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RESEARCH PAPER

Expression of *ASCORBATE PEROXIDASE 8* in roots of rice (*Oryza sativa* L.) seedlings in response to NaCl

Chwan-Yang Hong[1], Yi Ting Hsu[2], Yu-Chang Tsai[2,*] and Ching Huei Kao[2,†]

[1] *Department of Agricultural Chemistry and Institute of Biotechnology, National Taiwan University, Taipei, Taiwan, Republic of China*

[2] *Department of Agronomy, National Taiwan University, Taipei, Taiwan, Republic of China*

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Abstract

Reactive oxygen species are thought to play an important role in NaCl stress. Therefore, the expression patterns of the gene family encoding the H_2O_2-scavenging enzyme ascorbate peroxidase (APx; EC1.11.1.11) were analysed in roots of etiolated rice (*Oryza sativa* L.) seedlings in response to NaCl stress. Applying semi-quantitative RT-PCR, the mRNA levels were quantified for two cytosolic (*OsAPx1* and *OsAPx2)*, two peroxisomal (*OsAPx3* and *OsAPx4*), and four chloroplastic (*OsAPx5*, *OsAPx6*, *OsAPx7*, and *OsAPx8*) isoforms identified in the rice genome. NaCl at 150 mM and 200 mM increased the expression of *OsAPx8* and the activities of APx, but had no effect on the expression of *OsAPx1*, *OsAPx2*, *OsAPx3*, *OsAPx4*, *OsAPx5*, *OsAPx6*, and *OsAPx7* in rice roots. However, NaCl at 300 mM up-regulated *OsAPx8* expression, increased APx activity, and down-regulated *OsAPx7* expression, but had no effect on the expression of *OsAPx1*, *OsAPx2*, *OsAPx3*, *OsAPx4*, *OsAPx5*, and *OsAPx6.* The accumulation of abscisic acid (ABA) in response to NaCl was observed in rice roots. Exogenously applied ABA also specifically enhanced the expression of *OsAPx8* in rice roots. The accumulation of ABA in rice roots in response to NaCl was inhibited by fluridone (Flu), an inhibitor of carotenoid biosynthesis. Flu treatment also suppressed NaCl-enhanced *OsAPx8* expression and APx activity. The effect of Flu on the expression of *OsAPx8* and increase in APx activity was reversed by the application of ABA. It appears that NaCl-enhanced expression of *OsAPx8* in rice roots is mediated through an accumulation of ABA. Evidence is provided to show that Na^+ but not Cl^- is required for enhancing *OsAPx8* expression, APx activity, and ABA accumulation in rice roots treated with NaCl. H_2O_2 treatment resulted in an enhancement of *OsAPx8* induction but no accumulation of ABA. Diphenylene iodonium treatment, which is known to inhibit NaCl-induced accumulation of H_2O_2 in rice roots, did not suppress *OsAPx8* induction and ABA accumulation by NaCl. It appears that H_2O_2 is not involved in the regulation of NaCl-induced *OsAPx8* expression in rice roots.

Key words: Abscisic acid, ascorbate peroxidase, hydrogen peroxide, *Oryza sativa*, salt stress.

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. Salinity influences a number of physiological processes. These processes include photosynthesis, nutrient uptake, water absorption, root growth, and cellular metabolism (Werner and Finkelstein, 1995; Hasegawa *et al*., 2000; Lin and Kao, 2001*a*, Netondo *et al*., 2004; Niewiadomska *et al*., 2004; Chen *et al*., 2007).

Roots play a number of important roles during plant growth and development, and typically are the first and critical part of the plant to encounter soil salinity. When growing in saline soil, roots have to cope with two types of stress. The first of these is an osmotic stress resulting

* Present address: Department of Biochemistry and Cell Biology, Rice University, MS-140, Houston, TX 77005, USA.
† To whom correspondence should be addressed. E-mail: kaoch@ntu.edu.tw

Abbreviations: ABA, abscisic acid; APx, ascorbate peroxidase; DPI, diphenylene iodonium; ELISA, enzyme-linked immunosorbent assay; Flu, fluridone; ROS, reactive oxygen species.

from salt concentration in the soil that results in lowered water potential and a consequent loss of cell turgor in roots. The second is ionic stress induced by changes in the concentrations of Na^+, Cl^-, or both in the root growing medium and within root tissues. In addition to its known components of osmotic stress and ion toxicity, salt stress is also manifested as an oxidative stress, all of which contribute to its deleterious effects (Gueta-Dahan *et al*., 1997; Hernández *et al*., 2001; Shalata *et al*., 2001).

The increase in reactive oxygen species (ROS) seems to occur as a response to most, if not all, abiotic stresses including drought (Smirnoff, 1993) and salinity (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; Tsai *et al*., 2004). To minimize and/or to protect against the toxic effects of these damaging ROS, cells have evolved highly regulated enzymatic and non-enzymatic mechanisms to keep a balance between ROS production and destruction in order to maintain cellular redox homeostasis. ROS-scavenging enzymes include superoxide dismutase, ascorbate peroxidase (APx), glutathione reductase, and catalase (Scandalios, 2002; Mittler *et al*., 2004).

APx (EC 1.11.1.11) belongs to the class I haem-containing peroxidases found in higher plants (Takeda *et al*., 1998) and catalyses the conversion of H_2O_2 to H_2O and O_2 using ascorbate as the specific electron donor (Asada, 1999). It plays an important role in scavenging and in protecting cells against the toxic effects of H_2O_2 in higher plants (Shigeoka *et al*., 1980). The fact that APx has a high affinity for H_2O_2 and is able to detoxify low concentrations of H_2O_2, whereas catalase has a high reaction rate but a low affinity for H_2O_2, renders APx an ideal candidate for tight regulation of H_2O_2.

APx is located in different cellular compartments. Eight types of *APx* have been described for *Oryza sativa*: two cytosolic (*OsAPx1* and *OsAPx2*), two putative peroxisomal (*OsAPx3* and *OsAPx4*), and four chloroplastic isoforms (*OsAPx5*, *OsAPx6*, *OsAPx7*, and *OsAPx8*) (Teixeira *et al*., 2004). Using green fluorescent protein–APx fusion proteins in BY-2 cells, Teixeira *et al*. (2006) observed that OsAPx6 is located in mitochondia, in addition to a chloroplast location.

