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Salinity, oxidative stress and antioxidant responses in shoot cultures of rice

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Abstract

When shoot cultures derived from salt-sensitive *Oryza sativa* var. Taipei 309 were grown at 25 °C in medium containing 0.35 M NaCl, responses to possible oxidative stress in the early stages of exposure were observed. Overall levels of Mn-superoxide dismutase activity, Cu, Zn-superoxide dismutase activity and H_2O_2 were significantly elevated. After 1 d there was a notable decline in tissue concentrations of GSH and a corresponding increase in GSSG. However, after a further day, concentrations of GSH and GSSG returned to concentrations normally encountered in control cultures. Activities of ascorbate peroxidase and catalase were similar whether the shoots were grown in the presence or absence of NaCl. In contrast, there was an early increase in glutathione reductase activity in NaCl-exposed cultures, and no indication of extensive increases in lipid peroxidation. Thus although some indications of oxidative stress accompany exposure of this salt-sensitive rice variety to salinity, mechanisms appear to exist within its shoot tissue to permit the tolerance of such oxidative stress.

Key words: Salinity stress, hydrogen peroxide, glutathione, antioxidant enzymes, *Oryza sativa*.

Introduction

Most rice is grown in tropical and sub-tropical lowlands and most commonly in soils submerged under water. Salinity, however, is an important toxicity encountered by rice. On the other hand, rice is relatively tolerant to salinity and is widely grown in tidal swamps and in inland areas frequently subject to salinity (Greenland, 1990). Numerous metabolic changes have been noted to occur in plants exposed to ionic stress. For example Na^+/H^+ exchange processes are activated so that K^+ can pass across the cell membrane (Watad *et al.*, 1986) and Na^+ can be pumped into tonoplasts (Binzel *et al.*, 1988; Garbarino and DuPont, 1989). Additionally, the ratio of glycoproteins to phospholipids can increase to facilitate the entry of more solutes into cells (Hirayama and Mihara, 1987).

Overall concentrations of osmoprotectants including sugars, organic acids and proteins increase (Binzel *et al.*, 1987; La Rosa *et al.*, 1987) and other studies have demonstrated changes in cellular levels of a small number of proteins, such as osmotin in dicots (Singh *et al.*, 1985, 1987). In rice an osmotically regulated gene, *rab* 21, has been identified (Munday and Chua, 1988). Another gene *sal* T, encodes a protein whose mRNA accumulates in sheaths and roots of rice seedlings exposed to salt stress and correlates with patterns of Na^+ accumulation during salt stress (Claes *et al.*, 1990). In addition to these changes there have been some indications that salt stress can induce conditions of oxidative stress. For example, mitochondria from leaves of salt-sensitive peas exposed to salt stress exhibit increased activities of both Mn-superoxide dismutase and Cu, Zn-superoxide dismutase. Conversely, in salt-tolerant plants NaCl treatment brought about an increase in mitochondrial Mn-superoxide dismutase activity (Hernandez *et al.*, 1993). NaCl treatment also brought about increased superoxide generation by submitochondrial particles which was higher in the NaCl-sensitive compared with the NaCl-tolerant plants (Hernandez *et al.*, 1993).

In this study, some initial symptoms were observed of oxidative stress in shoot cultures derived from salt-sensitive *Oryza sativa* var. Taipei 309 grown in medium containing 0.35 NaCl. The response of antioxidant systems in these cultures has been studied and suggest that

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mechanisms exist whereby such salinity-induced oxidative stress is tolerated in rice.

