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REGULAR ARTICLE

NaCl-induced expression of glutathione reductase in roots of rice (*Oryza sativa* L.) seedlings is mediated through hydrogen peroxide but not abscisic acid

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Abstract Reactive oxygen species (ROS) play an important role in NaCl stress. Plants tolerant to NaCl stress may evolve certain strategies to remove these ROS, thus reducing their toxic effects. Therefore, the expression patterns of the gene family encoding glutathione reductase (GR, EC 1.6.4.2) were analyzed in roots of etiolated rice (*Oryza sativa* L.) seedlings in response to NaCl stress. Semi-quantitative RT-PCR was applied to quantify the mRNA levels for one cytosolic (*OsGR2*) and two chloroplastic (*OsGR1* and *OsGR3*) isoforms of glutathione reductase identified in the rice genome. The expression of *OsGR2* and *OsGR3* but not *OsGR1* was increased in rice roots treated with 150 mM NaCl. The *Rab16A* is an abscisic acid (ABA)-responsive rice gene. Increasing concentrations of ABA, from 1 to 12 μM, progressively increased the expression of *OsRab16A* in rice roots. In the present study, the ABA level was judged by the expression of *OsRab16A* in rice roots. Treatment with 150 mM NaCl induced the expression of *OsRab16A*, and the expression increased with increasing concentrations of ABA, which suggests that ABA may be involved in this response in rice roots. In fact, exogenous application of ABA enhanced the expression of *OsGR2* and *OsGR3* in rice roots. On inhibiting ABA accumulation with sodium tungstate (Tu), an inhibitor of ABA biosynthesis, the expression of *OsGR2* and *OsGR3* was still induced by NaCl; therefore, NaCl-triggered expression of *OsGR2* and *OsGR3* in rice roots is not mediated by accumulation of ABA. However, NaCl treatment could induce H_2O_2 production in rice roots, and H_2O_2 treatment resulted in enhanced *OsGR2* and *OsGR3* induction. On inhibiting the NaCl-induced accumulation of H_2O_2 with diphenylene iodonium, the expression of *OsGR2* and *OsGR3* was also suppressed. Moreover, the increase in H_2O_2 level was prior to the induction of *OsGR2* and *OsGR3* in NaCl-treated rice roots. Thus, H_2O_2, but not ABA, is involved in regulation of *OsGR2* and *OsGR3* expression in NaCl-treated rice roots.

Keywords Abscisic acid · Glutathione reductase · Hydrogen peroxide · *Oryza sativa* · Salt stress

Abbreviations

ABA	Abscisic acid
Asc	Ascorbic acid

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APX	Ascorbate peroxidase
CAT	Catalase
DCF-DA	2′, 7′-Dichlorofluorescein diacetate
DPI	Diphenylene iodonium
DW	Dry weight
ELISA	Enzyme-linked immunosorbent assay
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
ROS	Reactive oxygen species
SOD	Superoxide dismutase
Tu	Sodium tungstate

Introduction

Soil salinity is one of the major abiotic stresses affecting plant growth and productivity globally. 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 (Aydi et al. 2008; Meot-Duros and Magné 2008). When growing in saline soils, roots have to cope with two types of stresses, osmotic and ionic (Lin and Kao 2001b). These stresses, in turn, cause reduced water uptake and inhibition of root growth (Munns 1993).

Several reports demonstrated that salinity stress results in an excessive generation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide (H_2O_2) and hydroxyl radical (Dionisio-Sese and Tobita 1998; Lin and Kao 2001a; Hernández et al. 2001; Lee et al. 2001; Sudhakar et al. 2001; Hernández and Almansa 2002; Tsai et al. 2004). These ROS can damage DNA, protein, chlorophyll, and membrane functions. To mitigate and repair damage initiated by ROS, plants have developed a complex antioxidant system (Halliwell and Gutteridge 2007).

Glutathione (γ-glutamylcysteinylglycine) is widely distributed in eukaryotes and appears to be essential in plant cells. It is involved in detoxification of xenobiotics and heavy metals (Timmerman 1989; Steffens 1990; Cobbett 2000), in sulfur transport (Meister and Anderson 1983), in growth and developmental processes such as cell division (Vernoux et al. 2000) and flowering (Ogawa et al. 2001), in regulation of gene expression in response to abiotic and biotic stress (Wingate et al. 1983; Herouart et al. 1993; Wingsle and Karpinski 1995), and in antioxidation in both enzymatic and non-enzymatic reactions (Alscher 1989). For most of its functions, glutathione must be in its reduced form (GSH). Glutathione reductase (GR; EC 1.6.4.2) is a flavoprotein oxidoreductase that catalyzes the reduction of oxidized glutathione (GSSG) to GSH with the accompanying oxidation of NADPH. GR is thought to be an important component of the glutathione-ascorbate cycle (Noctor and Foyer 1998), the main mechanism for the detoxification of ROS in plants.

GR cDNAs from various plant species have been cloned and their sequences can be found in GenBank. In plants, GR is located in different cellular compartments. Three genes encoding GR have been described for *Oryza sativa*: a cytosolic (*OsGR2*) (Kaminaka et al. 1998) and two chloroplastic isoforms (*OsGR1* and *OsGR3*) (Chang et al. 2003; Bashir et al. 2007). *OsGR1*, *OsGR2* and *OsGR3* are located on chromosomes 3, 2, and 10, respectively.

The expression of *GR* is enhanced in plants by drought/desiccation (Contour-Ansel et al. 2006; Torres-Franklin et al. 2008) and salt (Kaminaka et al. 1998). Abscisic acid (ABA) and H_2O_2 accumulate in plants under salt stress (Moons et al. 1995; Montero et al. 1997; Tsai et al. 2005; Hong et al. 2007). Many stress-inducible genes are induced by exogenous ABA treatment. ABA application increased the expression of cytosolic *GR* of rice (Kaminaka et al. 1998) and cytosolic and chloroplast/mitochondria *GR* of *Vigna unguiculata* (Contour-Ansel et al. 2006). H_2O_2 could be a candidate for signal transduction. However, the failure of H_2O_2 to induce the *GR* gene has been reported (Xiang and Oliver 1998).

