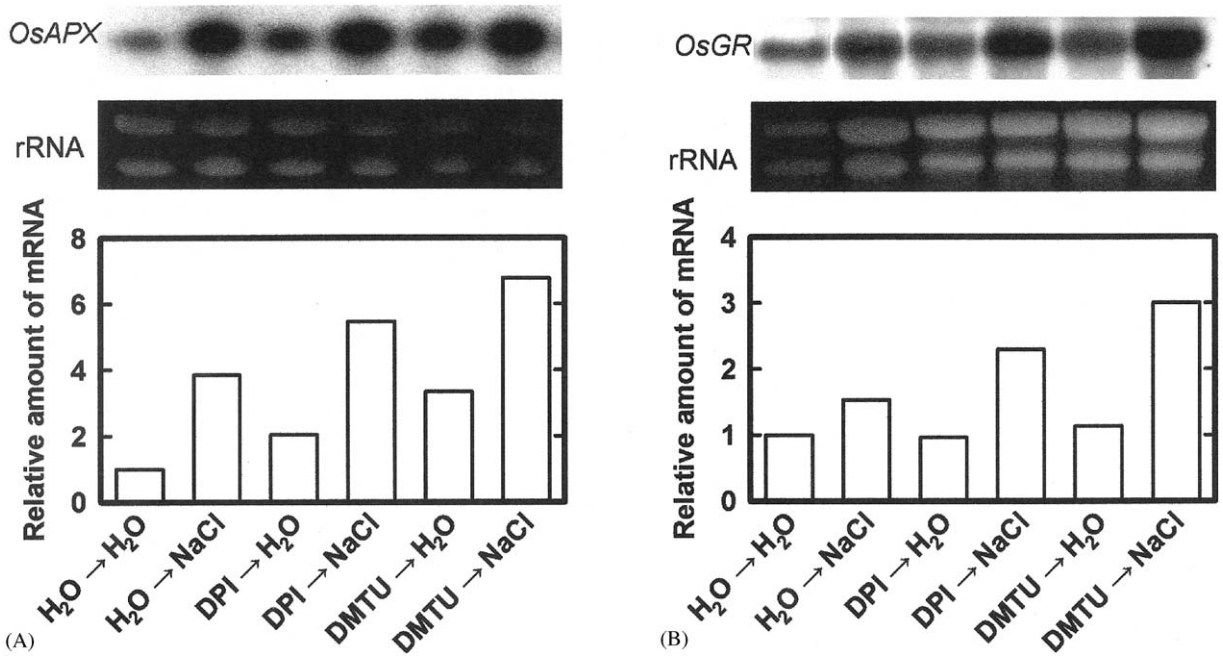

Figure 3. Effect of DPI and DMTU pre-treatments on mRNA abundance of *OsAPX* (A) and *OsGR* (B) in roots of rice seedlings in the presence or absence of NaCl. Two-day-old rice seedlings were pre-treated with distilled water, DPI (0.1 μmol L^{-1}), or DMTU (5 mmol L^{-1}) for 12 h and then transferred to distilled water and NaCl (150 mmol L^{-1}) for 24 h, respectively. Northern blots of total RNA samples were hybridized with *OsAPX* (A) and *OsGR* (B). The values of mRNA of *OsAPX* and *OsGR* were adjusted by its corresponding amount of rRNA for equality of loading. After the adjustment of rRNA, the reaction with roots in water was treated as the normalized reference, with a value of one, for determining the relative amount of mRNA of *OsAPX* and *OsGR*.

