Cell Death of Rice Roots under Salt Stress May Be Mediated by Cyanide-Resistant Respiration

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Treatment with solutions containing high concentrations of NaCl (200 or 300 mM) induced cell death in rice (*Oryza sativa* L.) roots, as well as the application of exogenous hydrogen peroxide (H_2O_2). Moreover, the pretreatment with dimethylthiourea (DMTU), a scavenger of H_2O_2, partially alleviated the root cell death induced by 200 mM NaCl. These observations suggest that the cell death of rice roots under high salt stress is linked to H_2O_2 accumulation *in vivo*. NaCl stress increased the level of cyanide-resistant respiration to some extent and enhanced the transcript levels of the alternative oxidase (AOX) genes *AOX1a* and *AOX1b* in rice roots. High-salt-stressed (200 mM NaCl) rice roots pretreated with 1 mM salicylhydroxamic acid (SHAM), a specific inhibitor of alternative oxidase, exhibited higher levels of cell death and H_2O_2 production than roots subjected to either 200 mM NaCl stress or SHAM treatment alone. These results suggest that cyanide-resistant respiration could play a role in mediating root cell death under high salt stress. Furthermore, this function of cyanide-resistant respiration could relate to its ability to reduce the generation of H_2O_2.

Key words: Cell Death, Cyanide-Resistant Respiration, Salt Stress

Introduction

Growing plants frequently are subjected to a stressful environment, such as soil salinity; particularly excess NaCl is the single most widespread soil toxicity problem presently faced by global crop production (Hong *et al*., 2007). The root is the primary organ that experiences soil salinity, and the effects of salinity stress in roots are generally associated with a low osmotic potential of the soil solution and a nutritional imbalance (Hasegawa *et al*., 2000). In particular, a high level of salt stress can induce the cell death of root tissues, causing the irreversible inhibition of root growth and seriously limiting the development, growth, survival, and productivity of the entire plant (Katsuhara and Shibasaka, 2000). Therefore, with the aim of enhancing the knowledge about the salt tolerance mechanisms of plant roots, investigations of the cell death induced by high salt stress have both fundamental and economic importance.

Abbreviations: AOX, alternative oxidase; DMTU, dimethylthiourea; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid.

The cellular and molecular events involved in plant cell death have been extensively studied. An important generalization is that the mitochondria are the first cellular compartments to demonstrate cell death responses and that these organelles play a central role in regulating the process of cell death (Rhoads *et al*., 2006). This regulation, in turn, seems to be intimately linked to the reactive oxygen species (ROS) produced by plant mitochondria (mtROS), which are initially generated from the mitochondrial electron transport chain and function as early signals in the induction of cell death (Yao *et al*., 2002; Rhoads *et al*., 2006; Love *et al*., 2008; and references cited therein).

The current understanding of how plants effectively control mtROS formation is focused on the plant-specific cyanide-resistant alternative pathway. It is well known that cyanide-resistant respiration is catalyzed by alternative oxidase (AOX), which is located in the mitochondrial inner membrane and transfers electrons directly from the ubiquinone pool to oxygen without energy conservation (Dutilleul *et al*., 2003; Millenaar and

Lambers, 2003). It has been confirmed that AOX can prevent the over-reduction of the ubiquinone pool and, therefore, has a function in limiting mtROS formation (Maxwell *et al.*, 1999). Many cell death-inducing substances or factors, such as O_3 (ozone), H_2O_2 (hydrogen peroxide), and pathogen infection, can strongly induce the expression of AOX or enhance the level of cyanide-resistant respiration (Amor *et al.*, 2000; Ederli *et al.*, 2006; Kiba *et al.*, 2007), suggesting that AOX plays a role in the process of cell death.

Recent work reported that treatment with high concentrations of NaCl leads to an increase in cyanide-resistant respiration and AOX expression in plant calli or cell suspension cultures (Ferreira *et al.*, 2008; Wang *et al.*, 2010). However, there is a remarkable lack of knowledge about the responses of AOX to salt stress in plant roots. In addition, it has not been extensively studied whether the high-salt-induced cell death of root tissues is related to cyanide-resistant respiration, an issue that should be considered important because the root is the first organ to experience salt stress and its viability has a significant influence on the other plant organs.