Previously, we demonstrated that *OsGR* gene expression was increased in response to NaCl and H_2O_2 in roots of etiolated rice seedlings (Tsai et al. 2005). These data, however, were obtained by use of a non-specific probe, so it was not possible to show precisely which member(s) of the *OsGR* gene family was induced in response to NaCl and H_2O_2 treatments. In this study, we used the 3′-untranslated region (3′-UTR)-specific primers for the *OsGR1*, *OsGR2*, and *OsGR3* genes from rice to examine (1) the effect of NaCl, ABA, and H_2O_2 on the expression of *OsGR* genes and (2) whether the induction of *OsGR* genes by NaCl is mediated through ABA or H_2O_2 in rice.

Materials and methods

Plant material and growth conditions

Rice (*Oryza sativa* L., cv. Taichung Native 1, an Indica type) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. To obtain more uniformly germinated seeds, rice seeds in Petri dish (20 cm) containing distilled water were pretreated at 37°C for 1 day 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 day. Two-day-old seedlings were then transferred to distilled water, NaCl, ABA, sodium tungstate (Tu), 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 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 seminal roots of rice seedlings at the times specified in the individual experiments were used for analyses the expression of *OsGR*, *OsRab16A*, and *Oryza sativa ascorbate peroxidase 8* (*OsAPX8*), GR activity and H_2O_2 detection.

Semi-quantitative RT-PCR analysis

Total RNA was isolated from root tissue of 2-day-old etiolated rice seedlings using the TRIzol reagent (Invitrogen, CA, USA), according to the supplier's recommendations. To prevent DNA contamination, RNA was treated with Turbo DNase I (Ambion, TX, USA) for 30 min at 37°C before RT-PCR analysis. Moreover, the control PCR amplifications were performed using RNA as template after the DNase I treatment to verify the complete elimination of contaminated DNA. The reverse transcription reactions were conducted with 200 ng of total RNA using the SuperScript III platinum one-step quantitative RT-PCR system (Invitrogen, CA, USA) according to the manufacturer's protocol.

The gene-specific primers were designed from the 3′-UTR of the rice *GR* and *OsAPX8* genes (Hong et al. 2007). The sequences used, the predicted amplicons, and the cycle numbers are listed in Table 1. The RT-PCR program initially started with a 50°C/30 min; 94°C denaturation for 5 min, followed by 94°C/30 s, 22 to 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 3% agarose gel, stained with ethidium bromide. The gel images were digitally captured with use of a SynGene gel documentation system and analyzed with the Genetools analysis software (Syngene, MD, USA). The rice *OsActin* gene was used as a reference for normalization. For some experiments, the ABA responsive gene, *OsRab16A*

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

Gene	Primer	Sequence (5′ to 3′)	Products (bp)	Cycles
OsGR1	GR1-5′	TCTCAGAGGGACTTCTCTACT	245	24
	GR1-3′	AGGCAGTGGTACTCACATGGT		
OsGR2	GR2-5′	GTGTACTCTGGTTTGCATCT	179	26
	GR2-3′	CTGCAGGCAGAACGAATGAT		
OsGR3	GR3-5′	CAACAGACAGATATCGGTA	244	24
	GR3-3′	TACTATCAACATCCTGAAGC		
OsAPX8	APx8-5′	TGGTCTGATGACCTCCTCTGA	222	28
	APx8-3′	CATGAGCCATGACAACTAGA		
OsRab16A	Rab16A-5′	CGACACACCACCACACCATG	294	28
	Rab16A-3′	TGTGTACATATGCACGATGA		
OsActin	Actin-5′	ATGCTCTCCCCCATGCTATC	465	20
	Actin-3′	TCTTCCTTGCTCATCCTGTC		

(Mundy and Chua 1988) and/or *OsAPX8* (Hong et al. 2007) were used as a control for each RT-PCR reaction.

GR assay

For extraction of GR, root tissues were homogenized with 0.1 M sodium phosphate buffer (pH 6.8) containing 0.1 mM EDTA, 1% (*v*/*v*) polyvinyl-pyrrolidone, and 0.5% (*v*/*v*) Triton X-100 with use of a chilled mortar. The homogenate was centrifuged at 12,000 g for 20 min, and the resulting supernatant was used for determination of GR activity as described by Foster and Hess (1980). One unit of GR was defined as the amount of enzyme that decreases 1 OD min^{-1}. The activity of GR was expressed on the basis of dry weight (DW).

Quantitation of H_2O_2

The H_2O_2 was determined by fluorescence microscopy. H_2O_2 imaging was conducted basically according to Sandalio et al. (2008). Two-day-old rice seedlings were treated with 150 mM NaCl for various incubation times as specified in the individual experiments. For negative controls, rice seedlings were incubated with 2 mM ascorbic acid (Asc; a H_2O_2 scavenger) and 150 mM NaCl. For DPI experiments, roots of rice seedlings were treated with 10 μM DPI for 1 h followed by incubation with 150 mM NaCl for 1 h. After NaCl, Asc, and DPI treatments, 2-cm root segments were cut and placed on a Petri dish containing 1 ml Tris–HCl buffer (10 mM, pH 7.2) with 2′,7′-dichlorofluorescein diacetate (DCF-DA) at a final concentration of 10 μM for 30 min. A Nikon SMZ 1500 stereoscopic fluorescent microscope was used for fluorescence images. Fluorescent dye emissions were recorded by use of a 505- to 530-nm band pass filter. Images were captured with use of an Evolution MP cooled color CCD Camera (Evolution MP 5.1, Media Cybernetics,MD, USA) and analyzed by use of the QCapture Pro 6.0 software (QImaging Corp., BC, Canada).

