Inabenfide-Induced Alleviation of Salt Stress in Rice as Linked to Changes in Salicylic Acid Content and Catalase Activity

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Abstract

The effect of inabenfide was investigated in rice seedlings subjected to salt stress in relation to changes in chlorophyll fluorescence (Δ F/Fm'), lipid peroxidation, salicylic acid (SA) content, and catalase (CAT) activity. A reduction in shoot growth of rice seedlings by 120 mM NaCl treatment was significantly alleviated by pretreatment with 30 μM inabenfide. Sodium ion content was not affected by pretreatment with inabenfide, suggesting that alleviation was not due to a reduction in sodium ion uptake by the rice seedlings. At three days after NaCl treatment, the rice seedlings pretreated with inabenfide showed a higher ΔF/Fm' (30%) and lower lipid peroxidation (28%) compared with the rice seedlings treated with NaCl alone. After NaCl treatment, CAT activity in the third leaf of rice seedlings decreased significantly but alleviated by pretreatment with inabenfide. Furthermore, pretreatment with inabenfide also reduced the level of SA which accumulated drastically in the third leaf of rice seedlings within a day after exposure to salt stress. These results suggest that inabenfide prevents SA accumulation in rice seedlings under salt stress which eventually induces the alleviation of salt stress damage.

Key words: inabenfide, rice, salt stress, catalase, salicylic acid, benzoic acid 2-hydroxylase, cytochrome P450

Introduction

Rice (*Oryza sativa* L.) is a staple food source for more than one-third of the world's population, and has a huge socio-economic impact on human existence, but its productivity is severely affected by salt stress - one of the major abiotic environmental constraints.

It has been known that rice growth is differentially affected by salt stress at each developmental stage of rice; in particular, in rice the seedling stage is known to be the most susceptible one to salt stress (Flowers and Yeo 1981; Zeng and Shannon 2000). It is generally accepted that salt stress is mainly caused by excessive concentrations of salt, especially sodium chloride (NaCl) in the soil solution (Levitt 1980). The detrimental effects of salt stress on plant growth are also associated with low osmotic potential of soil solution and nutritional imbalance (Hasegawa et al. 2000).

As a consequence of salt stress, caused by its hyperosmotic effect, stomatal closure reduces CO_2 availability in the leaves, which reduces the photosynthetic electron transport chain in the chloroplast (Dionisio-Sese and Tobita 1998). Furthermore, salt stress severely impairs the photosynthetic activities as well as the photosynthetic apparatus of plant (Singh and Dubey 1995; Dionisio-Sese and Tobita 2000). Under these circumstances, reactive oxygen species (ROS) such as superoxide radical, hydroxyl radicals and H_2O_2 are excessively produced in plants (Asada 1999; Dionisio-Sese and Tobita 1998; Menezes-Benavente et al. 2004). Insufficiently-reduced ROS is known to have potentially harmful effects on most cellular components (Smirnoff 1993). In a series of reactions, ROS is efficiently scavenged by superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT), which exist in different subcellular locations (Foyer et al. 1997; Breusegem et al. 2001). Several workers have demonstrated a vital role of SOD, APX, and GR under salt stress and their correlation with salt tolerance of rice (Tanaka et al. 1999; Lee et al. 2001; Vaidyanathan et al. 2003). However, in rice the activity of catalase (CAT), a major H_2O_2 scavenging enzyme, appeared to be either unaltered or even reduced in response to salt stress (Lee et al. 2001; Khan and Panda 2002; Shim et al. 2003; Tsai et al. 2004). Under this aspect, Chen et al. (1993, 1997) proposed that salicylic acid (SA) accumulation may cause a decrease in CAT activity in tobacco and rice. It was also suggested that the maintenance of catalase activity could be a key factor for determining stress tolerance of plants (Shim et al. 2003).

