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Na^+ but not Cl^- or osmotic stress is involved in NaCl-induced expression of *Glutathione reductase* in roots of rice seedlings

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Summary

Glutathione reductase (GR, EC 1,6.4.2) is an important reactive oxygen species-scavenging enzyme. The present study examined the relative importance of Na^+, Cl^-, and the osmotic component in NaCl-induced expression of *Oryza sativa* glutathione reductase (*OsGR*) genes in rice roots. Semi-quantitative RT-PCR was used to quantify the mRNA levels for one cytosolic (*OsGR2*) and two chloroplastic (*OsGR1* and *OsGR3*) isoforms of GR identified in the rice genome. The expression of *OsGR2* and *OsGR3* but not *OsGR1* was increased in rice roots treated with NaCl. Treatment with 150 mM NaCl and 150 mM $NaNO_3$ affected *OsGR2* and *OsGR3* induction similarly, which suggests that Na^+ but not Cl^- is required for the NaCl-induced expression of *OsGR2* and *OsGR3*. We also show that Na^+ but not Cl^- is required for NaCl-enhanced GR activity and hydrogen peroxide (H_2O_2) production in rice roots. In addition to its component of ion toxicity, salt concentration in soil results in an osmotic effect. Here, we show that *OsGR2* and *OsGR3* expression, GR activity, and H_2O_2 content were not affected at a concentration of mannitol iso-osmotic with 150 mM NaCl. NaCl-induced *OsGR2* and *OsGR3* in rice roots could be associated with Na^+ but not an osmotic component.

Introduction

Essential mineral elements are required for plant growth and development, but the excess presence of soluble salts in soil is harmful to most plants.

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; DW, dry weight; GR, glutathione reductase; GSSG, oxidized glutathione; GSH, reduced form glutathione; Man, mannitol; ROS, reactive oxygen species.

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More than 800 million ha of land are affected by salt throughout the world (Munns, 2005). Therefore, soil salinity can be considered the single most widespread toxicity problem that crop production faces at present. The main factors responsible for soil salination are the low quality of irrigated water, excess fertilization, and deficient drainage of some soils, which decreases the yield of crops.

The increase in reactive oxygen species (ROS) such as superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radicals seems to occur as a response to most, if not all, abiotic stresses, including salinity (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; Hong et al., 2009). 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 (Mittler, 2002).

Glutathione reductase (GR, EC 1.6.4.2), a flavoenzyme, has been found in all organisms investigated. This enzyme, which catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) with the accompanying oxidation of NADPH, is considered an important reaction of the ascorbate (AsA)–GSH cycle (Noctor and Foyer, 1998), the main mechanism for the detoxification of ROS in plants. Therefore, GR has been suggested to be regulated in response to various abiotic stresses and to contribute to stress tolerance in GR-overexpressing plants (Aono et al., 1991, 1993; Broadbent et al., 1995; Foyer et al., 1995; Fadzilla et al., 1997). The expression of *GR* in plants is enhanced by NaCl treatment (Kaminaka et al., 1998; Tsai et al., 2005; Hong et al., 2009).

For plants grown in saline soil, the roots must cope with two types of stress. The first is ionic stress induced by changes in the concentrations of Na^+, Cl^-, or both, in the root-growing medium and within root tissues (Cramer et al., 1994; Montero et al., 1998; Romero-Aranda et al., 1998; Sibole et al., 1998; Lin and Kao, 2001b; Moya et al., 2003; Tsai et al., 2004). The second is osmotic stress from salt concentration in the soil that results in lowered water potential and a consequent loss of cell turgor in roots (Lin and Kao, 2002).