In some experiments, the H_2O_2 content was measured spectrophotometrically after reaction with $TiCl_4$ (Tsai et al. 2004). The reaction mixture consisted of 2 ml of 50 mM phosphate buffer (pH 6.8) root extract supernatant and 1 ml reagent [0.1% (*v*/*v*) $TiCl_4$ in 20% (*v*/*v*) H_2SO_4]. The blank probe consisted of 50 mM phosphate buffer in the absence of root extract. The absorbance was measured at 410 nm. The amount of H_2O_2 was calculated by use of a standard curve prepared with known concentrations of H_2O_2. The H_2O_2 content was expressed on the basis of dry weight (DW).

Statistical analysis

Statistical differences between measurements (n=4) for different treatments or different times were analyzed following LSD test. A $P<0.05$ was considered statistically significant.

Results

NaCl increases the activity of GR

In the present study, 2-day-old rice seedlings were treated with 150 mM NaCl for 2, 4, 6, and 8 h. The activity of GR was increased by NaCl after 6 h of treatment in comparison with the control (Fig. 1).

ABA concentrations and the expression of *OsRab16A*

ABA-responsive rice gene *Rab16A* (initially called *Rab21*) has been characterized by Chua's group (Mundy and Chua 1988; Mundy et al. 1990). To examine the effect of ABA on the expression of *OsRab16A*, rice roots were treated with a series of ABA concentrations. Increasing the concentration of ABA from 1 to 12 μM progressively increased the expression of *OsRab16A* in roots (Fig. 2).

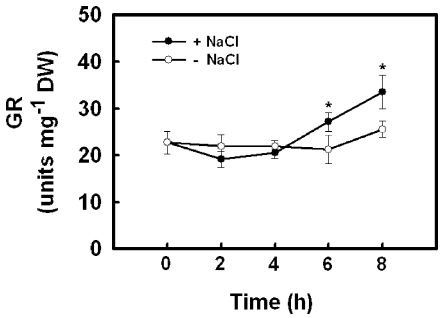

Fig. 1 Changes of GR activity in rice roots in the presence or absence of NaCl. Two-day-old rice seedlings were treated with or without NaCl (150 mM). One unit of GR was defined as the amount of enzyme that decreases 1 OD min^{-1}. * Represents values that are significantly different between −NaCl and +NaCl treatments ats $P<0.05$

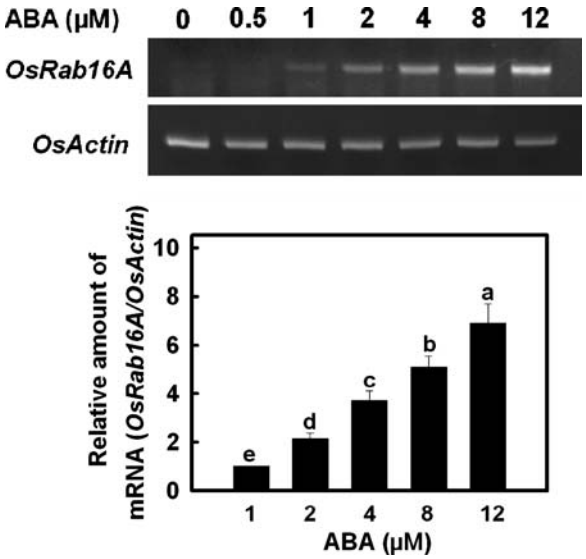

Fig. 2 Effect of ABA concentration on mRNA level of *OsRab16A* in rice roots. Two-day-old rice seedlings were treated with ABA (0–12 μM) for 1 h. Semi-quantitative RT-PCR of *OsRab16A* gene was performed as described in "Materials and methods". The values of *OsRab16A* mRNA were normalized by a corresponding amount of *OsActin*. After the adjustment by *OsActin*, the reaction with the roots in 1 μM ABA were treated as the normalized reference, with a value of one, for determining the relative amount of mRNA of *OsRab16A*. *Bars* show means±SE (n=4). Values with the *same letter* are not significantly different at P<0.05

NaCl induces the expression of *OsGR2, OsGR3,* and *OsRab16A*

To investigate the effect of NaCl on the expression of all three *OsGR* genes in roots, total RNA was extracted, and the expression dynamics of the three *OsGR* genes were examined by semi-quantitative RT-PCR analysis. When 2-day-old seedlings were subjected to 150 mM NaCl for 0.5, 1, and 1.5 h, transcripts of *OsGR2* and *OsGR3* genes increased at 1 and 0.5 h, respectively (Fig. 3). However, no significant increase due to NaCl could be detected in the expression of *OsGR1* (Fig. 3). The *OsRab16A* expression was notably increased 0.5 h after NaCl treatment (Fig. 3).

Exogenous application of ABA induces the expression of *OsGR2*, *OsGR3*, and *OsRab16A*

To test whether ABA is involved in the regulation of *OsGR* and *OsRab16A* genes, the effect of 9 μM ABA on the expression of *OsGR* and *OsRab16A* genes was examined. The expression of *OsGR2*, *OsGR3*, and *OsRab16A* was significantly increased by ABA after 0.5 h of treatment in comparison with control treatment (Fig. 4). However, ABA treatment had no effect on the expression of *OsGR1* (Fig. 4).