Expression of *APx* has been reported to be enhanced in plants by drought and salt (Smiroff and Colombe, 1988; Mittler and Zilinskas, 1992, 1994; Hernández *et al*., 1995; Savouré *et al*., 1999; Sreenivasulu *et al*., 2000; Kawasaki *et al*., 2001; Tsai *et al*., 2004, 2005). In contrast, Park *et al*. (2004) reported that treatment of sweet potato leaves with NaCl reduced the expression of *swAPx1* mRNA. Moreover, it has been demonstrated that the steady-state transcript level of cytosolic *APx* was not affected by NaCl stress (Lopez *et al*., 1996; Yoshimura *et al*., 2000; Menezes-Benavente *et al*., 2004). Recently, Teixeira *et al*. (2006) reported that three rice *APx* genes (*OsAPx*2, *OsAPx*7, and *OsAPx*8) showed altered transcript levels in response to NaCl treatment. The expression of *OsAPx*2 and *OsAPx*7 was increased, whereas the *OsAPx*8 transcript accumulation was strongly suppressed in plants subjected to salt stress (Teixeira *et al*., 2006).

The plant hormone abscisic acid (ABA) is a sesquiterpenoid derived from xanthophylls (Seo and Koshiba, 2002; Nambara and Marion-Poll, 2005) and appears to influence several physiological and developmental events (Zeevaart and Creelman, 1988; Seo and Koshiba, 2002). It has been shown that ABA accumulates in plants under salt stress (Moons *et al*., 1995; Montero *et al*., 1997). Many stress-inducible genes are induced by exogenous ABA treatment. It has been demonstrated that ABA application increased the expression of pea *APx1* (Mittler and Zilinskas, 1992), *OsAPx*1 and *OsAPx*2 (Agrawal *et al*., 2003), and *swAPx1* (Park *et al*., 2004), but had no effect on *APx* gene expression in *Brassica napus* (Vansuyt *et al*., 1997) and BY-2 cells (Bueno *et al*., 1998). Recently, the link between the induction of *APx2* expression and leaf water status has been suggested to be mediated by ABA in *Arabidopsis* (Fryer *et al*., 2003).

H_2O_2 is a major ROS generated in plants under stress, which is scavenged by a network of low molecular weight antioxidants and antioxidant enzymes (Asada, 1999). H_2O_2 has also been implicated in initiating defence responses to a diverse range of biotic and abiotic stresses. It has been shown previously that NaCl treatment increased the H_2O_2 level in roots of rice seedlings (Lin and Kao, 2001*a*). H_2O_2 induced the expression of a gene encoding *APx* in germinating rice embryos (Morita *et al*., 1999). However, the failure of H_2O_2 to induce the *APx* gene has also been reported (Vansuyt *et al*., 1997). It has been suggested that cytosolic *APx* transcripts can be up-regulated by increased levels of H_2O_2 in tobacco chloroplasts as a result of Cu-Zn-superoxide dismutase overexpression (Gupta *et al*., 1993). de Agazio and Zacchini (2001) demonstrated that dimethylthiourea, 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. Recent experiments indicate that H_2O_2 is the principal candidate ROS as a signal involved in the induction of *APx2* expression in *Arabidopsis* leaves by high light stress (Karpinski *et al*., 1997; Fryer *et al*., 2003; Chang *et al*., 2004).

It has been demonstrated previously that *OsAPx* gene expression was increased in response to NaCl and H_2O_2 in roots of etiolated rice seedlings (Tsai *et al*., 2004, 2005). These data were obtained using a non-specific probe, which meant it was not possible to show precisely which member(s) of the *OsAPx* gene family was induced in response to the NaCl and H_2O_2 treatments. In this study, using the 3′-untranslated region (UTR)-specific primers

for the *OsAPx1*, *OsAPx2*, *OsAPx3*, *OsAPx4*, *OsAPx5*, *OsAPx6*, *OsAPx7*, and *OsAPx8* genes from rice, the effect of NaCl, ABA, and H_2O_2, on the expression of *OsAPx* genes was first examined followed by an investigation of whether the induction of *OsAPx* genes by NaCl is mediated through ABA or H_2O_2.

Materials and methods

Plant material growth conditions

Rice (*O. 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 obtain more uniformly germinated seeds, rice seeds in a Petri dish (20 cm) containing distilled water were pre-treated at 37 °C for 1 d under dark conditions. Uniformly germinated seeds were then selected and transferred to a Petri dish (9.0 cm) containing two sheets of Whatman No.1 filter paper (Whatman, UK) moistened with 10 ml of distilled water for 2 d. Two-day-old seedlings were then transferred to distilled water, NaCl, ABA, fluridone (Flu), $NaNO_3$, H_2O_2, and diphenylene iodonium (DPI) at the desired concentration as specified in the individual experiments. Root growth of rice seedlings grown in distilled water is similar to that of those grown in medium containing inorganic salts, thus seedlings grown in distilled water were used as the controls. Each Petri dish contained 20 seedlings and each treatment was replicated four times. The seedlings were allowed to grow at 27 °C in darkness. The same part of the roots of rice seedlings was used for analyses of *OsAPx* gene expression, APx activity, and ABA level.

Semi-quantitative RT-PCR analysis

Total RNA was isolated from root tissue of 2-d-old etiolated rice seedlings using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), according to the supplier's recommendations. To prevent DNA contamination, RNA was treated with Turbo DNase I (Ambion, Austin, TX, USA) for 30 min at 37 °C before the RT-PCR analysis. The reverse transcription reactions were conducted using the SuperScript III platinum one-step quantitative RT-PCR system (Invitrogen) according to the manufacturer's protocol.

The gene-specific primers were designed from the 3′-UTR of the *OsAPx* genes (Teixeira *et al.*, 2006). The sequences used, the predicted amplicons, and the cycle numbers are listed in Table 1. The RT-PCR program initially started with 50 °C/30 min; 94 °C denaturation for 6 min, followed by 94 °C/30 s, and 22–32 cycles of 50 °C/30 s, 68 °C/30 s. The PCRs were optimized for a number of cycles to ensure product intensity within the linear phase of amplification. All tests were repeated at least three times, and one of the repeats is shown in the Results. For all treatments, three replicates of RT-PCR were conducted with three batches of total RNA samples isolated independently. PCR products were resolved by electrophoresis in a 3% agarose gel, stained with ethidium bromide. The gel images were digitally captured with a SynGene gel documentation system and analysed with the Genetools analysis software (Syngene, Frederick, MD, USA). The rice *OsActin* gene was used as a reference for normalization.