Materials and methods

Shoot cultures

Oryza sativa var. Taipei 309 seeds were supplied by the International Rice Research Institute, Manila, Philippines. For shoot culture, following the procedure of Finch *et al.* (1992), the seeds were dehusked and surface-sterilized in 30% commercial bleach solution (Domestos) for 30 min, then rinsed six times in sterile distilled water. The seeds were then inoculated to a depth of 2–3 mm in M50 agar medium containing Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 0.8% agar and adjusted to pH 5.8. The inoculated seeds were then incubated at 25 ± 1 °C with a 14/10 h (light/dark) period with a photon flux density of 100 μmol m^{-2} s^{-1}. After 14 d the germinated seedlings were trimmed of their endosperm and part of their root, leaving 5 mm of their roots intact. These seedlings were then transferred to shoot culture medium, MSB2A which comprised Murashige and Skoog medium supplemented with 2 mg l^{-1} benzyladenine, 6% (w/v) sucrose and 0.4% (w/v) agarose type 1 (Sigma Chemical Co. Poole, Dorset) and adjusted to pH 5.8. For experimental purposes triplicate shoot cultures, each represented by containers containing three shoots in MSB2A medium were used. At appropriate times the shoots from each culture were removed from the MSB2A medium, washed thoroughly of any attached pieces of agar medium and weighed immediately for fresh weight (FW). For determination of dry weight the shoots from each culture were placed in preweighed envelopes and dried to constant weights in a drying oven at 50 °C.

Determination of sodium in rice shoot tissue

Dried shoot tissue (50 mg) was placed in 1.5 ml concentrated sulphuric acid containing 20 g l^{-1} selenium dioxide. 6 ml hydrogen peroxide (30%, w/w) was carefully added and the mixture held at 340 °C for 60 min. After cooling and addition of 14 ml water, sodium was determined in the resulting digestion mixture at 589.59 nm using a Thermo Electron Plasma 100 inductively coupled emission spectrometer.

Lipid peroxidation breakdown products

Shoot samples were ground in liquid nitrogen with a pestle and mortar and suspended in 1 ml 20 mM TRIS-HCl (pH 7.4) and the suspension centrifuged at 2500 *g* for 10 min. Samples (200 μl) of the supernatant were used for the assay of decomposition products of oxidized polyunsaturated fatty acids (i.e. malonaldehyde and 4-hydroxyalkenals) using the LPO-586 colorimetric assay from Bioxytech SA, Bonneuil-sur-Marne, France by following their instructions.

Determination of reduced and oxidized glutathione

Glutathione (GSH) can be assayed by an enzymic recycling procedure (Griffith, 1980) in which it is sequentially oxidized by 5,5′-dithiobis (2-nitrobenzoic acid) [DTNB] and reduced by NADPH in the presence of glutathione reductase. The extent of 2-nitro-5-thiobenzoic acid formation is monitored at 412 nm. Besides GSH, the assay will also detect oxidized glutathione (GSSG). For specific assay of GSSG, the GSH can be masked by derivatization with 2-vinylpyridine. Shoot samples (3 shoots per sample) were ground in liquid nitrogen with pestle and mortar. The ground tissue was suspended in 0.5 ml 5% sulphosalicyclic acid and centrifuged at 12 000 *g* for 10 min. A 300 μl aliquot of supernatant was removed and neutralized by addition of 18 μl 7.5 M triethanolamine. One 150 μl sample was then used to determine concentrations of GSH plus GSSG. Another was pretreated with 3 μl 2-vinylpyridine for 60 min at 20 °C to mask the GSH by derivatization, to allow the subsequent determination of GSSG alone. In each case, 50 μl aliquots of the two types of sample were mixed with 700 μl 0.3 mM NADPH, 100 μl DTNB and 150 μl buffer containing 125 mM sodium phosphate–6.3 mM EDTA (pH 6.5). A 10 μl aliquot of glutathione reductase (5 U ml^{-1}; 1 unit will reduce 1 μmol oxidized glutathione min^{-1} at pH 7.6 at 25 °C) was then added and the change in absorbance at 412 nm monitored at 30 °C. A standard curve was prepared using solutions of GSH and GSSG. For any given concentration of GSSG, the concentration of GSH corresponds to twice that concentration.