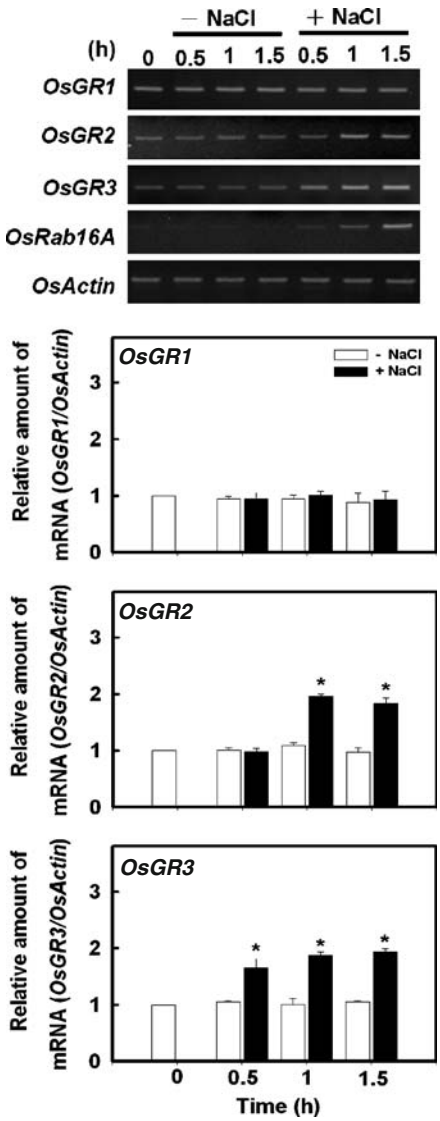

Fig. 3 Changes in mRNA levels of *OsGR* and *OsRab16A* genes in rice roots in the presence or absence of NaCl. Two-day-old rice seedlings were treated with or without NaCl (150 mM). Semi-quantitative RT-PCR analysis of *OsGR* and *OsRab16A* genes was performed as described in "Materials and methods". The values of mRNA of *OsGR* genes were adjusted by a corresponding amount of *OsActin*. After the adjustment by *OsActin*, the reaction of −NaCl in roots was treated as the normalized reference, with a value of one, for determining the relative amount of mRNA of *OsGR* genes. *Bars* show means± SE (n=4). * Represents values that are significantly different between −NaCl and +NaCl treatments at P<0.05

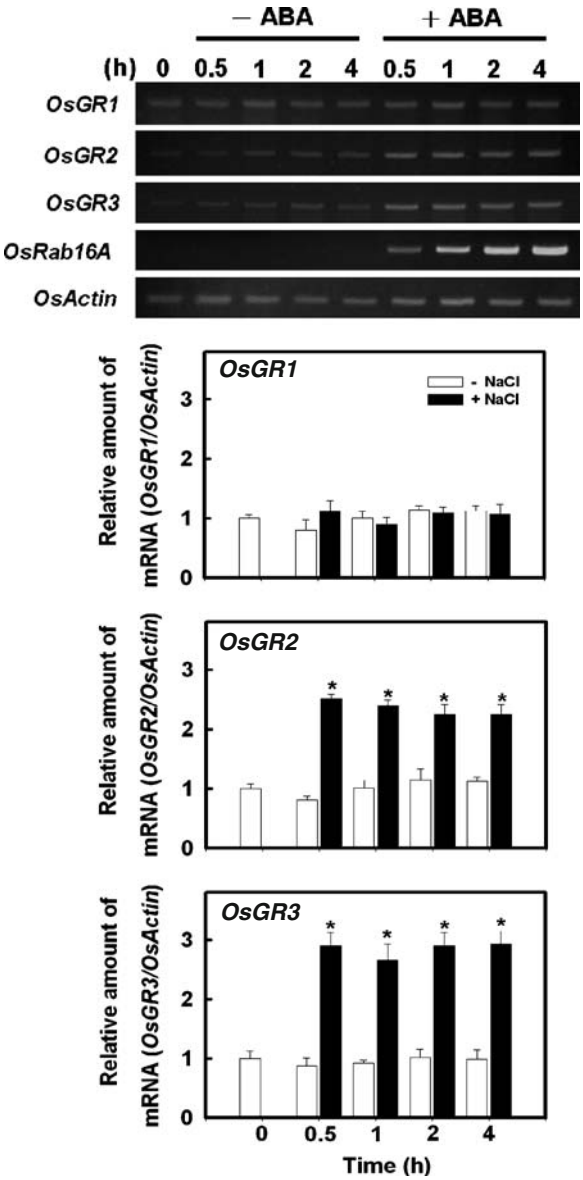

Fig. 4 Changes in mRNA levels of *OsGR* and *OsRab16A* genes in rice roots in the presence or absence of abscisic acid (ABA). Two-day-old rice seedlings were treated with distilled water or ABA (9 μM). Semi-quantitative RT-PCR for *OsGR* and *OsRab16A* genes was performed as described in "Materials and methods". The values of mRNA for *OsGR* genes were adjusted by corresponding amount of *OsActin*. After the adjustment by *OsActin*, the reaction with the roots in –ABA was treated as the normalized reference, with a value of one, for determining the relative amount of mRNA of *OsGR* genes. *Bars* show means± SE (n=4). * Represents values that are significantly different between –ABA and +ABA treatments at $P<0.05$

Tu effect

The role of ABA in NaCl-enhanced expression of the *OsGR* and *OsRab16A* genes was examined further by using Tu, which is known to block the formation of ABA from ABA aldehyde by impairing ABA-aldehyde oxidase (Hansen and Grossmann 2000). NaCl-enhanced *OsRab16A* expression in rice roots was significantly reduced by Tu pre-treatment (Fig. 5). Our previous results showed that NaCl-enhanced *OsAPX8* expression in rice roots is mediated through ABA (Hong et al. 2007). As a control, Tu pre-treatment reduced *OsAPX8* expression in rice roots caused by NaCl (Fig. 5). However, NaCl-enhanced expression of *OsGR2* and *OsGR3* in rice roots was not significantly suppressed by Tu (Fig. 5).