Extraction and assay of APx

For extraction of APx, root tissues were homogenized with 0.1 M sodium phosphate buffer (pH 6.8) containing 2 mM ascorbate in a chilled pestle and mortar. The homogenate was centrifuged at 12 000 *g* for 20 min and the resulting supernatant was used for the determination of APx 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 the optical density at 290 nm, and activity was calculated using the extinction coefficient (2.8 mM^{-1} cm^{-1} at 290 nm) for ascorbate. One unit of APx was defined as the amount of enzymes that breaks down 1 μmol of ascorbate min^{-1}.

Determinations of ABA

For extraction of ABA, roots were homogenized with a pestle and mortar in extraction solution (80% methanol containing 2% glacial acetic acid). To remove plant pigments and other non-polar compounds which could interfere in the immunoassay, extracts were first passed through a polyvinylpyrrolidone column and C18 (Sep-Pak Vac) cartridges (Waters, Milford, MA, USA). The eluates were concentrated to dryness by vacuum evaporation and resuspended in TRIS-buffered saline before enzyme-linked immunosorbent assay (ELISA). ABA was quantified by ELISA (Walker-Simmons, 1987). The ABA immunoassay detection kit (Phytodetek) was purchased from Agdia (Elkhart, IN, USA) and is specific for (+)-ABA. By evaluating [^{3}H]ABA recovery, [^{3}H]ABA

Table 1. *Primers used in semi-quantitative RT-PCR assay*

Gene	Primer	Sequence (5′ to 3′)	Products (bp)	Cycles
OsAPx1	APx1-5′	TAGTCTACTACTGCTAGTAC	160	22
	APx1-3′	TAACAGCCCACCGAGACATT		
OsAPx2	APx2-5′	AGAGTCAGTACGATCAAGAC	183	22
	APx2-3′	TCTTGACAGCAAATAGCTTGG		
OsAPx3	APx3-5′	GCATCGATCACAATGATCG	137	28
	APx3-3′	CAGCACTCACATATATATACC		
OsAPx4	APx4-5′	GATTGCTATGTTCTTCATCA	203	25
	APx4-3′	GAGCACACAGAAGACGGAATA		
OsAPx5	APx5-5′	GAGTGATAAACAAGATAATACCT	225	32
	APx5-3′	ACTGAGGTTGTGATGCATCT		
OsAPx6	APx6-5′	GCTACTATAGAGCATATTATG	104	32
	APx6-3′	CTAATGGAGAGCACAACTCA		
OsAPx7	APx7-5′	TGAGCCAGATCGCTGAAGTG	135	22
	APx7-3′	TCCAATATGACTCGTGGTCA		
OsAPx8	APx8-5′	TGGTCTGATGACCTCCTCTGA	222	28
	APx8-3′	CATGAGCCATGACAACTAGA		
OsActin	Actin-5′	ATGCTCTCCCCCATGCTATC		
	Actin-3′	TCTTCCTTGCTCATCCTGTC	465	20

loss was <3% by the method described here. ABA content is expressed on the basis of dry weight.

Statistical analysis

Statistical differences between measurements (n=4–6) on different treatments or on different times were analysed using the LSD test.

Results

NaCl induces OsAPx8 *expression and APx activity*

In a previous work, it was shown that increasing concentrations of NaCl from 50 mM to 150 mM progressively increased APx activity (Tsai *et al*., 2004). In the present study, 2-d-old rice seedlings were treated with 150, 200, and 300 mM NaCl for 8 h. The activity of APx of NaCl-stressed rice roots was higher than that of control (Fig. 1B). However, the increase in APx activities was higher in rice roots treated with 150 mM NaCl than in those treated with 200 mM and 300 mM NaCl (Fig. 1B). To investigate the effect of different concentrations of NaCl on the expression of all eight *OsAPx* genes in rice roots, the total RNA was extracted and the expression dynamics of eight *OsAPx* genes was examined by semi-quantitative RT-PCR analysis. After 8 h treatment with NaCl (150, 200, and 300 mM), the *OsAPx8* transcript was specifically increased (~2- and 3-fold) (Fig. 1A). Figure 1A also shows that the *OsAPx8* expression in rice roots induced by 200 mM and 300 mM NaCl was less than that induced by 150 mM NaCl. However, no significant increase due to NaCl (150, 200, and 300 mM) could be detected in the expression of *OsAPx1*, *OsAPx2*, *OsAPx3*, *OsAPx4, OsAPx5*, and *OsAPx6* (Fig. 1A). The expression of *OsAPx7* was not affected by 150 mM and 200 mM NaCl, but was decreased (~40%) by 300 mM NaCl (Fig. 1A).

When 2-d-old seedlings were subjected to 150 mM NaCl for 0.5, 1, 2, and 4 h, it was observed that the *OsAPx8* transcript was specifically increased (~2-fold) after 1 h treatment with NaCl (Fig. 2A). However, no significant increase due to NaCl could be detected in the expression of *OsAPx1*, *OsAPx2*, *OsAPx3*, *OsAPx4*, *OsAPx5*, *OsAPx6*, and *OsAPx*7 (Fig. 2A).

NaCl increases ABA level

It has been shown that ABA accumulates in plant tissues in response to salt stress (Moons *et al*., 1995; Montero *et al*., 1997). To understand if NaCl treatment results in accumulation of ABA in roots of rice seedlings, the level of ABA in rice roots was determined by ELISA. When 2-d-old rice seedlings were treated with 150 mM NaCl, the level of ABA in roots increased rapidly and peaked