Determination of hydrogen peroxide levels in shoot cultures

Shoot samples (3 shoots per sample) were ground with a mortar and pestle in liquid nitrogen. Samples were then suspended in 1 ml 0.2 M perchloric acid and centrifuged at 12 000 *g* for 10 min. To remove the perchloric acid, the supernatant was neutralized to pH 7.5 with 4 M potassium hydroxide and the solution centrifuged at 100 × *g* for 1 min to remove insoluble potassium perchlorate. A 200 μl aliquot of the supernatant was applied to a 1 ml column of anion exchange resin (AG 1-X2; Bio-Rad, Watford, UK) and the column washed with 800 μl of distilled water and the eluate used for the assay of hydrogen peroxide as outlined by Okuda *et al.* (1991). Reaction mixtures contained 1 ml of the eluate, 400 μl 12.5 mM 3-dimethylaminobenzoic acid (Sigma, Poole, Dorset, UK) in 0.375 M phosphate buffer (pH6.5), 80 μl 1.3 mM 3-methyl-2-benzothiazolinone hydrazone (Sigma) and 20 μl (0.25 units) horseradish peroxidase (Sigma) in a total volume of 1.5 ml. The reactions were initiated by the addition of the peroxidase at 25 °C and the increase in absorbance at 590 nm after 3 min at 23 °C was monitored and compared with increases elicited by standard samples of hydrogen peroxide.

Assays of total antioxidant enzyme activities

(i) *Catalase*: Shoot samples were homogenized in a mortar and pestle with liquid nitrogen. Samples were suspended in 50 mM potassium phosphate buffer (pH 7.4). Following centrifugation at 12 000 *g* for 5 min the supernatant was removed for assay of catalase activity at 25 °C using the method outlined by Clairborne (1985) in which consumption of hydrogen peroxide is determined.

(ii) *Glutathione reductase*: Shoot samples were homogenized in a mortar and pestle with liquid nitrogen and an extract prepared in 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 1% (w/v) polyvinylpolypyrrolidone and 1 mM ascorbate and centrifuged for 10 min at 12 000 *g*. The assay for glutathione reductase at 25 °C involved monitoring the oxidation of NADPH at 340 nm in 1 ml reaction solutions that contained 0.1 M TRIS–HCl (pH 7.8), 2 nM EDTA, 50 μM NADPH, 0.5 mM oxidized glutathione (GSSG), and 25 μl of the above enzyme extract (Sen Gupta *et al.*, 1993)

(iii) *Ascorbate peroxidase*: Shoot extracts prepared in medium containing ascorbate as for assay of glutathione reductase, were subsequently assayed for ascorbate peroxidase at 25 °C following the procedure of Nakamo and Asada (1981) in reaction mixtures containing 50 mM potassium phosphate (pH 7.0),

0.1 mM ascorbate, 0.1 mM EDTA, and 0.1 mM hydrogen peroxide.

(iv) *Superoxide dismutase*: Triplicate shoot samples were homogenized in an equal volume of ice-cold extraction buffer (50 mM potassium phosphate, pH 7.8, 0.1% (w/v) bovine serum albumin, 0.1% (w/v) ascorbate, 0.05% (w/v) β-mercaptoethanol, 0.2% (v/v) Triton-X-100), and clarified by centrifugation at 10 000 *g* for 12 min. Samples containing 25 mg protein were separated by electrophoresis through non-denaturing 10% polyacrylamide gels and superoxide dismutase activity localized using the photo-chemical method of Beauchamp and Fridovich (1971). Inhibitor studies as described by Sandalio *et al.* (1987) were carried out directly on the gels to distinguish between the Cu, Zn- and Mn-superoxide dismutase species (no Fe-superoxide dismutase was detected.). The stained gels were subsequently dried and scanned using an Epson BT-6500 scanner and the activities of superoxide dismutase were assessed from relative intensities as previously described (Burdon *et al.*, 1994).