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*: cytosolic (*OsGR2*) form (Kaminaka et al., 1998) and two chloroplastic forms (*OsGR1* and *OsGR3*) (Chang et al., 2004; Bashir et al., 2007). Previously, we demonstrated *OsGR* gene expression increased in response to NaCl in roots of etiolated rice seddlings (Tsai et al., 2005). These data, however, were obtained by use of a non-specific probe, so we could not show precisely which member(s) of the *OsGR* gene family showed increased expression in response to NaCl. Using the 3′-untranslated region (3′-UTR)-specific primers of *OsGR1*, *OsGR2*, and *OsGR3* from rice, we have recently shown that the expression of *OsGR2* and *OsGR3* but not *OsGR1* is increased in rice roots treated with NaCl (Hong et al., 2009). In this study, we investigated the relative importance of Na^+, Cl^- and the osmotic component in regulating the NaCl-induced expression of *OsGR2* and *OsGR*3 in rice roots.

Materials and methods

Plant material and growth conditions

Seeds of rice (*Oryza sativa* L., cv. Taichung Native 1, an Indica type) were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. To obtain uniformly germinated seeds, rice seeds in a Petri dish (20 cm) containing distilled water were pretreated 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 filter paper moistened with 10 mL distilled water for 2 d. Two-day-old seedlings were then transferred to distilled water, NaCl, $NaNO_3$, and mannitol (Man) at the desired concentration as specified in 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 individual experiments were used for analysis of *OsGR* gene expression, GR activity, and H_2O_2 content.

Semi-quantitative RT-PCR analysis

Total RNA was isolated from root tissue of 2-d-old etiolated rice seedlings with use of 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 involved use of RNA as a template after the DNase I

treatment to verify the complete elimination of contaminated DNA. The reverse-transcription reactions involved 200 ng of total RNA by use of 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 rice *GR* genes (Hong et al., 2009). The sequences used, the predicted amplicons, and the cycle numbers are listed in Table 1. The RT-PCR program initially started with 50 °C for 30 min; 94 °C denaturation for 5 min, followed by 94 °C for 30 s, 22–32 cycles of 50 °C for 30 s, and 68 °C for 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 figures. 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 and stained with ethidium bromide. The gel images were digitally captured with use of a SynGene gel documentation system and analyzed with use of Genetools (Syngene, MD, USA). The rice *OsActin* gene was used for normalization.

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 by 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 absorbance min^{-1}. The activity of GR was expressed on the basis of dry weight (DW).

Quantification of H_2O_2

H_2O_2 content was measured spectrophotometrically after reaction with $TiCl_4$ (Tsai et al., 2004).

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

Gene	NCBI accession no.	Primer	Sequence (5′–3′)	Products (bp)	Cycles
OsGR1	Os03g0163300	GR1-5′	TCTCAGAGGGACTTCTCTACT	245	24
		GR1-3′	AGGCAGTGGTACTCACATGGT		
OsGR2	Os02g0813500	GR2-5′	GTGTACTCTGGTTTGCATCT	179	26
		GR2-3′	CTGCAGGCAGAACGAATGAT		
OsGR3	Os10g0415300	GR3-5′	CAACAGACAGATATCGGTA	244	24
		GR3-3′	TACTATCAACATCCTGAAGC		
OsActin	Os03g0718100	Actin-5′	ATGCTCTCCCCCATGCTATC	465	20
		Actin-3′	TCTTCCTTGCTCATCCTGTC		

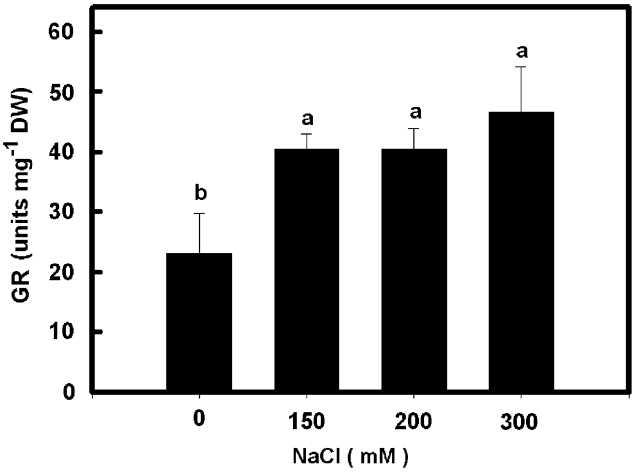


Figure 1. Effect of NaCl concentration on GR activity in rice roots. Two-day-old rice seedlings were treated with NaCl (0–300 mM) for 8 h. Bars show means ± SE ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$.