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SA is a well-known signaling molecule which is involved in the plant's defense response against pathogen attack (Malamy et al. 1990). It has also been found that endogenous SA content increases dramatically under various abiotic stresses, including salt stress (Yalpani et al. 1994; Shim et al. 2003; Scott et al. 2004). In plants, two different pathways have been described for SA biosynthesis beginning with either phenylalanine (Yalpani et al. 1993) or chorismate (Wildermuth et al. 2001). In the phenylalanine pathway, the conversion to SA from benzoic acid (BA) is catalyzed by benzoic acid 2-hydroxylase (BA2H), which is characterized as a soluble cytochrome P-450 (P450) monooxygenase (León et al. 1995).

Inabenfide [4'-chloro-2'-(α-hydroxybenzyl) isonicotinailide] was originally developed as a plant growth regulator, and is known to be effective in regulating stem elongation in rice (Rademacher 2000). This chemical was found to inhibit gibberellin biosynthesis by blocking P450 which catalyzes the steps of hydroxylation from ent-kaurene to ent-kaurenoic acid (Rademacher 2000). Based on these facts, it is reasonably assumed that inabenfide could inhibit the formation of SA which may be a reason for the decrease in CAT activity in rice under salt stress, which in turn could result in alleviating salt stress damage of rice.

Therefore, the present study was carried out to determine whether inabenfide in alleviating salt stress damage of rice.

Materials and Methods

Plant materials and treatment with inabenfide

As rice (*Oryza sativa* L.) cultivar Nipponbare has been used to release the almost complete genome sequence (International rice genome sequencing project 2005), 28,000 full-length cDNA sequences (Kikuchi et al. 2003), and more than 47,196 Tos17 mutant lines (Miyao et al. 2003), Nipponbare is clearly the preferred choice to study salt stress responses.

Rice seeds were surface sterilized and germinated at 30 °C in an incubator for 48 h. The uniformly-germinated seeds were transferred to a stainless steel net stretched over a six L plastic pot over distilled water, and placed in a growth chamber with photoperiod of 14 h light/12 h dark regime at 25/20 °C, light intensity of 180 μEm^{-2}s^{-1}, and 60% relative humidity. The seedlings were hydroponically-grown in a cultural medium that consisted of Kasugai's nutrient solution, pH 5.8 (Kasugai 1939) for two weeks until the third leaf were fully expanded. The nutrient solution was changed every two days. Seedlings were carefully selected and transferred to a 500 mL brown plastic bottle containing 30 μM inabenfide for three days prior to salt stress treatment.

Salt stress treatment

Ten uniformly-grown seedlings were carefully selected and transferred to a plastic bottle containing 500 mL of the culture medium containing 120 mM NaCl, which was added to the medium. After 0, 1, and 3 days of stress treatment, the fully expanded third leaves of each seedling were harvested, immediately frozen in liquid nitrogen, and stored at -80 °C until required for analysis.

Chlorophyll fluorescence measurement

Chlorophyll fluorescence (ΔF/Fm') was measured on the third leaf of the control and salt-treated rice seedlings using a portable chlorophyll fluorometer (Photosynthesis Yield Analyzer Mini-PAM, WALZ, Effeltrich, Germany) equipped with light-emitting diodes (emission maximum at 655 nm) and a halogen lamp (18,000 μmol m^{-2}s^{-1} PAR with saturation pulse).

Tissue sodium ion measurement

Leaves were oven dried for 48 h at 70 °C. A dried sample (0.1 g) was added to 10 mL of nitric acid in a beaker and then heated on a hot plate at 200 °C. After cooling, the suspension was filled with 25 mL distilled water and filtered through a 0.25 μm membrane filter. Sodium content was determined by inductively-coupled argon plasma emission spectrometry (ICAP-737V, Nippon Jarrell-Ash, Kyoto, Japan).