H_2O_2 in rice roots in response to NaCl

The detection in vivo of H_2O_2 in rice roots was carried out using a fluorescence probe (Figs. 6 and 7). DCF-DA was used to follow for H_2O_2 production. The probe specificity was checked by using Asc, an H_2O_2 scavenger. As shown in Fig. 6a, NaCl induced the accumulation of H_2O_2 in rice roots. The H_2O_2 production was reduced by DPI (Fig. 6b). The accumulation of H_2O_2 is a very fast process, occurring within the first 5 min of NaCl treatment (Fig. 7).

The H_2O_2 content shown in Fig. 8 was measured spectrophotometrically. As well, NaCl-induced accumulation of H_2O_2 in rice roots was reduced by DPI treatment (Fig. 8).

H_2O_2 is involved in NaCl-induced expression of *OsGR2* and *OsGR3*

The effect of 10 mM H_2O_2 on the expression of *OsGR* and *OsRab16A* genes is shown in Fig. 9. H_2O_2 treatment had no effect on the expression of the *OsGR1* in rice roots. In contrast, H_2O_2 significantly increased the expression of *OsGR2* and *OsGR3*. H_2O_2 treatment enhanced the expression of *OsGR2* and *OsGR3* in rice roots at about the same magnitude as NaCl treatment (Fig. 9). However, NaCl but not H_2O_2 increased *OsRab16A* expression in rice roots (Fig. 9). As well, pre-treatment with 0.1 μM DPI, an inhibitor of NADPH oxidase, decreased the expression of *OsGR2* and *OsGR3* but had no effect on the expression of *OsRab16A* and *OsAPX8* in NaCl-treated rice roots (Fig. 10). We have shown that

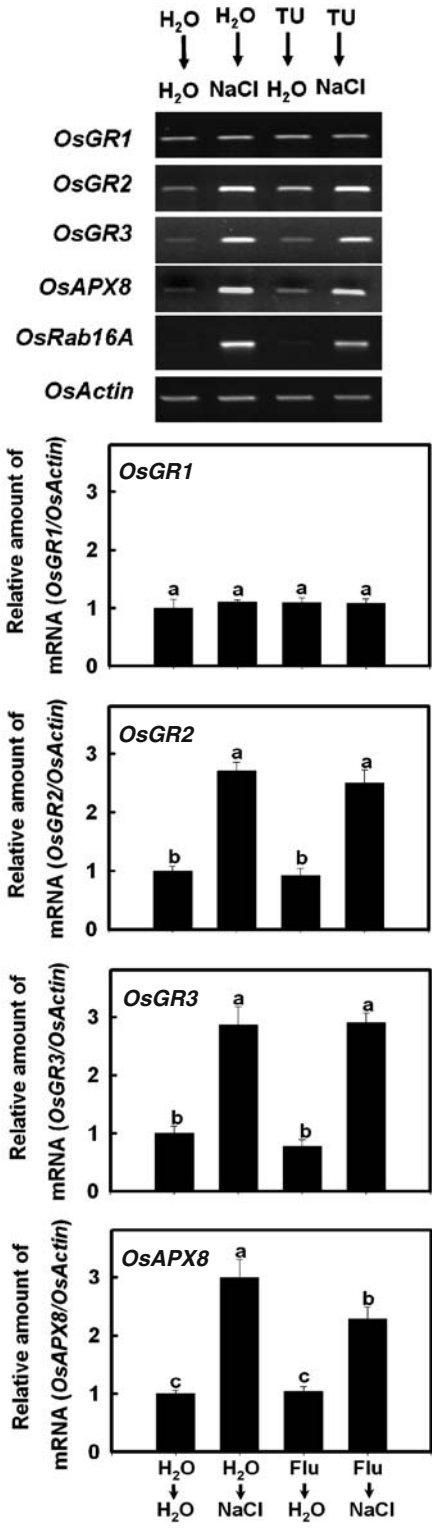

Fig. 5 Effect of sodium tungstate (Tu) on mRNA levels of *OsGR*, *OsRab16A*, and *OsAPX8* in roots of rice seedlings in the presence or absence of NaCl. Two-day-old rice seedlings were pre-treated with H_2O or Tu (1 mM) for 2 h and then transferred to H_2O and NaCl (150 mM) for 8 h, respectively. Semi-quantitative RT-PCR for *OsGR*, *OsRab16A* and *OsAPX8* genes was performed as described in "Materials and methods". The values of mRNA for *OsGR* genes were adjusted by a corresponding amount of *OsActin*. 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 determining the relative amount of mRNA of *OsGR* and *OsAPX8*. *Bars* show means±SE (n=4). Values with the *same letter* are not significantly different at $P<0.05$

NaCl-enhanced *OsAPX8* expression in rice roots is not mediated through H_2O_2 (Hong et al. 2007). As a control, DPI pre-treatment had no effect on NaCl-induced *OsAPX8* expression in rice roots (Fig. 10).

Discussion

Three *GR* genes exist in rice (Kaminaka et al. 1998; Chang et al. 2003; Bashir et al. 2007). In the present study, we demonstrated that NaCl increased the expression of *OsGR2* and *OsGR3* (Fig. 3). The time-course analyses of NaCl treatment clearly indicated that the expression of *OsGR2* and *OsGR3* increased first (1 and 0.5 h, respectively, after NaCl treatment; Fig. 3) and then GR activity increased (6 h after NaCl treatment; Fig. 1) in rice roots. These results have led to the conclusion that early expression of *OsGR2* and *OsGR3* during NaCl treatment is associated with an enhancement in its GR activity. Kaminaka et al. (1998) were the first to isolate a cDNA (*OsGR2*) encoding a cytosolic GR from rice; 10-day-old rice (cv. Nipponbare) seedlings grown in a growth cabinet were treated with 250 mM NaCl and showed that the expression of *OsGR2* increases during NaCl treatment.