Results

When shoots derived from *Oryza activa* Taipei 309 are cultured at 25 °C on medium containing 0.35 M NaCl they accumulate sodium ions (Fig. 1). Figure 2 shows that whilst tissue concentrations of H_2O_2 decline progressively with culture of these rice shoots at 25 °C, significantly higher concentrations of H_2O_2 were detected in shoots cultured in the presence of 0.35 M NaCl. Possible plant sources of H_2O_2 would include mitochondria and chloroplasts (Hernandez *et al.*, 1993) where superoxide generated could give rise to H_2O_2 through the action of organelle-associated superoxide dismutases. Whilst H_2O_2 can also arise from peroxisomes, these organelles can also be a source of superoxide although they contain superoxide dismutase activity (del Rio *et al.*, 1992). Table 1 shows that total Mn- and Cu, Zn-superoxide dismutase activities are elevated initially upon exposure of rice shoots to 0.35 M NaCl (i.e. by 24 h).

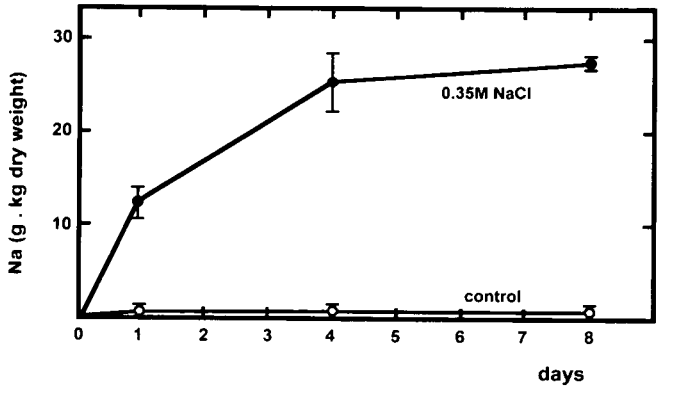

Fig. 1. The effect of growth in NaCl on levels of sodium in shoot cultures of *O. sativa*. After shoots had been grown at 25 °C in medium for the times indicated containing 0.35 M NaCl (●) or no added NaCl (○), samples were taken for the determination of tissue sodium. Data are means ± SD ($n = 3$).

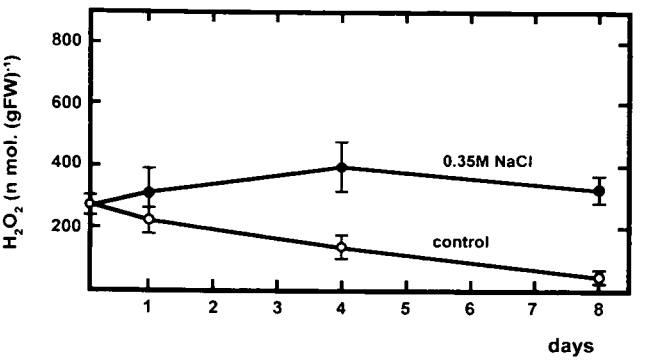

Fig. 2. The effect of growth in NaCl on levels of H_2O_2 in shoot cultures of *O sativa*. After shoots had been grown at 25 °C in medium for the times indicated containing 0.35 M NaCl (●) or no added NaCl (○), samples were taken for determination of tissue levels of H_2O_2. Data are means ± SD ($n = 3$).

Table 1. *Superoxide dismutase activities in rice shoot cultures exposed to 0.35 M NaCl*

Treatment	Superoxide dismutase (SOD) activity (arbitary units[a] per 25 mg sample)	
	Mn-SOD	Cu, Zn-SOD
None (1 d)	13.78 ± 1.08	29.11 ± 2.23
0.35 M NaCl (1 d)	34.55 ± 3.21	42.99 ± 3.90
0.35 M NaCl (8 d)	20.40 ± 2.15	29.03 ± 13.11

[a]Sandalio *et al.* (1987). Results are means ± SD ($n = 3$).