Lipid peroxidation measurement

Lipid peroxidation can be determined by the formation of malondialdehyde (MDA) as the decomposition product of polyunsaturated fatty acids (PUFA) of bio-membranes. MDA content was measured using the thiobarbituric acid (TBA) reaction as previously described (Sunohara and Matsumoto 2004). Tissue samples (0.5 g FW) from ca. 30-35 randomly selected third leaves were ground using a chilled pestle and mortar in the presence of liquid nitrogen, and then homogenized with 5 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000 x g for 20 min. For every 1 mL of the aliquot, 5 mL of 20% TCA containing 0.5% TBA was added. The mixture was heated at 95 °C for 30 min and immediately cooled in an ice bath to terminate the reaction. The mixture was centrifuged again at 10,000 x g for 10 min and the absorbance of the supernatant was measured at 532 nm using a spectrophotometer (DU-600, Beckman Coulter Inc., Fullerton, CA, USA). Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was calculated by using a molar extinction coefficient of 156 mM^{-1} cm^{-1}.

CAT activity measurement

Enzyme extraction was conducted according to the methods of Sunohara and Matsumoto (2004). Tissue samples (0.5 g FW) from ca. 30-35 randomly selected third leaves were homogenized with 25 mM potassium phosphate buffer (pH 7.8) containing 0.4 mM EDTA-4H, 1 mM ascorbic acid and 2% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 15,000 x g for 20 min at 4 °C, and the supernatant was filtered through Miracloth® (Calbiochem, San Diego, CA, USA). The filtrate was then used as an enzyme extract for CAT (EC 1.11.1.6) assay. Following the methods of Aebi (1984), CAT activity was assayed in a 1 mL reaction mixture containing 0.95 mL of 50 mM potassium phosphate buffer (pH 7.0, containing 10 mM H_2O_2) and 0.05 mL of enzyme extract. The subsequent decomposition of H_2O_2 was observed at 240 nm (UV-2450, Shimadzu, Kyoto, Japan).

Salicylic acid measurement

The extraction and determination of free salicylic acid were conducted according to the method of Malamy et al. (1992). Tissue samples (0.3 g FW) from the third leaves were ground with 7 mL of 90% methanol and centrifuged at 7000 x g for 15 min. The pellets were re-extracted with 2 mL of 90% methanol and centrifuged again as mentioned above. The supernatants from both centrifuge steps were

evaporated at 35 ℃ to remove methanol. Distilled water (5 mL) was added to the extracts to dissolve at 80 ℃ in a water bath for 2 min. For free SA extraction, half of the extract was added to an equal volume of 0.2 M sodium acetate (pH 4.5), and pH was adjusted to 1.5–2.0 using HCl. Untreated and glucosidase-treated fractions were extracted using two volumes of ethyl acetate/cyclopentane/2-propanol (50/50/1, v/v/v), and evaporated in a vacuum at 35 ℃ to complete dryness. The residues were dissolved in 23% (v/v) methanol/20 mM sodium acetate (pH 5.0) and were subjected to HPLC (LC-10AS; Shimadzu, Kyoto, Japan) using a column (μBondasphere 5 μ C-18-100A, 3.9 mm x 150 mm; Waters, Milford, MA, USA) at 35 ℃ and a 1.0 mL/min flow rate of eluent [23% (v/v) methanol/20 mM sodium acetate buffer; pH5.0]. Free SA was analyzed by using a spectrofluorescence detector (RF-10AXL; Shimadzu, Kyoto, Japan) at the excitation wavelength of 313 nm and emission wavelength of 405 nm.

Statistical analysis

All the data presented here were based on two independent experiments with three replications. Each experiment was conducted with three different pots for the same treatment. SPSS (Version 11.0J) software was used for the statistical analysis. To assess the statistical significance of treatment differences, a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (P < 0.05) was employed.

Results and Discussion

Effect of inabenfide on shoot growth and sodium ion uptake of riceseedlings under salt stress