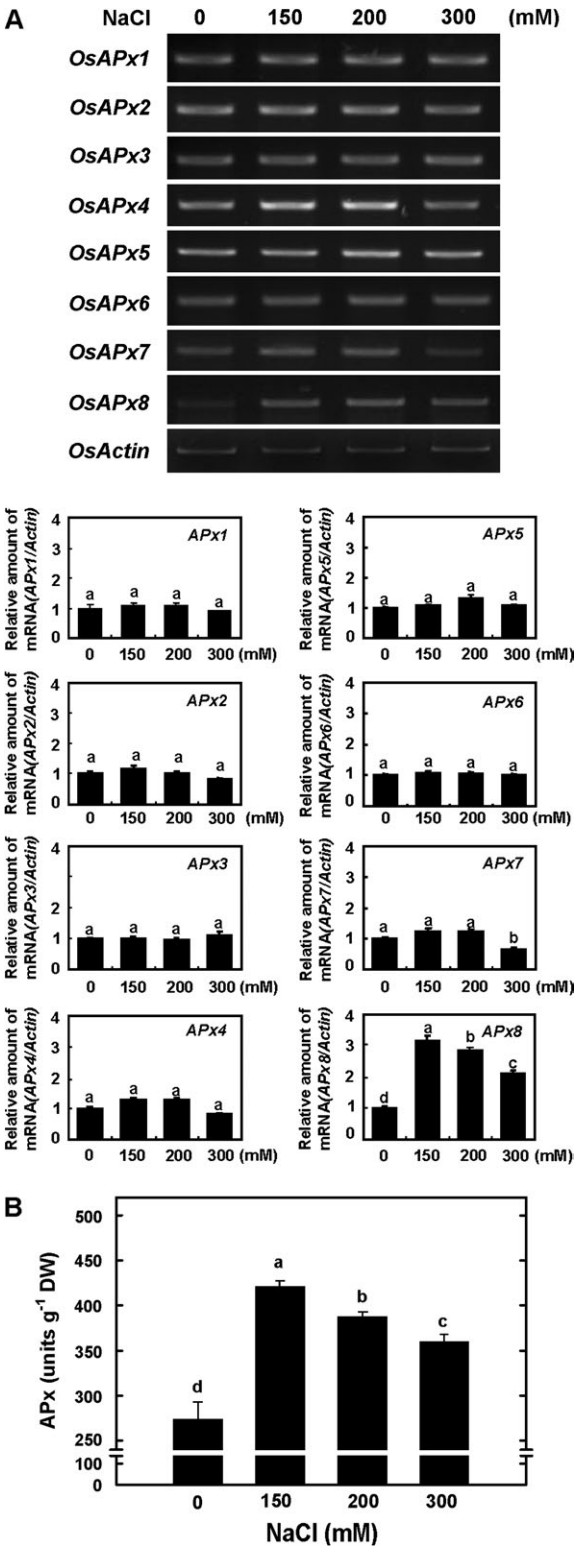


Fig. 1. Effect of NaCl concentration on mRNA levels for *OsAPx* genes (A) and APx activities (B) in root of rice seedlings. Two-day-old seedlings were treated with NaCl (0–300 mM) for 8 h. Semi-quantitative RT-PCR for *OsAPx* genes was performed as described in Materials and methods. The values of mRNA for the *OsAPx* genes were adjusted by the corresponding amount of *OsActin* mRNA for equality of loading. After the adjustment by *OsActin*, the reaction with the roots in 0 mM NaCl was treated as the normalized reference, with a value of one, for determination of the relative amount of mRNA of *OsAPx* genes. Bars show means ±SE (n=4–6). Values with the same letter are not significantly different at P <0.05.

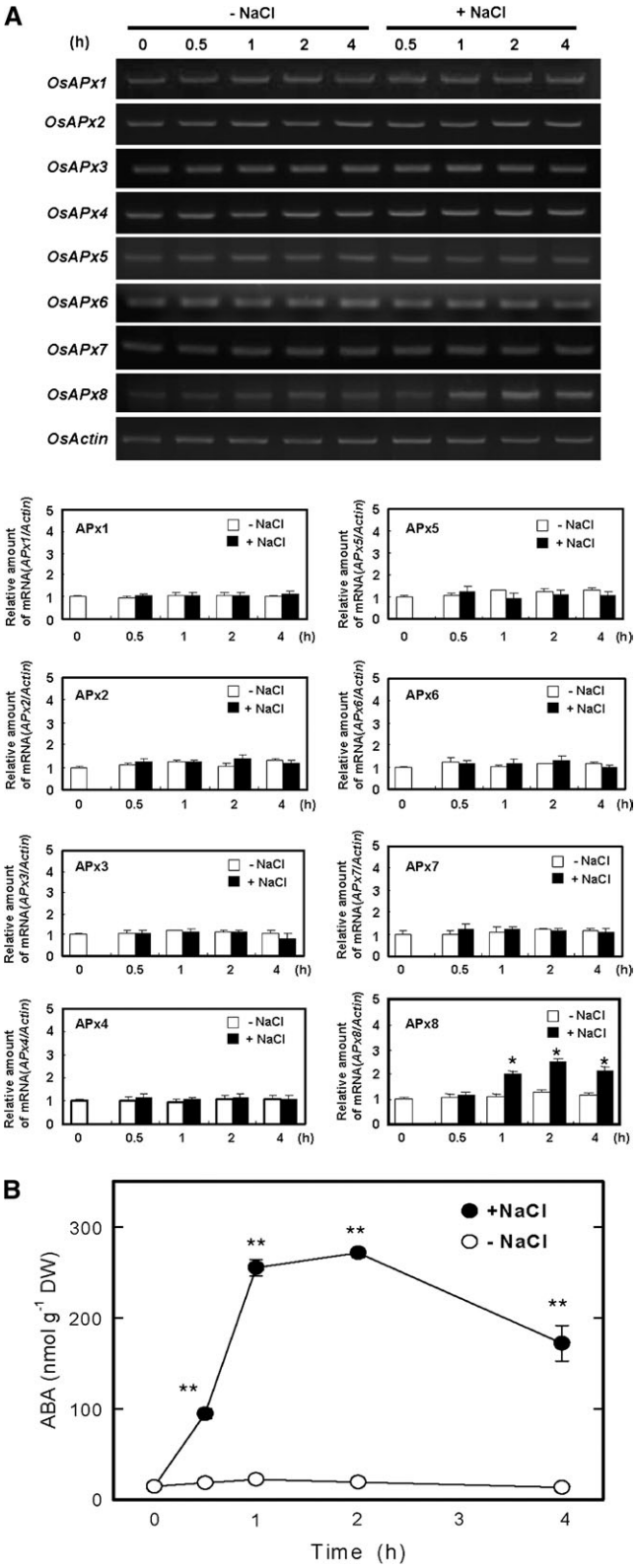


Fig. 2. Changes in mRNA level for *OsAPx* genes (A) and ABA levels (B) in rice roots in the presence or absence of NaCl. Two-day-old rice seedlings were treated either or not with NaCl (150 mM). Semi-quantitative RT-PCR for *OsAPx* genes was performed as described in Materials and methods. The values of mRNA for the *OsAPx* genes were adjusted by the corresponding amount of *OsActin* mRNA for equality of loading. After the adjustment by *OsActin*, the reaction with the roots without NaCl was treated as the normalized reference, with a value of one, for determination of the relative amount of mRNA of *OsAPx* genes. Bars show means ±SE (n=4–6). * and ** represent values that are significantly different between – NaCl and + NaCl treatments at P <0.05 and 0.01, respectively.

2 h after NaCl treatment, and then declined (Fig. 2B). The increase in ABA level due to NaCl (0.5 h after treatment) was observed to occur prior to the induction in *OsAPx8* expression (1 h after treatment) (Fig. 2A, B).