Excessive tissue levels of H_2O_2 could, in principle, be minimized through the activity of metabolizing enzymes such as ascorbate peroxidase and catalase. As the latter enzyme requires reduced glutathione (GSH) as co-factor, tissue concentrations of GSH and oxidized glutathione (GSSG) were examined in shoots grown in 0.35 M NaCl. From Fig. 3 it can be seen that although levels of GSH increased gradually with normal culture, exposure to salt results in a significant drop in GSH after 24 h. At that time there is a correspondingly notable rise in tissue levels of GSSG. Thereafter, however, concentrations of GSH and GSSG more closely parallel the situation in control cultures. The total activity of ascorbate peroxidase itself in the salt-exposed cultures is, however, quite similar to activities encountered in control cultures at the various times of exposure examined (Fig. 4). Although two forms of ascorbate peroxidase have been described (chloroplastic and cytosolic), it is likely that the total activity measured represents a mixture of both forms. Activities of catalase are also quite similar, although in the salt-stressed cultures they are moderately higher by 8 d (Fig. 5).

The recovery to normal tissue concentrations of GSH after 8 d of salt stress may be a function of glutathione reductase activity, which in normal cultures increases with growth at 25 °C. In salt-exposed cultures there is an early

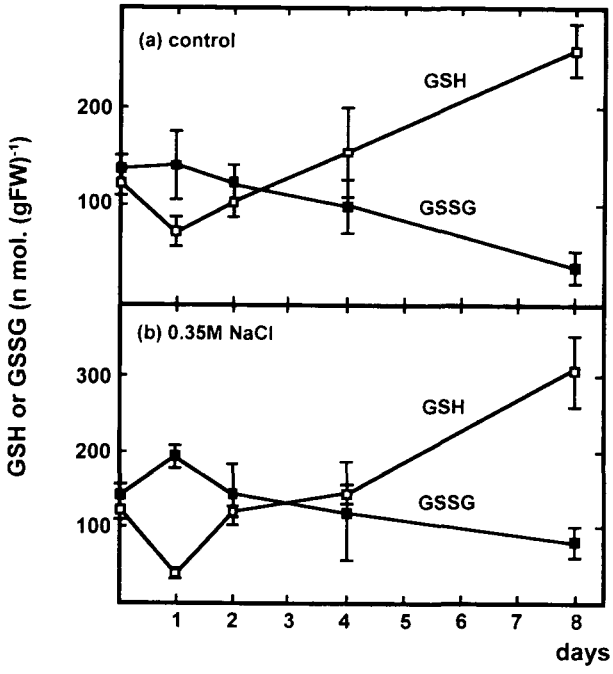

Fig. 3. Effect of growth in NaCl on levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) in shoot cultures of *O sativa*. After shoots had been grown at 25 °C in the presence (b) or absence (a) of 0.35 M NaCl for the times indicated, samples were taken for determination of tissue levels of GSH (□) and GSSG (●). Data are means ± SD ($n = 3$).

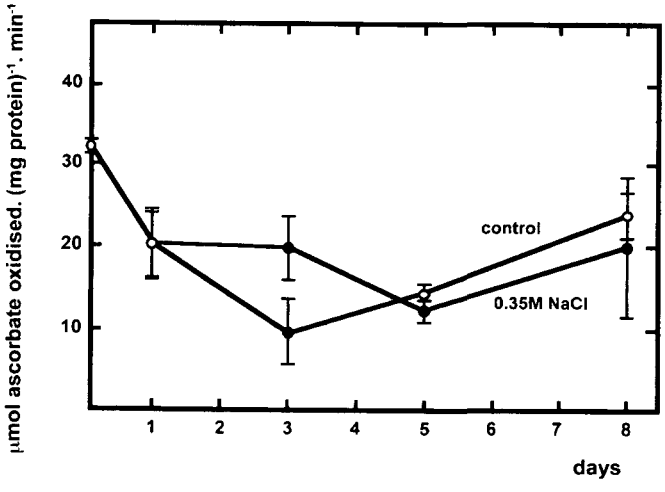

Fig. 4. Effect of growth in NaCl on levels of ascorbate peroxidase activity in shoot cultures of *O sativa*. After shoots had been grown at 25 °C in the presence (●) or absence (○) 0.35 M NaCl for the times indicated, samples were taken for determination of tissue levels of ascorbate peroxide activity Data are means ± SD ($n = 3$).

increase in tissue activities of glutathione reductase which, however, remain relatively unchanged thereafter (Fig. 6).