Shoot growth of rice seedlings pretreated with inabenfide for three days prior to NaCl treatment was slightly reduced comparing with the untreated control (Fig. 1a). After NaCl treatment, shoot growth was gradually reduced with wilting from the tip of the leaf blade, particularly in the older leaves, while it increased in the untreated control. On the other hand, the salt stress-induced reduction of shoot growth was 12% less when the rice seedlings were pretreated with inabenfide and showed less severe leaf desiccation. Furthermore, the newly expanding leaves were healthier under the salt stress condition when the seedlings were pretreated with inabenfide.
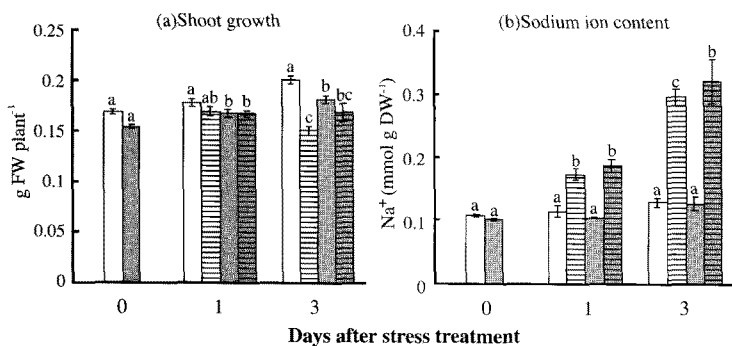

Fig. 1. Shoot growth (a) and sodium ion content (b) in the third leaves of rice seedlings as affected by NaCl treatment and inabenfide pretreatment; untreated control (□), 120 mM NaCl (▤), 30 μM inabenfide pretreatment (▨), and 30 μM inabenfide pretreatment + 120 mM NaCl treatment (▦). Different letters indicate significant differences at P < 0.05 according to Tukey's multiple comparison test. Vertical bars indicate the mean ± standard error (n=10).

Salinity has a deleterious effect on plant growth causing ion toxicity and ionic imbalance. Salt tolerance is affected by the uptake of Na^+ into the plants and its cytoplasmic concentration (Greenway and Munns 1980). To know whether inabenfide pretreatment affects the Na^+ uptake of rice seedlings, the changes in Na^+ content of the third leaf of seedlings subjected to 120 mM NaCl for three days were investigated (Fig. 1b).

As expected, Na^+ content in the third leaf of rice seedlings increased linearly after NaCl treatment. However, regardless of inabenfide pretreatment, the content of Na^+ of the third leaf after NaCl treatment did not show a notable difference. This indicates that inabenfide pretreatment did not significantly affect the Na^+ uptake of rice seedlings under salt stress. Therefore, we confirmed that alleviation of salt damage by inabenfide pretreatment observed in the shoot growth was not due to the reduction of Na^+ uptake to the shoots of rice seedlings.

Effect of inabenfide on chlorophyll fluorescence and lipid peroxidation in rice seedlings under salt stress.

Chlorophyll fluorescence has been used as a rapid and nondestructive method to study the photosynthetic performance of plants under stress conditions, including salt stress (Dionisio-Sese and Tobita 2000; Souza et al. 2004). Chlorophyll fluorescence severely decreased with time after NaCl treatment while it was almost constant in the untreated control during the experimental period. However, the salt stress-induced decrease of chlorophyll fluorescence was significantly alleviated by 30% in the rice seedlings pretreated with inabenfide at three days after NaCl treatment (Fig. 2a). Tiwari et al. (1997) also reported that salt stress reduced PSII activity of chloroplast isolated from rice leaves and the reduction was more severe in the salt-sensitive rice cultivars.
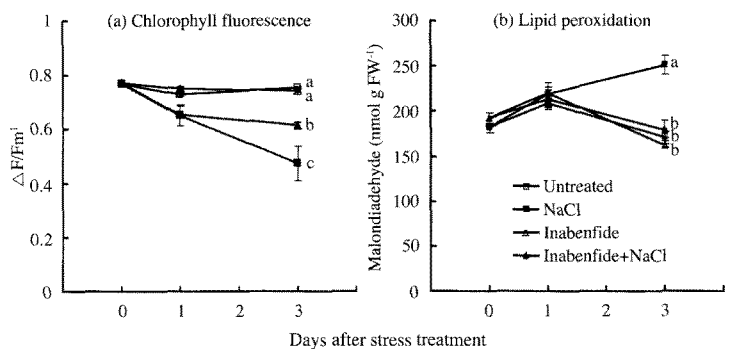

Fig. 2. Chlorophyll fluorescence (a) and malondealdehyde (b) content in the third leaves of rice seedlings as affected by NaCl treatment and inabenfide pretreatment. Different letters indicate significant differences at P < 0.05 according to Tukey's multiple comparison test. Vertical bars indicate the mean ± standard error (n=3).