ABA content increases in plants under salinity (Skriver and Mundy 1990; Moons et al. 1995; Montero et al. 1997; Hong et al. 2007). *OsRab16A* mRNA is known to increase in rice embryos, leaves, roots, and callus-derived suspension cells on treatment with NaCl or ABA (Mundy and Chua 1988; Mundy et al. 1990). In rice roots, increasing the ABA concentration from 1 to 12 μM progressively increased the expression of *OsRab16A* (Fig. 2). Thus, the level of ABA reported in this study was judged by the transcripts of *OsRab16A*. Figure 3 shows that

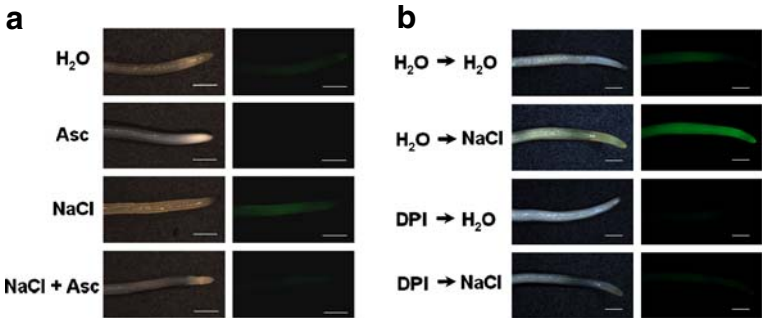

Fig. 6 Imaging of H_2O_2 production in rice roots. **a** Root segments cut from 2-day-old rice seedlings were treated with or without NaCl (150 mM). As a negative control, roots were incubated with 2 mM ascorbic acid (Asc), which acts as an H_2O_2 scavenger. After 1-h incubation, roots were incubated with 10 μM DCF-DA for 30 min at 37°C in the dark. Panels on the *left* and the *right* show bright-field image and H_2O_2-dependent DCF-DA fluorescence, respectively. *Bar*: 1 mm. **b** DPI inhibited NaCl-induced H_2O_2 production in rice roots. Two-day-old rice seedlings were pre-treated with 10 μM DPI for 1 h and then treated with or without 150 mM NaCl. One hour after treatment, roots were incubated with 10 μM DCF-DA for 30 min at 37°C in the dark. Panels on the *left* and the *right* show a bright field image and H_2O_2-dependent DCF-DA fluorescence, respectively. *Bar*: 1 mm

OsRab16A expression was increased in rice roots on exposure to NaCl. As well, on ELISA, NaCl treatment resulted in an accumulation of ABA in rice roots (Hong et al. 2007).

ABA is generally thought to be a candidate signal transducer in stress-induced gene expression. ABA application significantly enhances the expression of *GR* genes in rice (Kaminaka et al. 1998) and cowpea (Contour-Ansel et al. 2006). In the present study, exogenous ABA enhanced the expression *OsGR2* and *OsGR3* in rice roots (Fig. 4). Tu is an inhibitor of ABA biosynthesis through the carotenoid pathway (Hansen and Grossmann 2000). The addition of Tu resulted in an inhibition of ABA accumulation (Fig. 4) but had no effect on the expression of *OsGR2* and *OsGR3* in rice roots exposed to NaCl (Fig. 5). However, Tu pre-treatment reduced the NaCl-enhanced *OsRab16A* expression, as well as NaCl-induced *OsAPX8* expression (Fig. 5). NaCl-enhanced expression of *OsGR2* and *OsGR3* seems not to be mediated by ABA accumulation in rice roots.

Environmental stresses are known to cause oxidative stress within plant cells. H_2O_2 accumulates in response to NaCl in plants (Hernández et al. 2001; Lee et al. 2001; Sudhakar et al. 2001; Mittova et al. 2004; Tsai et al. 2004). In the present study, NaCl treatment resulted in an accumulation of H_2O_2-dependent DCF-DA fluorescence in rice roots (Fig. 6a). NaCl-induced accumulation of H_2O_2 in rice roots could be due to NaCl-enhanced superoxide dismutase (SOD) and NaCl-deactivated catalase (CAT) activities (Lee et al. 2001), but in the roots of rice seedlings, NaCl had no effect on SOD and CAT activities (Tsai et al. 2004). In several model systems

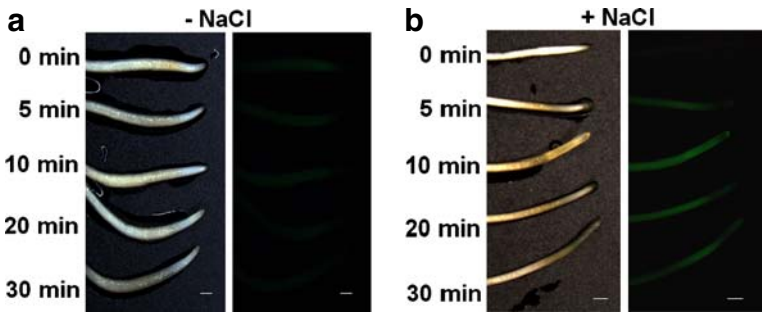

Fig. 7 Changes in H_2O_2 production in rice roots treated with or without NaCl. Two-day-old rice roots were treated with distilled water (−NaCl, **a**) or 150 mM NaCl (+NaCl, **b**) for 0–30 min. Root segments (2 cm) were cut and labeled with 10 μM DCF-DA for 30 min at 37°C in the dark. Panels on the *left* and the *right* show bright-field image and H_2O_2-dependent DCF-DA fluorescence, respectively. *Bar*: 1 mm