Rice (*Oryza sativa* L.) is a staple food source for more than one-third of the world's population, and rice production, which is severely affected by salt stress, has a huge socio-economic impact on human existence (Sawada *et al.*, 2006). The present work demonstrates that cyanide-resistant respiration could play a role in mediating the high-salt-induced cell death of rice roots. In addition, this function of cyanide-resistant respiration may relate to its ability to reduce the generation of H_2O_2.

Material and Methods

Plant material and treatment

Rice (*Oryza sativa* L. ty. 559) seeds (Shenzhou Seed Co., Ltd., Nanjing, China) were treated with 1% NaOCl for 10 min and then washed clean with distilled water. The seeds were imbibed in distilled water for 24 h at 23 °C and then germinated at 26 °C for 24 h. Germinated seeds were placed on the surface of gauze discs placed in open Petri dishes containing distilled water, and the growing roots were allowed to pass through the gauze discs into the distilled water. The seedlings were grown at 26 °C in the dark until the lengths of their roots had reached approximately 2 cm.

In the first set of the experiments, the distilled water in the Petri dishes was replaced with 100 mM, 200 mM, or 300 mM NaCl solution; then the seedlings were kept in the dark for 12 h at 26 °C. In the second set of the experiments, the distilled water in the Petri dishes was replaced with 50 mM, 100 mM, 200 mM, or 400 mM H_2O_2 solution; then the seedlings were kept in the dark for 12 h at 26 °C. Seedlings exposed to distilled water under the same conditions were used as the controls for both sets of experiments.

In the third set of the experiments, prior to the salt stress treatment, the distilled water in the Petri dishes was replaced with 1 mM salicylhydroxamic acid (SHAM), a specific inhibitor of cyanide-resistant respiration, or 5 mM dimethylthiourea (DMTU), a scavenger of H_2O_2. The concentrations of the chemical inhibitors used here are based on previous reports (Jiang and Zhang, 2002; Bartoli *et al.*, 2005), and control experiments showed that the solvent alone had no significant effects on any of the experimental parameters measured. After 4 h of incubation with these chemical inhibitors under dark conditions at 26 °C, the solutions of the chemicals were replaced with 0 or 200 mM NaCl solution, and the seedlings were kept in the dark for 12 h at 26 °C. Seedlings exposed to distilled water under the same conditions for the entire period were used as the control.

Cell death analysis

Cell death was quantified using the Evans blue staining method (Hung *et al.*, 2007): cells with damaged membranes take up Evans blue dye, whereas viable cells that retain intact plasma membranes can exclude the dye (Kawai and Uchimiya, 2000; Hung *et al.*, 2007). The roots were submerged in 0.25% (w/v) aqueous Evans blue solution for 20 min at room temperature and then washed twice for 15 min with distilled water to remove the excess and unbound dye. Thereafter the roots were incubated in distilled water overnight. For the quantitative assessment, the roots were excised from the seedlings, followed by the extraction of the dye in a solution containing 50% methanol and 1% SDS (sodium dodecyl sulfate) for 1 h at 50 °C. The extracted dye was determined spectrophotometrically at 595 nm.

Root respiration

The roots were detached, weighed, and cut into small pieces using razor blades. The pieces placed in assay buffer (20 mM Hepes, 0.2 mM $CaCl_2$, pH 7.2; Simons *et al*., 1999) were incubated for 10 min to allow the wound respiration to subside. After these treatments, the pieces were transferred to a 3-ml air-tight cuvette. The oxygen uptake of the roots in the assay buffer was monitored using a Clark-type oxygen electrode (SP-2 type; constructed by the Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, China). The rates of total respiration (V_t), cyanide-resistant respiration (V_{alt}), and residual respiration were measured and calculated using the method described by Bingham and Farrar (1989).