Whilst some symptoms of oxidative stress are elicited in rice shoot cultures exposed to 0.35 M NaCl, such as reduced concentrations of GSH and elevated H_2O_2, there also appear to be rapid, but transient, responses in terms of cellular anti-oxidant enzyme activities. There is a

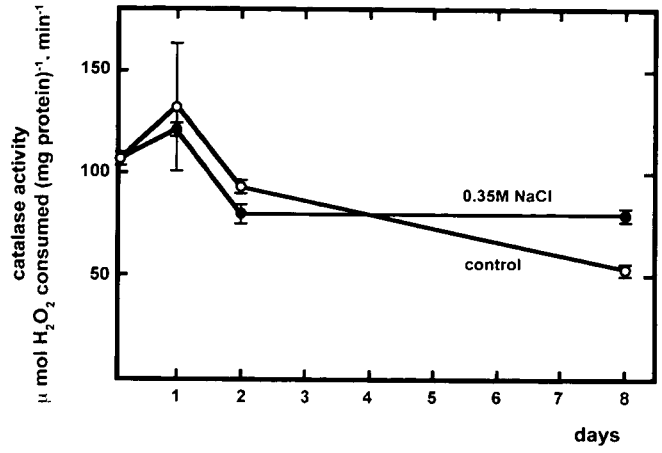

Fig. 5. Effect on growth in NaCl on levels of catalase activity in shoot cultures of *O sativa*. After shoots had been grown at 25 °C in the presence (●) or absence (○) of 0 35 M NaCl for the times indicated, samples were taken for determination of tissue levels of catalase activity. Data are means ± SD ($n = 3$)

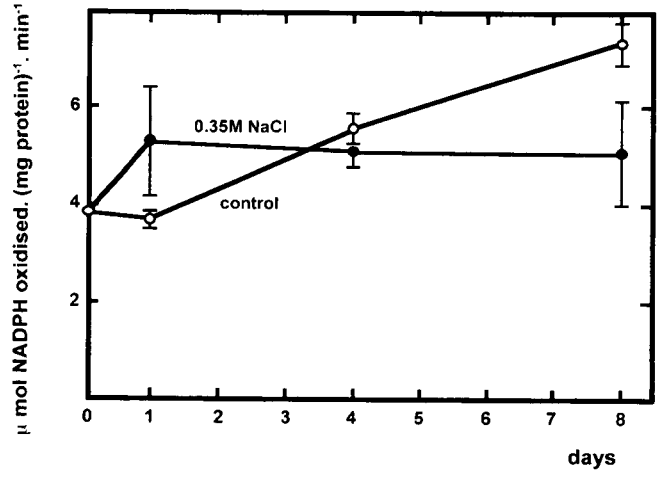

Fig. 6. Effect on growth in NaCl on levels of glutathione reductase activity in shoot cultures of *O. sativa*. After shoots had been grown at 25 °C in the presence (●) or absence (○) of 0.35 M NaCl for the times indicated, samples were taken for determination of tissue levels of glutathione reductase activity. Data are means ± SD ($n = 3$).

question concerning the general effectiveness of such responses. Figure 7 attests to their efficiency in as much as tissue levels of lipid peroxidation breakdown products are only marginally higher in the salt-exposed cultures.