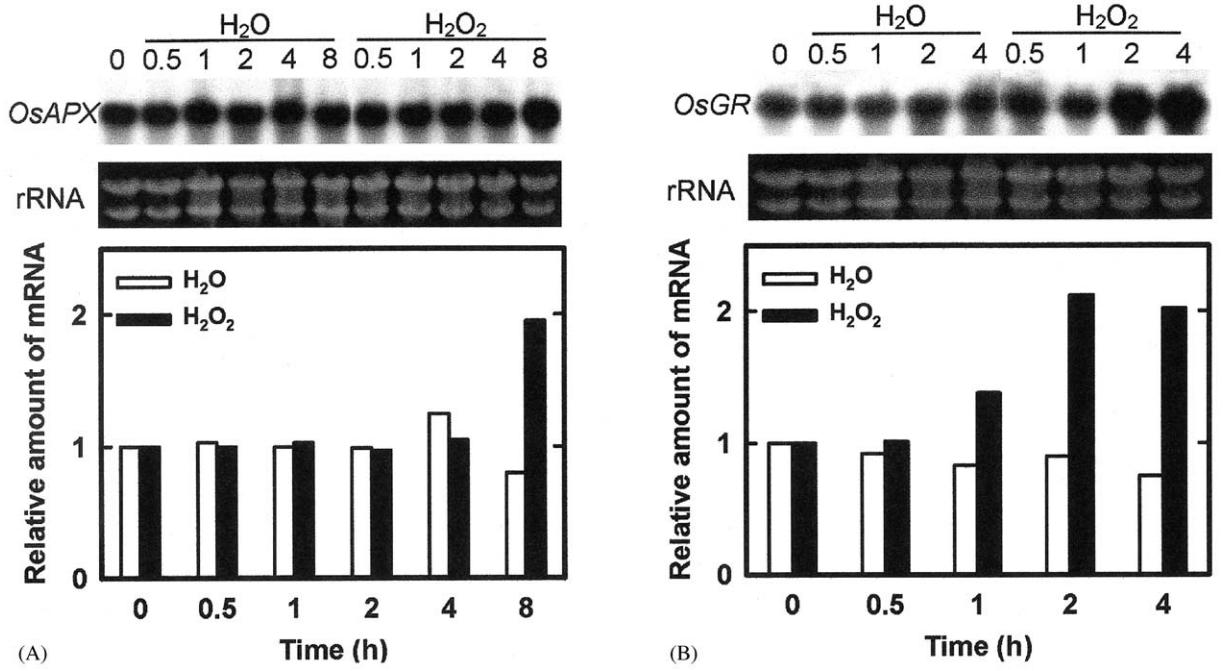

Figure 4. Changes in mRNA abundance of *OsAPX* (A) and *OsGR* (B) in roots of rice seedlings in the presence or absence of H_2O_2. Two-day-old rice seedlings were treated either with distilled water or H_2O_2 (10 mmol L^{-1}). Northern blots of total RNA samples were hybridized with *OsAPX* (A) and *OsGR* (B). The values of mRNA of *OsAPX* and *OsGR* were adjusted by its corresponding amount of rRNA for equality of loading. After the adjustment of rRNA, the reaction with roots in water was treated as the normalized reference, with a value of one, for determining the relative amount of mRNA of *OsAPX* and *OsGR*.

et al. (1996) reported that APX activity, not the mRNA level, was enhanced by NaCl-stressed *R. sativus* plants. In the present study, we observed that NaCl treatment resulted in an enhancement of *OsAPX* and *OsGR* in the roots of rice seedlings (Figs. 3A and B). Our results reveal that *OsAPX* and *OsGR* gene expression is upregulated by NaCl in rice roots.

Induction of *APX* and *GR* expression by H_2O_2 has been reported (Karpinski et al., 1999; Morita et al., 1999). In agreement with these findings, both *OsAPX* and *OsGR* expression in rice roots were enhanced by H_2O_2 (Figs. 4A and B). It has been suggested that cytosolic *APX* transcripts can be upregulated by increased levels of H_2O_2 in tobacco chloroplasts as results of Cu-Zn-superoxide dismutase overexpression (Gupta et al., 1993). de Agazio and Zacchini (2001) showed that induced-of *APX* expression in spermidine-treated maize roots was mediated though H_2O_2. However, to our knowledge, the involvement of H_2O_2 in regulating GR expression has not been examined. In the present study, we observed that DPI and IMD, which reduced NaCl-induced accumulation of H_2O_2 (Fig. 2A), did not inhibit NaCl-enhanced activities of APX and GR (Figs. 2B and C) and expression of *OsAPX* and *OsGR* (Figs. 3A and B). Similarly, NaCl-enhanced activities of APX and GR and expression of *OsAPX* and *OsGR* were not reduced by DMTU, a chemical trap for H_2O_2 (Figs. 2B, C, 3A, and B). Our results suggest that NaCl-enhanced activities of APX and GR and expression of *OsAPX* and *OsGR* are not mediated through H_2O_2 in rice roots. We have measured total root H_2O_2 levels; however, different subcellular activities of antioxidant enzymes could interact the cell to create local differences of H_2O_2 levels in different cellular compartments and therefore, we cannot exclude the involvement of H_2O_2 in this signaling pathway in rice roots during NaCl stress.

Acknowledgements

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