Extraction of total RNA and Northern hybridization

Total RNA was extracted using the Total RNA Trizol Extraction Kit (Sangon Inc., Shanghai, China). Equal amounts of RNA (based on OD_{260}) were separated by electrophoresis using an 1.0% agarose gel with 1 × TAE (Tris-acetate-EDTA) buffer. The specific probes for rice *AOX1a*, *AOX1b*, and *AOX1c* were obtained according to previous reports (Saika *et al*., 2002; Feng *et al*., 2009). Northern hybridization was performed using the ECL DNA Labelling and Detection Kit (Enzo Diagnostics Inc., Little Chalfont, Buckinghamshire, UK) following the manufacturer's instructions.

Determination of H_2O_2

The H_2O_2 content was measured according to the method described by Brennan and Frenkel (1977), with some modifications. H_2O_2 was extracted by homogenizing 0.5 g root tissue with 3 ml of cold acetone. The homogenate was centrifuged at 10,000 x *g* for 10 min. An 1-ml aliquot of the supernatant was mixed with 0.1 ml of 5% (w/v) $Ti(SO_4)_2$ and 0.2 ml of NH_4OH. The mixture was then centrifuged at 2,500 x *g* for 10 min at room temperature, and the precipitate was solubilized in 5 ml of 2 M H_2SO_4. The optical absorption of the supernatant was measured spectrophotometrically at 415 nm to determine the H_2O_2 content, and the absorbance values were calibrated to a standard graph generated with known concentrations of H_2O_2.

Statistical analysis

The results are expressed as mean values ± standard deviation (SD). The data were analysed using the Kruskall-Wallis one-way analysis of variance test. $P < 0.05$ was considered statistically significant.

Results

Effects of NaCl stress on cyanide-resistant respiration and rice AOX1 genes expression

The roots of rice seedlings were exposed to solutions of 100, 200, or 300 mM NaCl for 12 h. Compared with the control (0 mM NaCl), the treatment with 100 mM NaCl did not significantly affect the levels of total respiration (V_t) and cyanide-resistant respiration (V_{alt}) (Table I). In contrast, treatment with 200 mM NaCl significantly decreased the value of V_t, but increased the value of V_{alt} compared with the control (Table I). The value of V_{alt} under 300 mM NaCl stress was slightly (but not significantly) higher than that in the control, whereas the value of V_t was significantly decreased (Table I). The values of residual respiration were very low (less than 5% of the total oxygen uptake) and are not shown here.

Specific probes for rice *AOX1a*, *AOX1b*, and *AOX1c* were used to investigate the effects of NaCl stress on the expression of members of the rice *AOX1* multigene family (Fig. 1). It was observed that 100 mM NaCl enhanced the level of *AOX1a* transcripts but had no effect on the *AOX1b* transcripts; NaCl at 200 and 300 mM further enhanced the level of *AOX1a* mRNA and slightly increased the *AOX1b* transcript level. Transcripts for *AOX1c* were barely detectable under our conditions (Fig. 1).

Table I. The effects of NaCl stress on the rates of total respiration (V_t) and cyanide-resistant respiration (V_{alt}). Each value represents the mean ± SD of three independent experiments. Asterisks indicate statistically significant differences from the control (0 mM NaCl). $P < 0.05$ was considered.

NaCl concentration [mM]	Respiratory rate	
	V_t [μmol O_2 g^{-1} FW min^{-1}]	V_{alt} [μmol O_2 g^{-1} FW min^{-1}]
0	0.180 ± 0.010	0.033 ± 0.006
100	0.187 ± 0.012	0.043 ± 0.021
200	0.157 ± 0.006*	0.053 ± 0.006*
300	0.137 ± 0.015*	0.040 ± 0.010

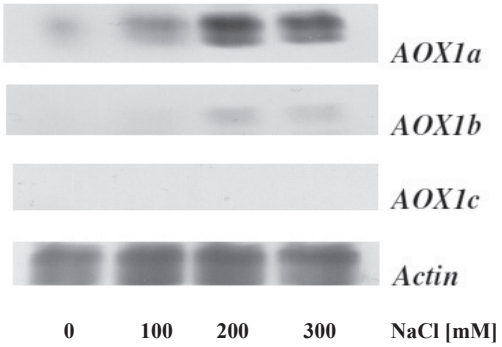

Fig. 1. The effects of NaCl stress on the expression of rice *AOX1a*, *AOX1b*, and *AOX1c* transcript levels. Actin was used as the RNA loading control.