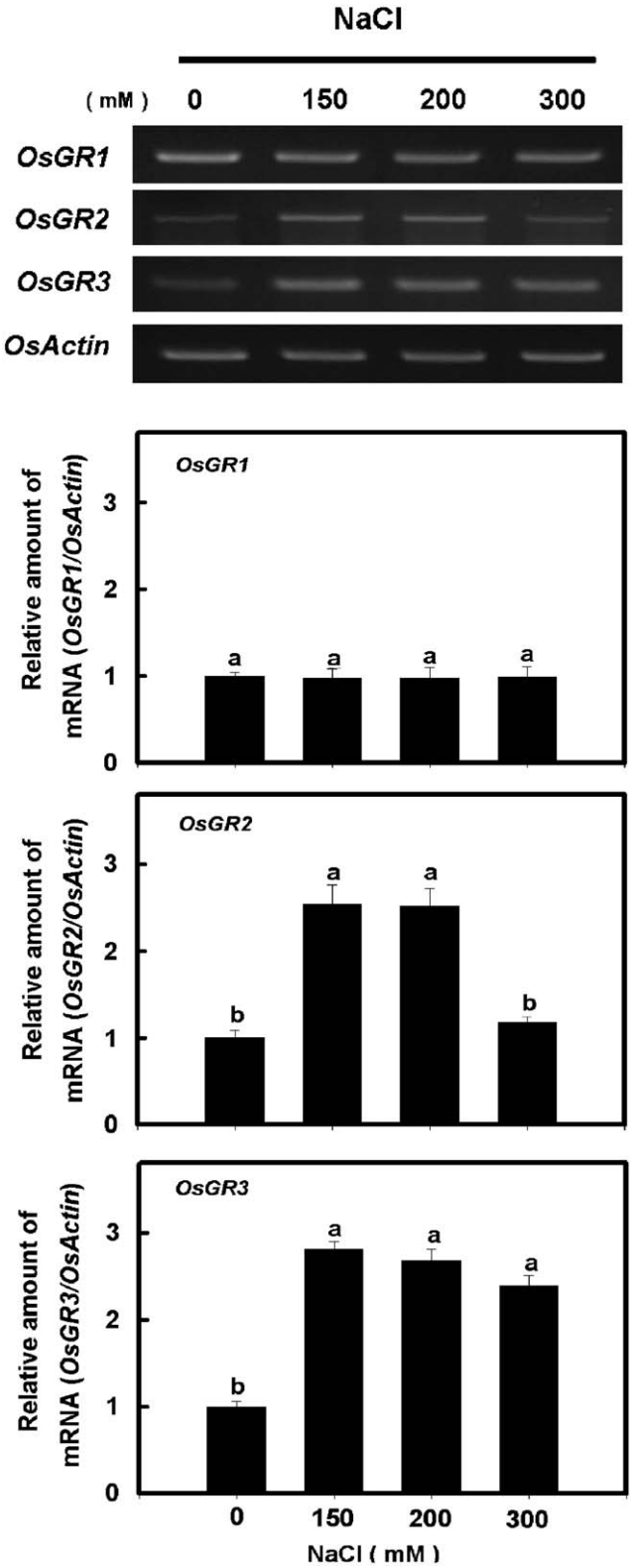