Discussion

Whilst plants exposed to salinity stress are known to alter their lipid composition (Hirayama and Mihara, 1987), or to increase levels of osmoprotectants such as sugars, organic acids and proline (Binzel *et al.*, 1987; La Rosa *et al.*, 1987) or cellular proteins like osmotin (Singh *et al.*, 1985, 1987) and the products of *rab* 21 or *sal* T (Munday and Chua, 1988; Claes *et al.*, 1990), responses to

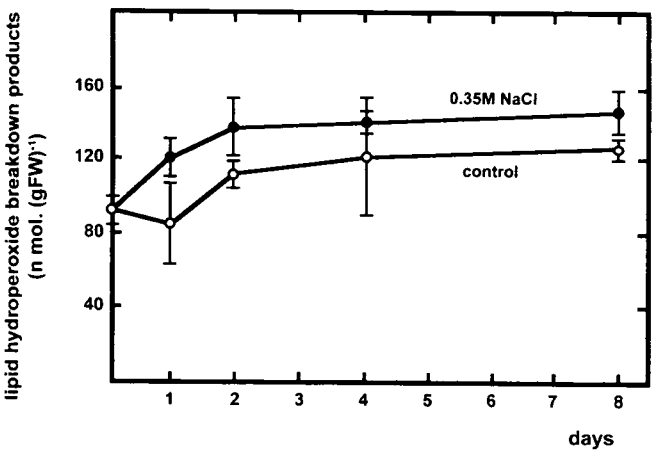

Fig. 7. Effect on growth in NaCl on levels of lipid hydroperoxide breakdown products in shoot cultures of *O. sativa*. After shoots had been grown at 25 °C in the presence (●) or absence (○) of 0.35 M NaCl for the times indicated, samples were taken for determination of tissue levels of breakdown products of lipid hydroperoxide (i.e. malondialdehyde and 4-hydroxyalkenals). Data are means ± SD ($n = 3$).

symptoms of 'oxidative stress' are also evident in certain species. For instance in mitochondria from NaCl-sensitive cultivars of *Pisum sativum* (Hernandez *et al.*, 1993), salinity brought about decreased levels of Mn-superoxide dismutase. Conversely, in salt-tolerant plants NaCl treatment brought about an increase in mitochondrial Mn-superoxide dismutase activity. Salt treatment also increased the generation of superoxide by sub-mitrochondrial particles, more so from salt-sensitive than salt-tolerant cultivars (Hernandez *et al.*, 1993).

Previous work of Sen Gupta *et al.* (1993) on transgenic tobacco plants suggested that overexpression of superoxide dismutase could protect plants from oxidative stress. Other studies (Herouart *et al.*, 1993) suggest that expression of the Cu, Zn-superoxide dismutase gene is redox-activated in transgenic tobacco plants. In shoot cultures derived from *Oryza sativa* var. Taipei 309 it is clear that exposure to 0.35 M NaCl leads to increased activities of both Mn-superoxide dismutase and Cu, Zn-superoxide dismutase. However, it should be emphasized that the elevated activities were only encountered in the initial stages of salt exposure as both returned to activities more similar to those detected in control cultures after prolonged salt exposure (i.e. 8 d). Although increased activity of superoxide dismutase would be a means of removing any excessive amount of superoxide anions generated, for example, in mitochondria (Boveris and Chance, 1973; Loschen *et al.*, 1973; Nohl and Heger, 1988), or chloroplasts (Asada and Takahashi, 1987) under conditions of salt stress, the H_2O_2 produced by such superoxide dismutase would still present a potential hazard to plant tissues. For example, it could directly oxidize vital cellular proteins or it could have deleterious effects on cell macromolecules including lipids, proteins and nucleic acids (Halliwell and Gutteridge, 1989). In these studies, tissue concentrations of H_2O_2 in rice shoot cultures exposed to 0.35 M NaCl are significantly higher than in control cultures. In addition to mitochondria and chloroplasts, peroxisomes are also a potential source of H_2O_2 through flavin oxidases (del Rio *et al.*, 1992) although they could also be a source of superoxide through xanthine oxidase as well as NADH-requiring systems (del Rio *et al.*, 1992). These organelles often have Cu, Zn-superoxide dismutase activity as well, thus possibly serving as a source of H_2O_2 (Sandalio *et al.*, 1987). H_2O_2 could also be derived from cellular peroxidase activity (Peng and Koo, 1992).