NaCl stress induces cell death of rice roots

In the present work, cell death induced by NaCl stress was examined using an Evans blue staining assay. As shown in Fig. 2, 100 mM NaCl did not affect the viability of root cells, whereas NaCl at 200 and 300 mM induced obvious cell death in the rice roots. By quantitatively measuring the Evans blue uptake, 300 mM NaCl caused a higher level of cell death when compared with 200 mM NaCl (Fig. 2).

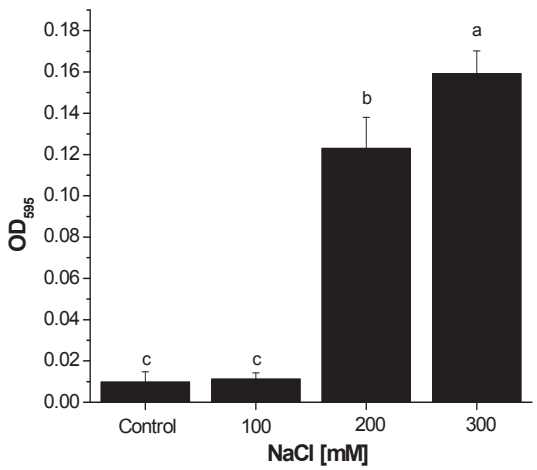

Fig. 2. The induction of cell death in rice roots under NaCl stress examined using an Evans blue staining assay. The roots of rice seedlings were exposed to 0 mM (control), 100 mM, 200 mM, or 300 mM NaCl stress. The induction of cell death was monitored by measuring the absorbance of extracted dye at 595 nm. Each value represents the mean ± SD of four independent experiments. The means denoted by the same letter did not significantly differ at $P < 0.05$.

Cell death of rice roots under NaCl stress is linked to H_2O_2 accumulation

Compared with the control, no significant increase in the content of H_2O_2 was detected in the rice roots exposed to 100 mM NaCl stress, whereas NaCl at 200 or 300 mM significantly increased the H_2O_2 level. There was no significant difference in the H_2O_2 level between the rice roots treated with 200 and 300 mM NaCl (Fig. 3).

The roots were treated with exogenous H_2O_2 from 0 to 400 mM, and the level of cell death was found to increase with the increase in the concentration of exogenous H_2O_2 (Fig. 4). To study further whether the induction of cell death by high NaCl stress could be linked to the accumulation of H_2O_2 *in vivo*, we used a scavenger of H_2O_2, DMTU, to limit the level of H_2O_2 in the NaCl-stressed rice roots. We concentrated on the 200 mM NaCl-stressed roots, because 200 mM NaCl seemed to cause a higher oxidative stress in the rice roots, as shown in Fig. 3. The concentration of the inhibitor used here was 5 mM, based on previous reports (Jiang and Zhang, 2002). The pretreatment with DMTU at this concentration inhibited the H_2O_2 content in the 200 mM NaCl-stressed roots by nearly 46% (data not shown). In contrast, the treatment with 5 mM DMTU partially alleviated the root cell death induced by 200 mM NaCl

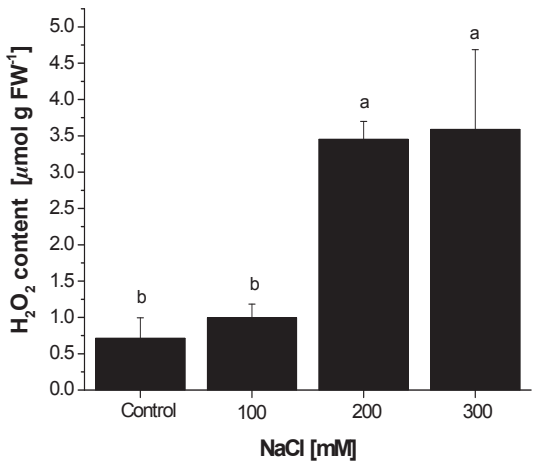

Fig. 3. The endogenous contents of H_2O_2 in rice roots under NaCl stress. The roots of rice seedlings were exposed to 0 mM (control), 100 mM, 200 mM, or 300 mM NaCl stress for 12 h. Each value represents the mean ± SD of four different experiments. The means denoted by the same letter did not significantly differ at $P < 0.05$.