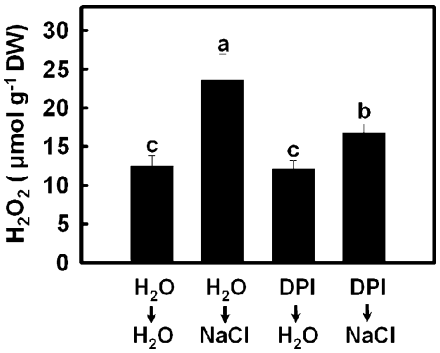

Fig. 8 Effect of diphenylene iodonium (DPI) on the content of H_2O_2 in roots of rice seedlings in the presence or absence of NaCl. Two-day-old rice seedlings were pre-treated with H_2O or DPI (0.1 μM) for 12 h and then transferred to H_2O and NaCl (150 mM) for 24 h, respectively. The H_2O_2 content was determined spectrophotometrically. *Bars* show means±SE (n= 4). Values with the *same letter* are not significantly different at P<0.05

investigated in plants, the accumulation of H_2O_2 appears to be mediated by activation of a plasma-membrane-bound NADPH oxidase complex (Pei et al. 2000; Orozco-Cardénas et al. 2001; Zhang et al. 2001; Tsai et al. 2005). DPI is known to inhibit plasma-membrane NADPH oxidase (Orozco-Cardénas et al. 2001). DPI (0.1 μM) can prevent the increased production of H_2O_2 in roots induced by NaCl (Tsai et al. 2005). Our findings provide further evidence that DPI pre-treatment significantly reduced the H_2O_2-dependent DCF-DA fluorescence and H_2O_2 content in rice roots caused by NaCl (Figs. 6b and 8).

It has been shown that high concentration of DPI can affect other enzymes potentially involved in generation of ROS, including cell wall peroxidase and nitric oxide synthase (Stuehr et al. 1991; Frahry and Schopfer 1998; Orozco-Cádenas et al. 2001). The fact that NaCl-induced H_2O_2 accumulation in rice roots can be inhibited by low concentration DPI (0.1 μM) and can be inhibited by imidazole (10 mM), another inhibitor of NADPH oxidase (Cross 1990; Tsai et al. 2005), strongly suggested that NaCl-induced accumulation of H_2O_2 was mediated through the activation of NADPH oxidase in rice roots. Figure 8 shows that the basal level of H_2O_2 is insensitive to DPI pre-treatment. It appears that the basal H_2O_2 is unlikely generated through NADPH oxidase.

Two genes endcoding GR have been identified in Arabidopsis: one, *gr2*, encodes a plastidic iosform (Kubo et al. 1993), and the other, *gr1*, encodes a cytosolic enzyme (Xiang and Oliver 1998). Xiang and Oliver (1998) demonstrated that the transcript level of *gr1* is induced by jasmonic acid but not by H_2O_2. However, they did not show the data concerning the effect of H_2O_2 on the expression of *gr2*. Here, we demonstrated that the expression of *OsGR2* and

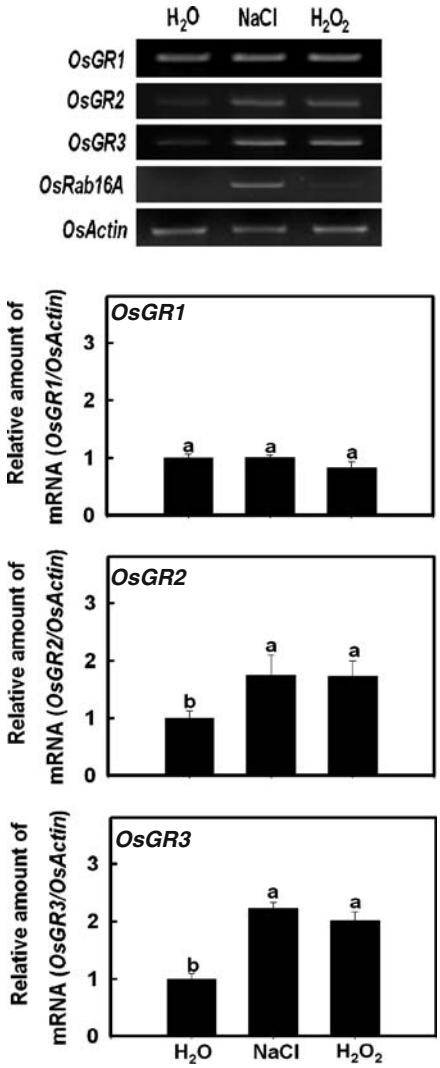

Fig. 9 Effect of NaCl and H_2O_2 on mRNA levels for *OsGR* and *OsRab16A* genes in roots of rice seedlings. Two-day-old rice seedlings were treated with H_2O, NaCl (150 mM) or H_2O_2 (10 mM) for 8 h. Semi-quantitative RT-PCR for *OsGR* and *OsRab16A* was performed as described in "Materials and methods". The values for mRNA of *OsGR* genes were adjusted by a corresponding amount of *OsActin*. 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 determining the relative amount of mRNA of *OsGR*. *Bars* show means±SE (n= 4). Values with the *same letter* are not significantly different at P<0.05