Increased concentrations of H_2O_2 would, nevertheless, be expected to be alleviated by the combined activities of ascorbate peroxidase and catalase. In the salt-stressed rice cultures, the total activities of both these key enzymes initially remained quite similar to those in control cultures. It has been suggested that H_2O_2 could act as a cellular signal for the induction of catalase genes in maize (Prasad *et al.*, 1994), however, despite increased tissue levels of H_2O_2 in salt-stressed rice cultures, a slight increase in catalase activity was only observed after 8 d of salt exposure. As judged from the analysis of tissue concentrations of GSH and GSSG it seems that ascorbate peroxidase may be playing some role in the metabolism of H_2O_2 in the salt-stressed rice cultures, as an early drop in the substrate for this enzyme GSH is balanced by a concomitant rise in its product GSSG.

By the second day of salt stress, concentrations of both GSH and GSSG, however, return to concentrations similar to those in control shoot cultures. By 8 d of salt stress, GSH concentration is even slightly higher than in control cultures. This could be a function of glutathione reductase activity. Increased activity of glutathione reductase is an early occurrence in the salt-stressed cultures (Fig. 6). A pivotal role for glutathione reductase in resisting 'oxidative stress' is suggested from the experiments of Foyer *et al.* (1991) and Aono *et al.* (1991). Both groups observed resistance to active oxygen toxicity in transgenic tobacco that expressed the gene for glutathione reductase from *Escherichia coli*. Additionally, recent experiments have shown that tobacco plants transgenic for pea glutathione reductase were more resistant to paraquat (Creissen *et al.*, 1994).

Whilst the transient changes in overall activity of superoxide dismutases and glutathione reductase as well as in concentrations of GSH and GSSG, suggest that in the period immediately following exposure of the rice shoots to 0.35 M NaCl, any problems of oxidative stress are being overcome, there is always the potential hazard from the higher than normal concentrations of H_2O_2 encountered. However, tissue concentrations of H_2O_2 do not rise any further after 4 d of salt exposure. A problem might be that under the conditions of salt exposure, some delocalization of intracellular iron might take place to

release iron capable of catalysing the formation of hydroxyl radicals from the H_2O_2, which, in principle, could initiate the peroxidation of critical cell membranes (Halliwell and Gutteridge, 1989). On balance, however, as judged indirectly by the determination of tissue levels of lipid hydroperoxide breakdown products (Fig. 7), any increase in lipid peroxidation in these rice shoots brought about by the salt exposure are likely to have been only marginal. Although salt-tolerant varieties of rice are known (Hernandez *et al.*, 1993), the studies reported here have been carried out on shoot cultures derived from rice variety Taipei 309, which does not show salt-tolerance *in vivo*. Nevertheless, the shoots from Taipei 309 can be maintained *in vitro* in medium containing 0.35 M NaCl for periods of up to 8 d, although they accumulate sodium ions (Fig. 1) and exhibit signs of necrosis by then. Thereafter such salt-exposed shoots deteriorate rapidly.

In summary, it appears that although exposure of Taipei 309 shoots to salt does elicit some symptoms of oxidative stress, such as elevated superoxide dismutase activities, increasing tissue concentrations of H_2O_2 and depressed ratios of GSH to GSSG, these seem to be only short-term. After 4 d of salt-stress, H_2O_2 concentrations do not increase further and by 8 d ratios of GSH/GSSG and activities of superoxide dismutase have returned to more normal values. Thus although steps to tolerate oxidative stress may be a component of plant responses to high salinity, they are unlikely to be a major determinant of overall salt-tolerance.

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