Exogenous application of ABA induces OsAPx8 *expression*

To test whether ABA is involved in the regulation of *OsAPx* genes, the effect of 9 μM ABA on the expression of *OsAPx* genes was examined. It was observed that *OsAPx8* mRNA was significantly increased by ABA after 0.5 h of treatment in comparison with the control (Fig. 3A). However, ABA treatment had no effect on the expression of *OsAPx1*, *OsAPx2*, *OsAPx3*, *OsAPx4*, *OsAPx5*, *OsAPx6*, and *OsAPx7* (Fig. 3A). Figure 3B also shows that the increase in ABA level could be detected at 0.5 h after ABA treatment.

Fluridone effect

The role of ABA in NaCl-enhanced expression of the *OsAPx8* gene was tested further by using Flu, which is known to inhibit the conversion of phytoene to phytofluene in the carotenoid biosynthesis pathway (Kowalczyk-Schröder and Sandmann, 1992). The data revealed that NaCl-enhanced ABA accumulation in rice roots was significantly reduced by Flu pre-treatment (Fig. 4C). NaCl-enhanced *OsAPx8* expression and APx activity in rice roots was also observed to be suppressed by Flu (Fig. 4A, B). The effect of Flu on the expression of *OsAPx8* and APx activity can be reversed by the application of ABA (Fig. 4A, B).

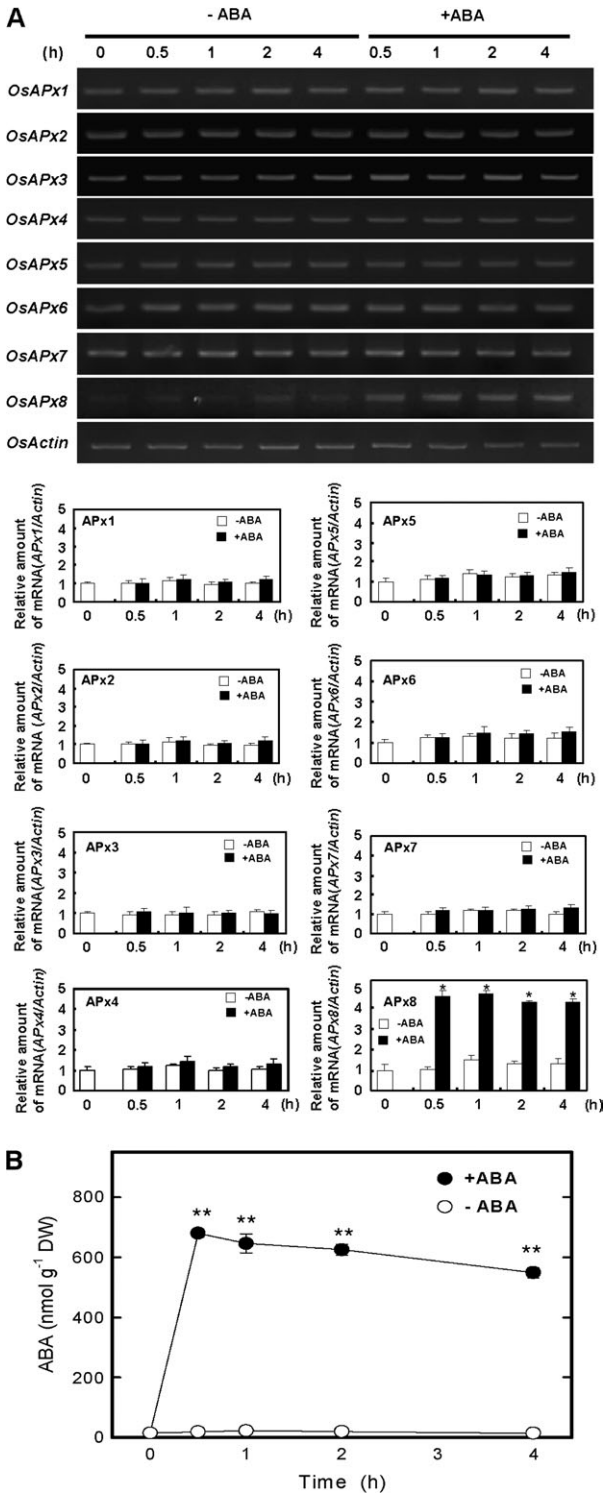
Na^+ but not Cl^- is required for increasing OsAPx8 *expression, APx activity, and ABA level*

To test whether Cl^- is involved in enhancing the expression of *OsAPx8*, experiments were performed to compare the effect of NaCl (150 mM) with that of $NaNO_3$ (150 mM). The effect of $NaNO_3$ and NaCl on the expression of *OsAPx8*, APx activity, and ABA level is shown in Fig. 5A–C. Clearly, *OsAPx8* transcript, APx activity, and ABA level in roots treated with $NaNO_3$ are similar to those in roots treated with NaCl.

NaCl-induced OsAPx8 *expression is not controlled by H_2O_2*

The effect of 10 mM H_2O_2 on the expression of the *OsAPx* genes is shown in Fig. 5A. H_2O_2 treatment had no effect on the expression of the *OsAPx1*, *OsAPx2*, *OsAPx3*,

OsAPx4, *OsAPx5*, *OsAPx6*, and *OsAPx7* in rice roots. In contrast, H_2O_2 significantly increased the expression of *OsAPx8*. H_2O_2 treatment enhanced the expression of *OsAPx8* in rice roots at about the same magnitude (~2-fold increase) as NaCl treatment (Fig. 6A). However, NaCl, but not H_2O_2, increased the ABA level in rice roots (Fig. 6B). In the present study, it was also observed that 0.1 μM DPI pre-treatment had no effect on the expression of *OsAPx8* (Fig. 7A) and the level of ABA (Fig. 7B) in NaCl-treated rice roots.



Discussion

There are eight *APx* genes in rice (Morita *et al*., 1997, 1999; Agrawal *et al*., 2003; Teixeira *et al*., 2004, 2006). Here, it is shown that the transcripts of eight *OsAPx* genes were detectable in roots of 2-d-old etiolated rice seedlings (Figs 1A, 2A). The expression profile of individual *APx* genes of plants in response to NaCl has been reported (Savouré *et al*., 1999; Menezes-Benavente *et al*., 2004; Park *et al*., 2004). Teixeira *et al*. (2006) were the first to conduct a systematic study of the expression patterns of *OsAPx* genes in response to NaCl. In their experiments, 2-week-old greenhouse-grown rice plants (cv. Taim7) were treated with 250 mM NaCl for a much longer time (24–96 h). They demonstrated that the expression of *OsAPx2* and *OsAPx7* increased during NaCl treatment, whereas the expression of *OsAPx8* was drastically down-regulated by NaCl stress. In the present study, 2-d-old etiolated rice seedlings (cv. Taichung Native 1) were exposed to 150–300 mM NaCl for 8 h. It is shown that *OsAPx8* expression in rice roots was specifically enhanced by all concentrations of NaCl tested and *OsAPx7* expression was down-regulated by 300 mM NaCl (Fig. 1A). Thus, the discrepancy in the regulation of the *OsAPx* genes of rice plants in response to NaCl between the present results and the results of Teixeira's group is unlikely to be due to different NaCl concentrations, but is more probably due to differences in cultivars, plant age, organs, and growing conditions.