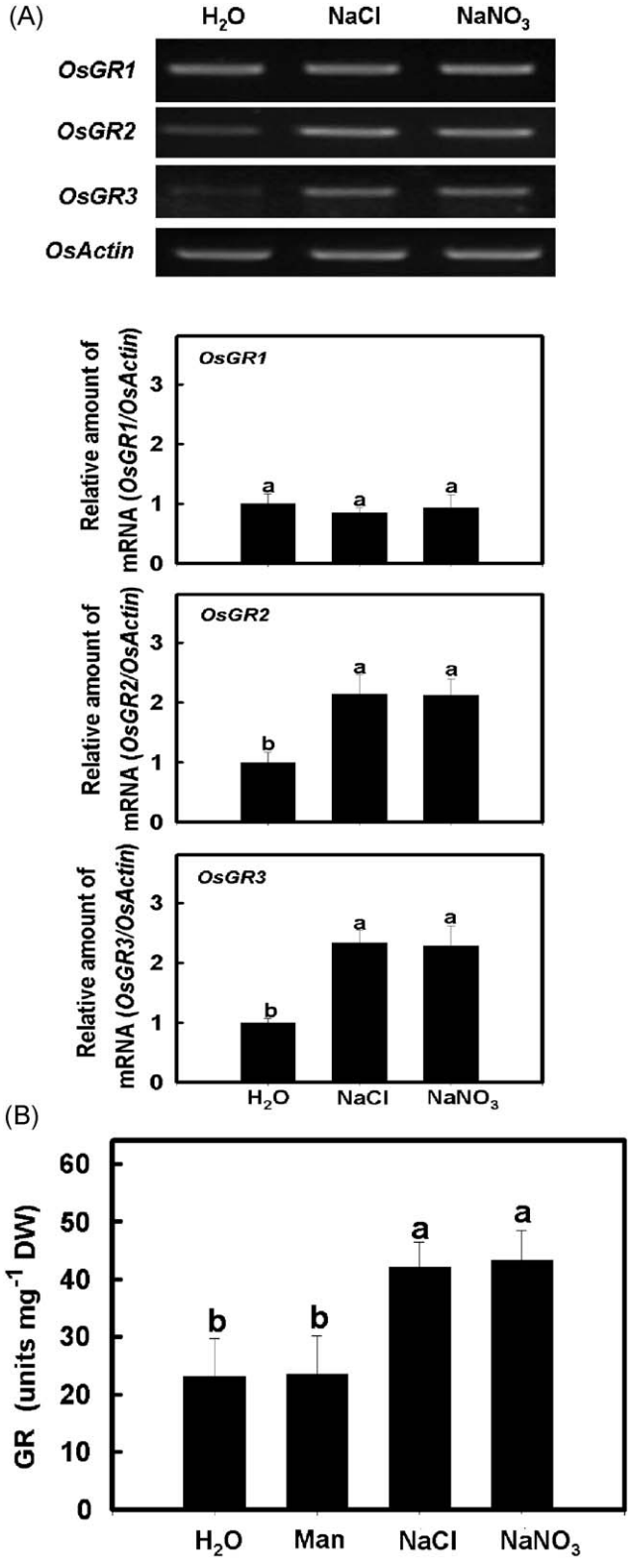