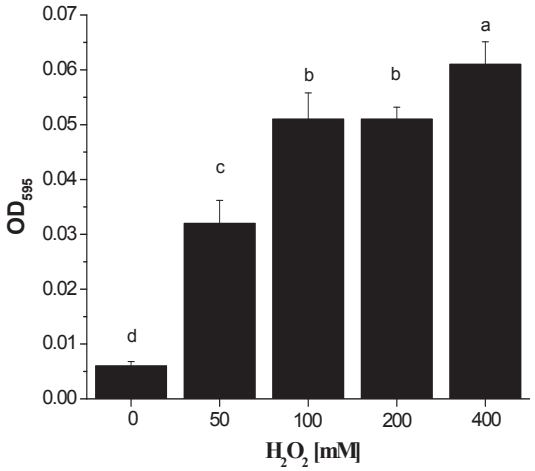

Fig. 4. The induction of root cell death by exogenous H_2O_2 (0 to 400 mM) examined by an Evans blue staining assay. The induction of cell death was monitored by measuring the absorbance of extracted dye at 595 nm. Each value represents the mean ± SD of four independent experiments. The means denoted by the same letter did not significantly differ at $P < 0.05$.

(Fig. 5). These observations suggest that the cell death of rice roots under a high level of salt stress may be linked to H_2O_2 accumulation *in vivo*.

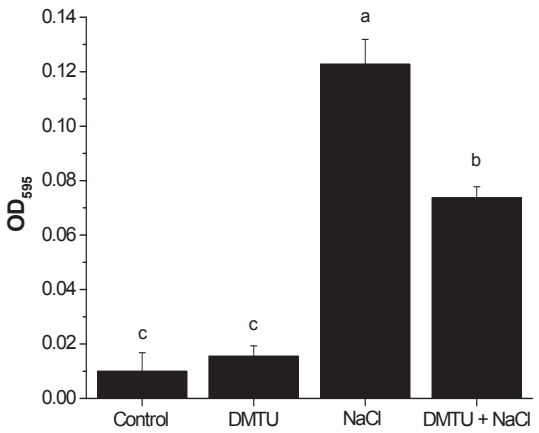

Fig. 5. The effects of DMTU on the cell death of rice roots under 200 mM NaCl stress examined by an Evans blue staining assay. The plants were treated as follows: the roots without chemical pretreatments were exposed to 0 mM or 200 mM NaCl (Control and NaCl); the roots pretreated with 5 mM DMTU were exposed to 0 mM or 200 mM NaCl (DMTU and DMTU + NaCl). The induction of cell death was monitored by measuring the absorbance of extracted dye at 595 nm. The results are the mean values ± SD of four different experiments. The means denoted by the same letter did not significantly differ at $P < 0.05$.

Cyanide-resistant respiration could play a role in mediating root cell death under NaCl stress

SHAM, a specific inhibitor of alternative oxidase, was used to inhibit cyanide-resistant respiration. We used 1 mM SHAM, because this concentration is sufficiently low to minimize the possible side effects observed with a higher level of this AOX inhibitor or during the relatively long duration of the assay (Møller *et al.*, 1988; Bartoli *et al.*, 2005). Treatment with 1 mM SHAM inhibited the cyanide-resistant respiration in the rice roots exposed to 200 mM NaCl by approximately 65%, whereas 1 mM SHAM had no significant effect on either the H_2O_2 content or the extent of cell death of the rice roots under the control condition (0 mM NaCl) (Figs. 6A and B). Compared to the control (roots without any treatment), it was observed that 200 mM NaCl resulted in a 2.3-fold and 11.7-fold increase, respectively, in the *in vivo* H_2O_2 content and cell death. By comparison, the combined treatment with 200 mM NaCl and 1 mM SHAM increased the H_2O_2 content and cell death 3.0-fold and 14.5-fold, respectively, compared to the control (Figs. 6A and B). These results suggest that the inhibition of cyanide-resistant respiration under the condition of high salt stress caused more H_2O_2 production and further decreased the viability of the root cells of rice.