The level of ABA in plants increases upon their exposure to environmental stress (Zeevaart and Creelman, 1988), such as drought (Tian *et al*., 2004) and salinity (Moons *et al*., 1995; Montero *et al*., 1997). Here, it is shown that ABA accumulation in rice roots was induced by NaCl stress (Fig. 2B). It is now well established that ABA in higher plants is derived from C_{40}-carotenoids (Seo and Koshiba, 2002; Nambara and Marion-Poll, 2005). As Flu is an inhibitor of ABA biosynthesis through the

Fig. 3. Changes in mRNA levels of *OsAPx* genes (A) and ABA levels (B) in rice roots in the presence or absence of abscisic acid (ABA). Two-day-old rice seedlings were treated with distilled water or ABA (9 M). Semi-quantitative RT-PCR for *OsAPx* genes was performed as described in Materials and methods. The values of mRNA for the *OsAPx* genes were adjusted by the corresponding amount of *OsActin* mRNA for equality of loading. After the adjustment by *OsActin*, the reaction with the roots without ABA was treated as the normalized reference, with a value of one, for determination of the relative amount of mRNA of *OsAPx* genes. Bars show means ±SE (n=4–6). * and ** represent values that are significantly different between – ABA and + ABA treatments at P <0.05 and 0.01, respectively.

carotenoid pathway (Kowalczyk-Schröder and Sandmann, 1992), the effects of this inhibitor on the reduction of ABA accumulation in NaCl-treated rice roots (Fig. 4C) may imply that the ABA biosynthetic pathway in response to NaCl appears to be the same as that established in other stress conditions (Zeevaart and Creelman, 1998; Seo and Koshiba, 2002).

It has been shown the ABA application increased the expression of *APx* genes in pea, rice, and sweet potato (Mittler and Zilinska, 1992; Agrawal *et al*., 2003; Park *et al*., 2004), but had no effect on *APx* gene expression in *Brassica napus* (Vansuyt *et al*., 1997) and BY-2 cells (Bueno *et al*., 1998). Recently, the link between the expression of *APx2* and leaf water status has been suggested to be mediated by ABA in *Arabidopsis* (Fryer *et al*., 2003). In the present study, it is shown that exogenous ABA specifically induced the expression of *OsAPx8* in rice roots (Fig. 3A).

In stress-induced gene expression, ABA has been thought to be a candidate for a signal transducer. The present study indicated that ABA was involved in regulating the expression of *OsAPx8* in rice roots by NaCl. This conclusion was based on the following observations: (i) NaCl treatment resulted in an increase in the endogenous level of ABA (Fig. 2B) and the induction of *OsAPx8* expression in rice roots (Fig. 2A); (ii) the expression of *OsAPx8* in rice roots was enhanced by exogenous ABA (Fig. 3A); (iii) the increase in ABA levels due to NaCl preceded the enhancement of *OsAPx8* expression (Fig. 2); (iv) Flu treatment reduced the ABA level, as well as NaCl-induced *OsAPx8* expression (Fig. 4); and (v) the effect of Flu on the reduction of *OsAPx8* expression caused by NaCl can be reversed by the application of ABA (Fig. 4A). The present results suggest that NaCl-enhanced *OsAPx8* expression is mediated through ABA accumulation in rice roots.

In previous work, it was shown that increasing concentrations of NaCl from 50 mM to 150 mM progressively increased both Na^+ and Cl^- levels in roots of rice seedlings (Lin and Kao, 2001*b*). Of particular interest in the present study are the findings that Na^+ but not Cl^- is required for the NaCl-enhanced expression of *OsAPx8*, APx activity, and ABA level in rice roots (Fig. 5).

Induction of *APx* expression by H_2O_2 has been reported before (Karpinski *et al*., 1999; Morita *et al*., 1999). In agreement with these findings, *OsAPx8* expression in rice roots was enhanced by H_2O_2 (Fig. 5A). Recently, de Pinto *et al*. (2006) reported that the level and timing of H_2O_2