Figure 2. Effect of NaCl concentration on mRNA levels of *OsGR* genes in rice roots. Two-day-old rice seedlings were treated with NaCl (0–300 mM) for 8 h. Semi-quantitative RT-PCR for *OsGR* genes was performed as described in Materials and methods. The expression of *OsGR* genes was normalized to that of *OsActin*. After the adjustment by *OsActin* expression, the reaction with the roots in 0 mM NaCl was treated as the normalized reference, with a value of one, for determining the relative amount of mRNA of *OsGR* genes. Bars show means $\pm$ SE ($n = 3$). Values with the same letter are not significantly different at $P < 0.05$.

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. H_2O_2 content was expressed on the basis of DW.



Statistical analysis

Data were expressed as mean ±SE. Statistical differences between measurements ($n = 4$) for different treatments or different times were analyzed following Duncan's multiple range test or Student's *t*-test. A $P < 0.05$ was considered statistically significant.

Results

NaCl induces activity increase of GR and the expression of *OsGR2* and *OsGR3*

Previous work showed that concentrations of NaCl from 50 to 150 mM progressively increased the activity of GR (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 GR in NaCl-stressed rice roots was higher than that in controls (Figure 1). We used semi-quantitative RT-PCR to investigate the effect of different concentrations of NaCl on the expression of *OsGR* genes in rice roots. After 8-h treatment with NaCl (150, 200, and 300 mM), *OsGR3* expression was specifically increased (Figure 2). The expression of *OsGR2* was also increased by 150 and 200 mM NaCl but not 300 mM NaCl; however, no significant increase due to NaCl (150, 200, and 300 mM) could be detected in the expression of *OsGR1* (Figure 2).

Na^+ but not Cl^- is involved in increasing the expression of *OsGR2* and *OsGR3*

To test whether Cl^- is involved in enhancing the expression of *OsGR* genes, we compared the effect of 150 mM NaCl and 150 mM $NaNO_3$ on the expression *OsGR1*, *OsGR2*, and *OsGR3* in rice roots (Figure 3A). The expression of *OsGR2* and *OsGR3* but not *OsGR1* was increased by NaCl and $NaNO_3$ treatments. *OsGR2* and *OsGR3* expression with $NaNO_3$ treatment was similar to that with NaCl treatment.

An osmotic effect is not involved in NaCl-induced expression of *OsGR2* and *OsGR3*

To understand the role of the osmotic effect in NaCl-induced expression of *OsGR2* and *OsGR3* 2-d-old rice seedlings were treated with 276 mM Man, a concentration iso-osmotic with 150 mM NaCl, but the expression of *OsGR1*, *OsGR2*, and *OsGR3* in rice roots was not enhanced by Man (Figure 4).

Na^+ but not Cl^- or osmotic stress is responsible for NaCl-increased GR activity

To examine the role of ionic and osmotic effects in NaCl-increased GR activity in rice roots, 2-d-old rice seedlings were treated with H_2O, 150 mM NaCl, 150 mM $NaNO_3$, and 276 mM Man for 8 h. Both NaCl and $NaNO_3$ increased the GR activity in rice roots to a similar extent; however, GR activity in rice roots was not increased by Man (Figure 3B).

Na^+ but not Cl^- or osmotic stress is responsible for NaCl-induced H_2O_2 accumulation

H_2O_2 is increased in level in rice roots in response to NaCl (Tsai et al., 2005). Recently, we demonstrated that NaCl-induced expression of *OsGR2* and *OsGR3* is mediated through H_2O_2 (Hong et al., 2009). To examine the role of ionic and osmotic effects in NaCl-induced H_2O_2 accumulation in rice roots, 2-d-old seedlings were treated with 150 mM NaCl, 150 mM $NaNO_3$, and 276 mM Man. Both NaCl and $NaNO_3$ increased H_2O_2 content in rice roots to a similar extent; however, Man treatment had no effect on H_2O_2 content in rice roots (Figure 5).

Discussion

Gene expression in response to abiotic 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. The expression of *GR* genes is enhanced by NaCl treatment in plants (Kaminaka et al., 1998; Tsai et al., 2005; Hong et al., 2009). In

Figure 3. (A) Effect of NaCl and $NaNO_3$ on the mRNA levels of *OsGR* genes 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 *OsGR* genes was performed as described in Materials and methods. The expression of *OsGR* genes was normalized to that 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 ($n = 3$). Values with the same letter are not significantly different at $P < 0.05$. (B) Effect of mannitol (Man), NaCl, and $NaNO_3$ on GR activity in roots of rice seedlings. Two-day-old rice seedlings were treated with H_2O, Man (276 mM), NaCl (150 mM), or $NaNO_3$ (150 mM) for 8 h. Bars show means ±SE ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$.