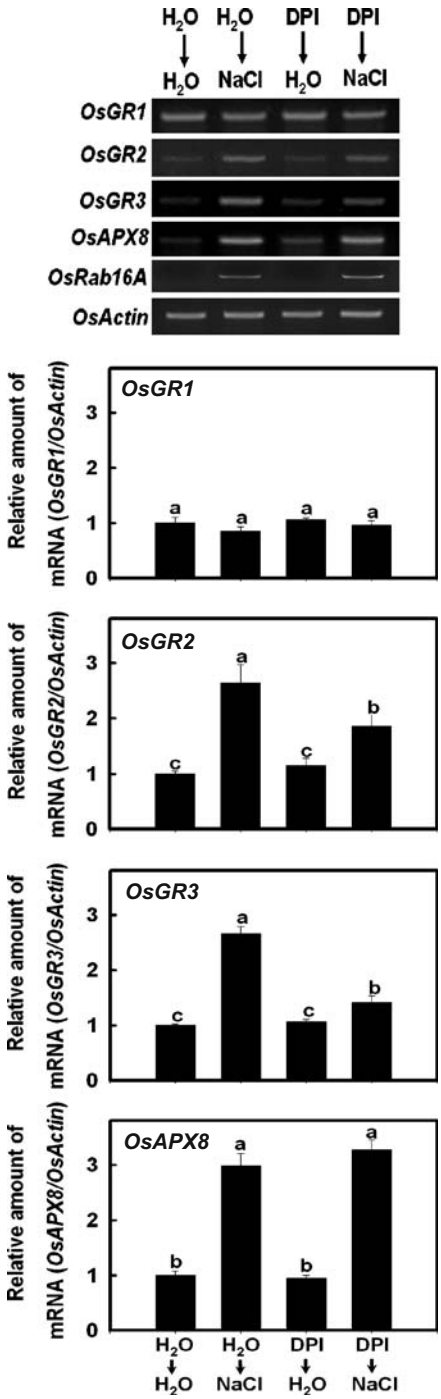

Fig. 10 Effect of diphenylene iodonium (DPI) on mRNA levels of *OsGR*, *OsRab16A*, and *OsAPX8* genes in roots of rice seedlings in the presence or absence of NaCl. Two-day-old rice seedlings were pre-treated with H_2O or DPI (0.1 μM) for 12 h and then transferred to H_2O and NaCl (150 mM) for 24 h, respectively. Semi-quantitative RT-PCR for *OsGR*, *OsRab16A* and *OsAPX8* genes was performed as described in "Materials and methods". The values of mRNA for *OsGR* genes were adjusted by a corresponding amount of *OsActin*. 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 determining the relative amount of mRNA of *OsGR* and *OsAPX8* genes. *Bars* show means±SE (n=4). Values with the *same letter* are not significantly different at $P<0.05$

OsGR3 but not *OsRab16A* in rice roots was enhanced by H_2O_2 (Fig. 9). We also observed that DPI pre-treatment reduced the expression of *OsGR2* and *OsGR3* but had no effect on *OsRab16A* and *OsAPX8* in NaCl-treated rice roots (Fig. 10). Thus, the expression of *OsGR2* and *OsGR3* induced by NaCl is likely mediated through H_2O_2 in rice roots. This conclusion was supported further by the observations that the accumulation of H_2O_2 preceded the expression of *OsGR2* and *OsGR3* in NaCl-treated rice roots (Figs. 3 and 7).

In addition to being an endogenous oxidant, H_2O_2 has also been implicated as a diffusible signal for selective induction of defense mechanisms in plant cells (Dat et al. 2000). H_2O_2 generated in the apoplast appears to trigger the expression of *OsGR2* and *OsGR3* in NaCl-treated rice roots. Because apoplast has only a small proportion of the cell's antioxidant capacity, H_2O_2 will rapidly move into the cell to exert its effect on the expression of *OsGR2* and *OsGR3*. Peroxiporins or water channels (aquaporins) may serve as conduits for trans-membrane H_2O_2 transport (Neill et al. 2002). Since H_2O_2 is able to diffuse freely through membranes (Levin et al. 1994), it likely diffuses to the cytoplasm, the nucleus, and/or the plastid. This would explain why H_2O_2 generated in the apoplast is able to trigger the expression of cytosolic *OsGR2* and plastidic *OsGR3* in NaCl-treated rice roots. However, whether H_2O_2 is the sole signal remains to be determined. NADPH oxidase does not seem to be the only source of H_2O_2 generation in NaCl-treated rice roots, because NaCl-induced cell wall-bound NADH peroxidase and diamine oxidase activities, devoted to H_2O_2 production, have been detected in the roots of rice seedlings (Lin and Kao 2001b). This finding would explain why DPI pre-treatment cannot completely reduce NaCl-enhanced content of H_2O_2 (Fig. 8) and expression of *OsGR2* and *OsGR3* in rice roots (Fig. 10).

Reports have shown that ABA usually works together and upstream of H_2O_2 in signaling (Hung and Kao 2004; Pei et al. 2000; Zhang et al. 2001).

However, in NaCl-treated rice roots, the expression of *OsGR2* and *OsGR3* is regulated by H_2O_2 but not ABA. It appears that H_2O_2 can work alone in signaling the expression of *OsGR2* and *OsGR3*.

From previously reported results (Tsai et al. 2005) and from this study (Figs. 1 and 3), the increase in the expression of *OsGR2* and *OsGR3* is indeed associated with enhanced GR activity. The present results suggest that *OsGR2* and *OsGR3* induced by NaCl may affect ROS scavenging properties in rice roots. Tolerance to oxidative stress is enhanced in transgenic plants by overexpressed bacterial *GR* genes in the chloroplast (Aono et al. 1991, 1993; Broadbent et al. 1995; Foyer et al. 1995). Clearly, more experiments regarding knockout mutants or plants with overexpressed *OsGR2* and *OsGR3* are needed for our understanding of the function of *OsGR2* and *OsGR3* in rice roots under stress conditions.

ROS are thought to play an important role in NaCl stress. 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 balance ROS production and destruction and maintain cellular redox homeostasis (Halliwell and Gutteridge 2007). APx and GR are two important ROS-scavenging enzymes (Halliwell and Gutteridge 2007). Of note, in NaCl-treated rice roots, the expression of *OsAPX8* is regulated by ABA (Hong et al. 2007), whereas that of *OsGR2* and *OsGR3* is regulated by H_2O_2.

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