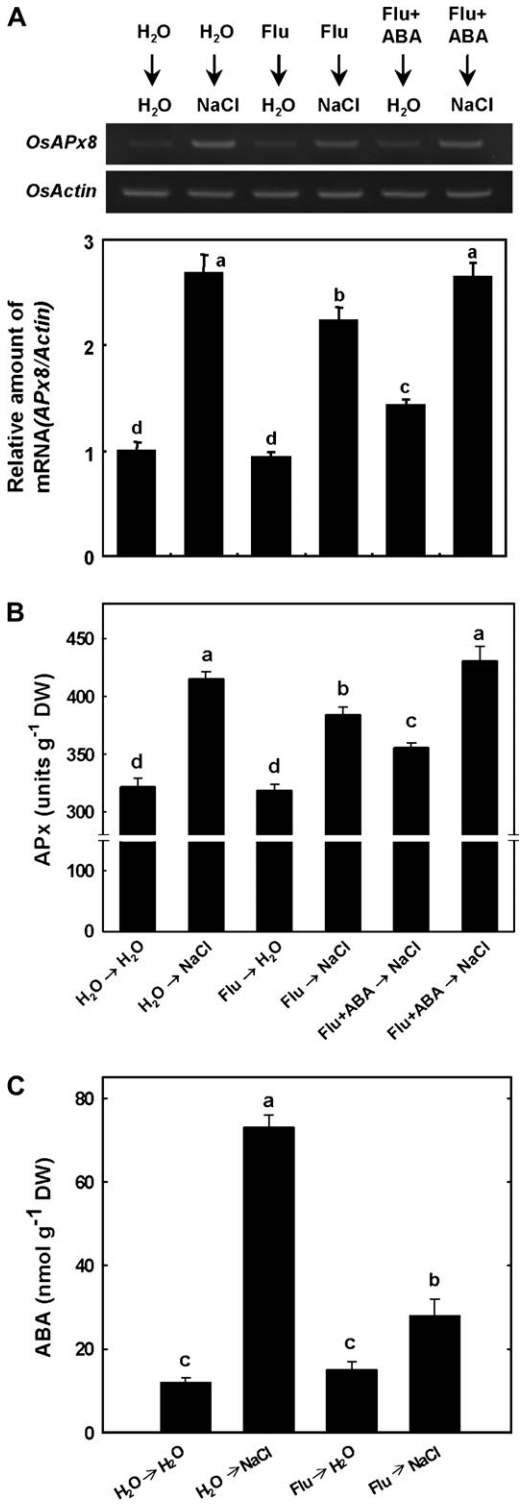


Fig. 4. Effect of fluridone (Flu) and abscisic acid (ABA) on mRNA levels for *OsAPx8* (A), APx activities (B), and ABA levels (C) in roots of rice seedlings in the presence or absence of NaCl. Two-day-old rice seedlings were pre-treated with H_2O, Flu (0.2 mM), or Flu (0.2 mM) + ABA (9 μM) for 2 h and then transferred to H_2O_2 and NaCl (150 mM), respectively. for 8 h. Semi-quantitative RT-PCR for *OsAPx8* was performed as described in Materials and methods. The values of mRNA for *OsAPx8* were adjusted by the corresponding amount of *OsActin* mRNA for equality of loading. After the adjustment by *OsActin*, the reaction with the roots in H_2O was treated as the normalized reference, with a value of one, for determination of the relative amount of mRNA of *OsAPx8*. Bars show means ±SE (n=4–6). Values with the same letter are not significantly different at P <0.05.

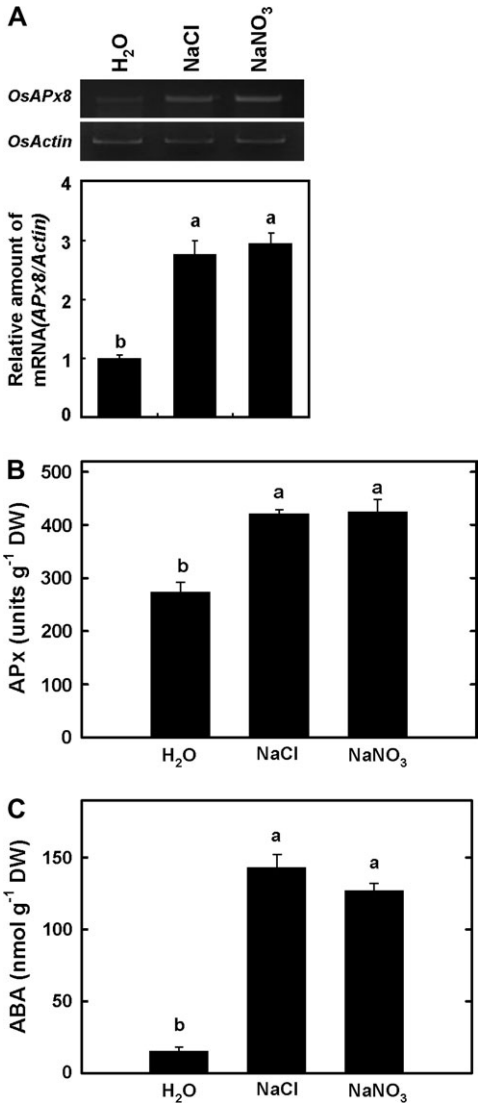


Fig. 5. Effect of NaCl and $NaNO_3$ on the mRNA levels for *OsAPx8* (A), APx activities (B), and ABA levels (C) in roots of rice seedlings. Two-day-old rice seedlings were treated with H_2O, NaCl (150 mM), or $NaNO_3$ (150 mM) for 8 h. Semi-quantitative RT-PCR for *OsAPx8* was performed as described in Materials and methods. The values of mRNA for *OsAPx8* were adjusted by the corresponding amount of *OsActin* mRNA for equality of loading. After the adjustment by *OsActin*, the reaction with the roots in H_2O was treated as the normalized reference, with a value of one, for determination of the relative amount of mRNA of *OsAPx8*. Bars show means ±SE (n=4–6). Values with the same letter are not significantly different at P <0.05.