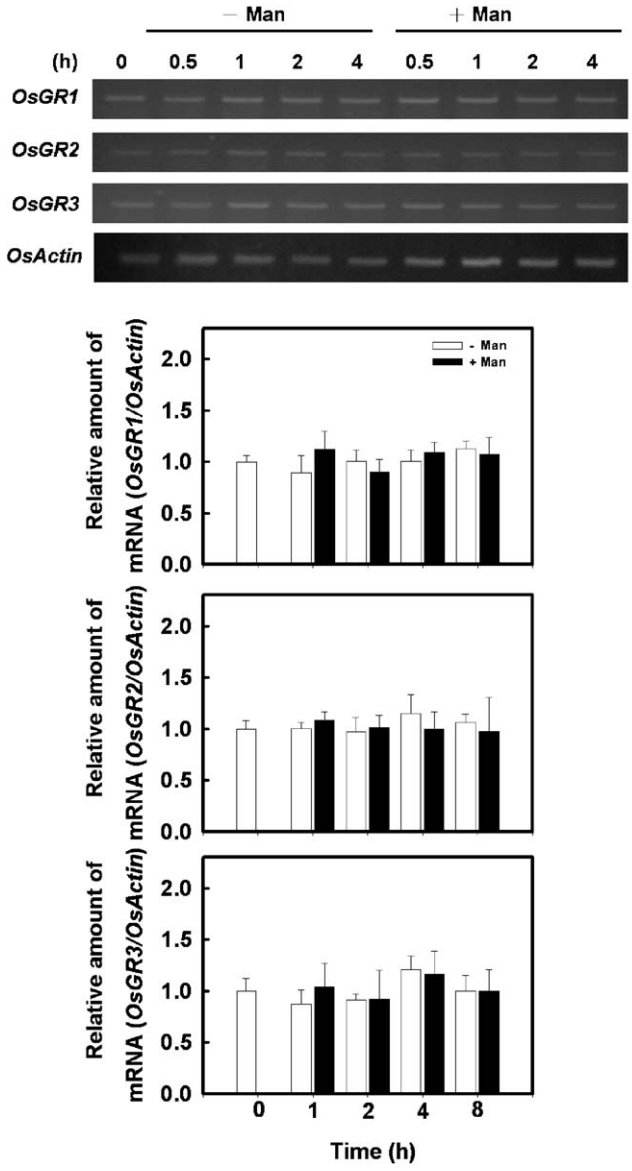


Figure 4. Effect of mannitol (Man) on mRNA levels of *OsGR* genes in rice roots. Two-day-old rice seedlings were treated with or without Man (276 mM). Semi-quantitative RT-PCR for *OsGR* genes was performed as described in Materials and methods. The expression of *OsGR* genes was normalized to that of *OsActin*. After the adjustment by *OsActin*, the reaction with the roots without Man was treated as the normalized reference, with a value of one, for determination of the relative amount of mRNA of *OsGR* genes. Bars show means $\pm$SE ($n = 3$).

the present study, NaCl treatment resulted in enhanced *OsGR2* and *OsGR3* expression (Figure 2).

It has bee shown that NaCl treatment causes an increase in GR activity and has significant effect on the activation of two isozymes of GR in rice isozymes of GR in rice roots (Tsai et al., 2004). From the results reported previously (Tsai et al., 2005; Hong et al., 2009) and in the present study (Figures 1–3), the increase in the expression of *OsGR2* and *OsGR3* is indeed associated with enhanced GR activity. The expression of *OsGR2* and *OsGR3* induced by NaCl may affect ROS-scavenging properties in rice roots. Both *OsGR1* and *OsGR3* are genes encoding GR of chloroplastic forms in rice roots. The fact that the expression of *OsGR3* but not *OsGR1* was induced by NaCl is most likely due to the differential sensitivity to NaCl in rice roots.