As an aditional biochemical indicator of salt stress damage, we examined the changes of lipid peroxidation (Fig. 2b). The content of MDA was not significantly changed until one day after NaCl treatment. The content of MDA, however, increased ca. 46% at three days after NaCl treatment, indicating that NaCl treatment causes the oxidative damage to the rice seedlings. During the same periods of normal condition, rice seedlings pretreated with inabenfide did not show any surplus increase of MDA content compared with the untreated con-

trol. Additionally, the increase of MDA contents in rice seedlings pretreated with inabenfide was not observed under the salt stress conditions. This indicates that membrane lipid peroxidation of rice seedlings subjected to salt stress was prevented by inabenfide pretreatment resulting in alleviating the decrease of chlorophyll fluorescence and the reduction of growth.

Among the deleterious effects of ROS is lipid peroxidation caused by the reaction between ·OH and the methylene groups of polyunsaturated fatty acids, which are the main components of membrane lipids (Blokhina et al. 1999). Lipid peroxidation of cell membrane is caused by ROS accumulated when a dark reaction of photosynthesis is inhibited, especially under the oxidative stress conditions in which an accumulated ROS is not properly scavenged by the antioxidant system including a series of enzymes (Asada and Takahashi 1987). Therefore, our present results implored us to continue our investigation into the activity of antioxidant enzymes, particularly focusing on the effect of inabenfide on CAT activity under salt stress conditions.

Effect of inabenfide on CAT activity in rice seedlings under salt stress

Shim et al. (2003) has argued that CAT activity is a determining factor of salt tolerance in rice. However, CAT activity in rice leaf is often found to be reduced in response to salt stress (Tanaka et al. 1999; Lee et al. 2001; Khan and Panda 2002; Demiral and Türkan 2004). It was also reported that the salt-sensitive cultivars showed more severe decrease in CAT activity compared with the salt-tolerant cultivars (Shim et al. 1999, 2003).

In Fig. 3, CAT activity did not change significantly in the untreated control, and its level was almost unchanged by pretreatment with inabenfide during the experimental period. On the other hand, CAT activity in the rice seedlings treated with NaCl alone severely decreased by 32.6% at three days after stress treatment compared with the untreated control. Interestingly, at three days after NaCl treatment, CAT activity in the seedlings pretreated with inabenfide was 30.8 % higher than the seedlings treated with NaCl alone.
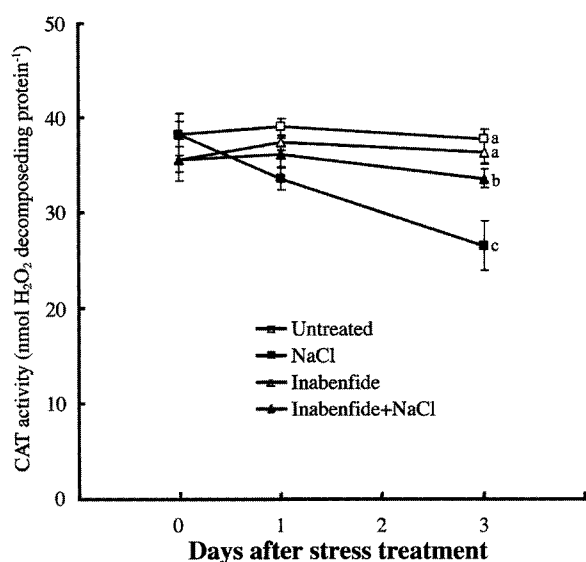
Related to these results, it should be noticed that the seedlings pretreated with inabenfide also showed a higher chlorophyll fluorescence and lower lipid peroxidation in response to NaCl treatment comparing with the seedlings treated with NaCl alone (Fig. 2).