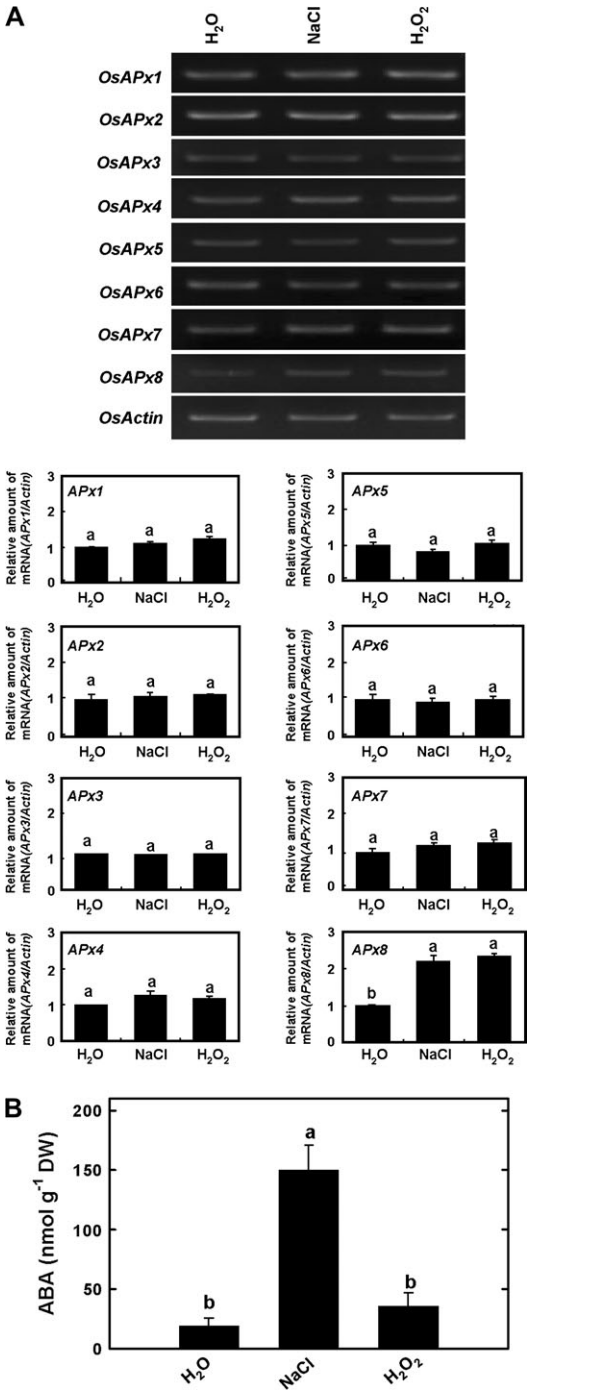


Fig. 6. Effect of NaCl and H_2O_2 on the mRNA levels for *OsAPx* genes (A) and ABA levels (B) in roots of rice seedlings. Two-day-old rice seedlings were treated with H_2O, NaCl (150 mM), or H_2O_2 (10 mM) for 8 h. Semi-quantitative RT-PCR for *OsAPx* genes was performed as described in Materials and methods. The values for mRNA of *OsAPx* genes were adjusted by the corresponding amount of *OsActin* mRNA for equality of loading. After the adjustment by *OsActin*, the reaction with the roots in H_2O was treated as the normalized reference, with a value of one, for determination of the relative amount of mRNA of *OsAPx8*. Bars show means ±SE (n=4–6). Values with the same letter are not significantly different at P <0.05.

production in tobacco BY-2 cells are critical points for APx regulation. The constant production of low amounts of H_2O_2, which was ineffective in inducing cell death, determines a transient, modest increase in APx activity. DPI is an inhibitor of NADPH oxidases and other flavoenzymes (Cross and Jones, 1986; O'Donnell *et al*., 1993; Moulton *et al*., 2000). In previous work, it could be shown that NaCl-induced H_2O_2 accumulation was significantly inhibited by pre-treatment of rice roots with 0.1 μM DPI (Tsai *et al*., 2005). This observation has led

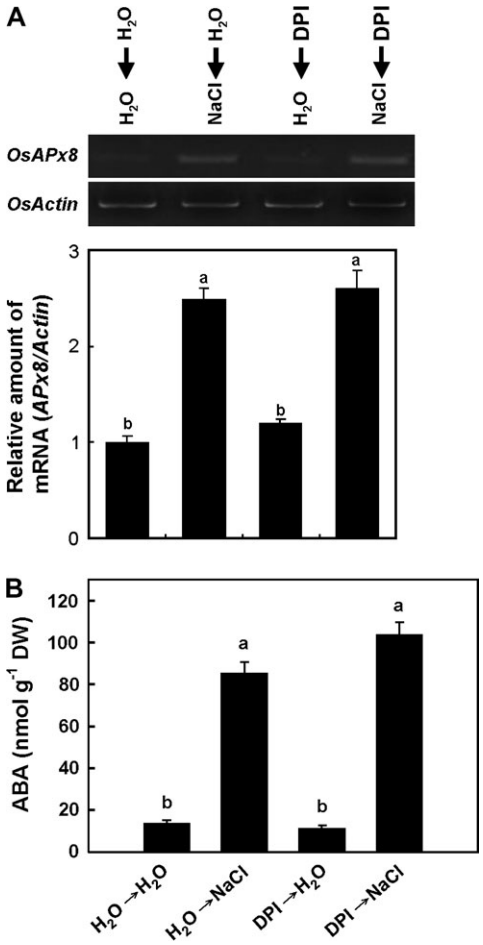


Fig. 7. Effect of diphenylene iodonium (DPI) on mRNA levels for *OsAPx8* (A) and ABA levels (B) in roots of rice seedlings in the presence or absence of NaCl. Two-day-old rice seedlings were pre-treated with H_2O or DPI (0.1 M) fror 12 h and then transferred to H_2O and NaCl (150 mM) for 24 h, respectively. Semi-quantitative RT-PCR for *OsAPx8* was performed as described in Materials and methods. The values of mRNA for *OsAPx8* were adjusted by the corresponding amount of *OsActin* mRNA for equality of loading. After the adjustment by *OsActin*, the reaction with the roots in $H_2O \rightarrow H_2O$ was treated as the normalized reference, with a value of one, for determination of the relative amount of mRNA of *OsAPx8*. Bars show means ±SE (n=4–6). Values with the same letter are not significantly different at P <0.05.

to the proposal that NaCl-induced H_2O_2 accumulation may be catalysed by NADPH oxidase (Orozoco-Cárdenas *et al*., 2001). Here, it is shown that DPI pre-treatment had no effect on the expression of the *OsAPx8* and accumulation of ABA in NaCl-treated rice roots (Fig. 7). Based on the present and previous results (Fig. 7; Tsai *et al*., 2005), it is suggested that *OsAPx8* expression and APx activity induced by NaCl are not mediated through H_2O_2 in rice roots. Total root H_2O_2 levels have been measured; however, different activities of antioxidant enzymes could interact in the cell to create local differences in H_2O_2 levels in different cellular compartments and, therefore, the involvement of H_2O_2 in this signalling pathway in rice roots during NaCl stress cannot be excluded. The fact that ABA accumulation was enhanced by NaCl but not by H_2O_2 in rice roots (Figs 2B, 6B) indicates that the signalling pathway for *OsAPx8* induction in rice roots by NaCl differs from that by H_2O_2. Ethylene, salicylic acid, and jasmonic acid have also been thought to be candidates for signal transducers. Further work is needed to determine the role of each of these candidates in *OsAPx8* gene expression.

A mutant of hexaploid wheat with reduced thylakoid-bound APx (tAPx) has been shown to exhibit impaired electron transport and photosynthetic activity (Danna *et al*., 2003). Transgenic tobacco plants overexpressing tAPx showed increased tolerance to oxidative stress caused by application of methylviologen and by chilling stress under light conditions (Yabuta *et al*., 2002). The time-course analyses of NaCl (150 mM) treatment clearly indicated that *OsAPx8* expression occurs first (1–4 h after NaCl treatment; Fig. 2A) and then APx activity (8 h after NaCl treatment; Tsai *et al*., 2005) in rice roots. These results have led to the conclusion that early expression of *OsAPx8* during NaCl treatment results in an increase in APx activity in rice roots. In the present study, evidence is also provided to show that the increase in the expression of *OsAPx8* is indeed associated with an enhancement in its APx activity (Figs 1, 4, 5). Although *OsAPx8* is a putative thylakoid isoform (Teixeira *et al*., 2004), the present results suggest that *OsAPx8* expression by NaCl may affect ROS scavenging properties in rice roots. Clearly, more experiments concerning *OsAPx8* knockout mutants and overexpression plants are required for our understanding of *OsAPx8* function in rice roots under stress conditions.

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