Discussion

In the present study, the treatment with 100 mM NaCl did not significantly affect the value of V_t, whereas NaCl at 200 or 300 mM led to a significant decrease in the V_t value. The response of cyanide-resistant respiration to NaCl stress was different from that of total respiration: compared with the control, the value of V_{alt} increased under the varying levels of NaCl stress and was significantly higher at 200 mM NaCl (Table I). Similar to our observations, Jolivet *et al.* (1990) found that, compared to the dramatic decreases of both the total respiration and the activity of the cytochrome pathway, the level of cyanide-resistant respiration in mitochondria isolated from NaCl-stressed barley seedlings was only weakly affected or increased.

Ferreira *et al.* (2008) found that the treatment of *Citrus* cell suspensions with a high concentration of NaCl led to an increase in the amount of the AOX protein. However, in a wide variety

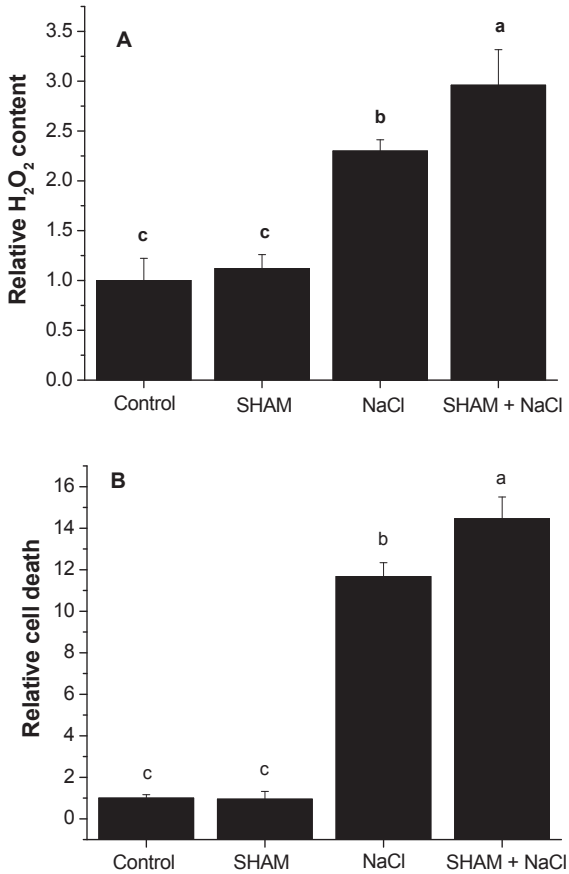

Fig. 6. The effects of SHAM on (A) the H_2O_2 content and (B) the cell death of rice roots. The plants were treated as follows: the roots without chemical pretreatments were exposed to 0 mM or 200 mM NaCl (Control and NaCl); the roots pretreated with 1 mM SHAM were exposed to 0 mM or 200 mM NaCl (SHAM and SHAM + NaCl). The results are the mean values ± SD of four different experiments. The values in the control were set to 1.0 to facilitate the comparison among the different treatments. The means denoted by the same letter did not significantly differ at $P < 0.05$.

of monocotyledon and eudicotyledon plants, the AOX protein is encoded by a small family of nuclear genes (Considine *et al.*, 2002). Because the members of the gene family encoding the AOX protein have been isolated and characterized from rice (*AOX1a*, *AOX1b*, and *AOX1c*; Saika *et al.*, 2002), we used probes specific for each gene to reveal the effects of NaCl stress on their expression at the transcriptional level. The present results showed that the transcript abundances of *AOX1a* and *AOX1b*, but not of *AOX1c*, were increased under the conditions of NaCl stress (Fig. 1), with the *AOX1a* mRNA presenting higher levels than the *AOX1b* mRNA (Fig. 1). Moreover, it was also noted that the roots exposed to 200 mM NaCl stress had the highest level of *AOX1* mRNA (particularly *AOX1a* mRNA) among the roots treated with different concentrations of NaCl. Thus, it could be concluded that the enhanced expression of the *AOX1a* and *AOX1b* genes is involved in the responses of plant roots to NaCl stress.