Based on our results as well as on previous reports, it was considered that maintaining CAT activity by inabenfide pretreatment might contribute, at least partially, to a more proper scavenging of ROS, which resulted in alleviating salt stress damage of rice seedlings. A further study might be needed to find out whether inabenfide also has an alleviating effect on plant damage caused by other types of stress.

Effect of inabenfide on the SA content of rice seedlings under salt stress

SA content of rice seedlings was drastically increased by more than four-folds at one day after NaCl treatment alone compared with the untreated control. However, the level of SA content was also increased by NaCl treatment in the seedlings pretreated with inabenfide, but its level was 27% lower than the seedlings treated with NaCl alone (Fig. 4). At three days after NaCl treatment, the level of SA content decreased in the seedlings pretreated with inabenfide or treated with NaCl alone.
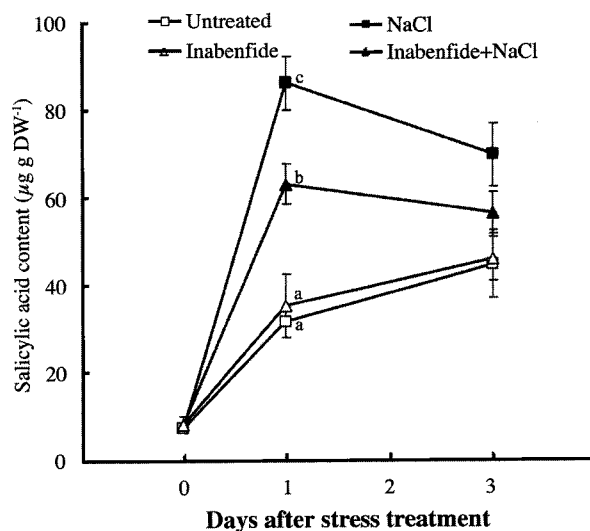
Fig. 4. Salicylic acid contents in the third leaves of rice seedlings as affected by NaCl treatment and inabenfide pretreatment. Different letters indicate significant differences at P < 0.05 according to Tukey's multiple comparison test. Vertical bars indicate the mean ± standard error (n=3).

Inabenfide has been known to inhibit P450 catalyzing the hydroxylation of ent-kaurene in the gibberellin biosynthesis process. Salicylic acid is formed from the hydroxylation of BA, and this step of biosynthesis is catalyzed by BA2H, a different type of P450 (Rademacher 2000; León et al. 1995). It was found that BA2H activity and SA accumulation in tobacco were induced by exposure to both UV irradiation and O_3 (Yalpani et al. 1994). Moreover, treatment with H_2O_2 promoted accumulation of BA and SA, and induction of BA2H in tobacco leaves (León et al. 1995). Therefore, it can be thought that inabenfide nonspecifically inhibits not only P450 catalyzing the hydroxylation of ent-kaurene but also BA2H, resulting in the reduction of SA synthesis from BA under salt stress.

In addition, SA content also increased gradually in the seedlings that were not exposed to salt stress, but its level was not significantly

Fig. 3. Catalase (CAT) activity in the third leaves of rice seedlings as affected by NaCl treatment and inabenfide pretreatment. Different letters indicate significant differences at P < 0.05 according to Tukey's multiple comparison test. Vertical bars indicate the mean ± standard error (n=3).

changed by inabenfide pretreatment. It has been reported that SA content increased during leaf senescence in leaves of *Arabidopsis thaliana* (Morris et al. 2000). Therefore, in the present study, the increase of SA content in the untreated seedlings might be related to leaf senescence.

In the present study, inabenfide was found to be effective to prevent SA accumulation, which might lead to maintaining CAT activity in rice seedlings under salt stress. Therefore, it could be concluded that inabenfide alleviated the salt stress damage of rice seedlings probably by the more effective scavenging of ROS. However, inabenfide which was originally developed for regulating plant growth is not the unique chemical for preventing SA accumulation. Therefore, further studies are needed to find a chemical specifically inhibiting BA2H, which would effectively reduce SA formation and lead to more effective alleviation of stress damages.

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