The physiological disturbances produced by salinity in citrus and maize plants are associated with Cl^- build-up rather than Na^+ accumulation (Cramer et al., 1994; Romero-Aranda et al., 1998; Moya et al., 2003). Results from Montero et al. (1998) and Sibole et al. (1998) strongly suggest that

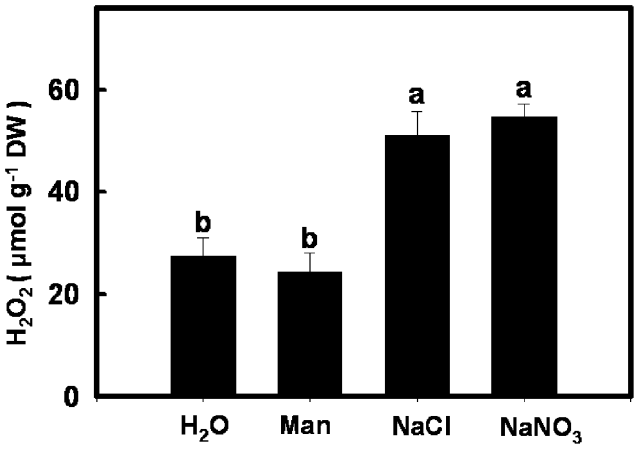


Figure 5. Effect of mannitol (Man), NaCl, and $NaNO_3$ on H_2O_2 levels in roots of rice seedlings. Two-day-old rice seedlings were treated with H_2O, Man (276 mM), NaCl (150 mM), or $NaNO_3$ (150 mM) for 8 h. Bars show means ± SE ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$.

bean is extremely sensitive to Na^+. Our own data also demonstrated that NaCl-inhibited growth of rice roots is mainly associated with Na^+ (Lin and Kao, 2001b). However, little is known about the relation of Na^+ and Cl^- accumulation to induction of antioxidant systems. Of particular interest in the present study are the findings that Na^+ but not Cl^- is required for the NaCl-enhanced expression of *OsGR2* and *OsGR3* and activity of GR in rice roots (Figures 3A and B).

NaCl treatment increases H_2O_2 content in rice roots (Tsai et al., 2004; Hong et al., 2007). In the present study, increased H_2O_2 content in rice roots was similar with $NaNO_3$ and NaCl treatment (Figure 5). These results strongly suggest that NaCl-increased H_2O_2 accumulation is associated with Na^+. In addition to its known component of ion toxicity, salt concentration in soil results in an osmotic effect. Here we show that the content of H_2O_2, the expression of *OsGR2* and *OsGR3*, and the activity of GR were not affected at a concentration of Man (276 mM) iso-osmotic with 150 mM NaCl (Figures 3–5), which suggests that NaCl-enhanced H_2O_2 accumulation, *OsGR2* and *OsGR3* expression, and GR activity are not associated with the osmotic component.

Recently, we demonstrated that H_2O_2 is involved in regulation of *OsGR2* and *OsGR3* expression in NaCl-treated rice roots and that NaCl-induced expression of *OsGR2* and *OsGR3* occurs before the NaCl-enhanced increase in GR activity in rice roots (Hong et al., 2009). The following sequence of events may take place for NaCl-induced GR activity in rice roots: $Na^+ \rightarrow H_2O_2 \rightarrow$ *OsGR2, OsGR3* $\rightarrow$ GR activity. ROS are thought to play an important role in NaCl stress. To scavenge the toxic effects of these damaging ROS, cells have developed highly regulated enzymatic and non-enzymatic mechanisms (Mittler, 2002). Ascorbate peroxidase (APX) and GR are two important ROS-scavenging enzymes (Noctor and Foyer, 1998). The expression of *O. sativa APX8* (*OsAPX8*) is enhanced by NaCl in rice roots (Hong et al., 2007) and NaCl-induced *OsAPX8* expression in rice roots is associated with Na^+ but not Cl^- and osmotic component (Hong et al., 2007; Hong and Kao, 2008). In conclusion, an ion-specific effect caused by Na^+ in rice roots may account for NaCl-induced expression of *OsGR2*, *OsGR3*, and *OsAPX8*.

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