In the present work, 100 mM NaCl did not reduce the viability of the cells, whereas NaCl at 200 or 300 mM induced obvious cell death (Fig. 2). A recent study by Lin *et al.* (2006) found that salt-stress-induced cell death in tobacco protoplasts could be dependent on the H_2O_2 production. We first investigated whether H_2O_2 can induce cell death in rice roots. The results showed that the concentration of endogenous H_2O_2 was significantly enhanced when NaCl stress at 200 or 300 mM induced cell death in the rice roots (Figs. 2 and 3). Furthermore, the exogenous application of H_2O_2 caused notable cell death in a dose-dependent manner (Fig. 4). We also found that the treatment with a scavenger of H_2O_2, DMTU, partially alleviated the cell death induced by 200 mM NaCl stress (Fig. 5). These observations suggest that the death of rice root cells under a high level of salt stress is linked to H_2O_2 accumulation *in vivo*.

Although we did not precisely define the subcellular location of H_2O_2 accumulation in the salt-stressed cells of the rice roots, it is considered that the mitochondria are the major source of ROS in non-photosynthetic plant organs (Maxwell *et al.*, 1999). Because the AOX gene has been proposed to be a 'reporter gene' for the evaluation of whether mitochondrial oxidative stress occurs during abiotic and biotic stresses (Arnholdt-Schmitt *et al.*, 2006; Amirsadeghi *et al.*, 2007; Van Aken *et al.*, 2009), the enhanced expression of the rice *AOX1a* and *AOX1b* genes, as observed in the salt-stressed rice roots, could indicate that the H_2O_2 accumulation under high levels of salt stress could be partially generated by the mitochondria. The work of Lin *et al.* (2006) also showed that the H_2O_2 production in salt-stressed tobacco protoplasts could originate from the mitochondria.

We used SHAM to inhibit cyanide-resistant respiration. The treatment with 1 mM SHAM inhibited cyanide-resistant respiration by approximately 65% in roots exposed to 200 mM NaCl. When the roots pretreated with 1 mM SHAM

were exposed to 200 mM NaCl, it was observed that they had higher levels of H_2O_2 than those subjected either to 200 mM NaCl stress or SHAM treatment alone (Fig. 6A). Although SHAM has been reported to also inhibit the peroxidase activity at higher concentration (20 mM), the effect of SHAM on peroxidases was found to be minimal at low concentration (2 mM) (Amor *et al.*, 2000). SHAM may also stimulate the NADH oxidase reaction (also potentially increasing ROS production), but this usually occurs only in illuminated green tissue (Askerlund *et al.*, 1987; Diethelm *et al.*, 1990). Therefore, we suggest that, under NaCl stress, the additional H_2O_2 production in the presence of 1 mM SHAM was the result of the inhibition of cyanide-resistant respiration, indicating that cyanide-resistant respiration could play a role in limiting excess H_2O_2 production in salt-stressed root cells.

Many studies have revealed that the increase in the H_2O_2 level of mitochondria is an important factor in triggering the cell death responses induced by biotic or abiotic stress (Lin *et al.*, 2006; Rhoads *et al.*, 2006; Hano *et al.*, 2008). Thus, if cyanide-resistant respiration has a function in limiting the H_2O_2 production of NaCl-stressed mitochondria, it is expected that cyanide-resistant respiration should have the ability to attenuate the root cell death induced by salt stress. This expectation is supported by our finding that the salt-stressed (200 mM NaCl) roots pretreated with 1 mM SHAM had higher levels of cell death than those subjected either to 200 mM NaCl stress or SHAM treatment alone (Fig. 6B).

Altogether, these results indicate that cyanide-resistant respiration may be involved in the response of rice roots to salt stress and may play a role in mediating the process of cell death induced by salt stress.

Acknowledgements

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