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Original Article

Mechanism of growth amelioration of NaCl-stressed rice (*Oryza sativa* L.) by δ-aminolevulinic acid

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The mechanism of growth amelioration of NaCl-stressed rice (*Oryza sativa* L. cv. Nipponbare) by δ-aminolevulinic acid (ALA) was investigated. Rice growth recovered when plants were pretreated with 0.1 or 1 μM ALA before treatment with 50 mM NaCl. There was no increase in chlorophyll content by ALA treatment, indicating that the growth recovery was not due to increased chlorophyll content although ALA is a precursor of chlorophyll biosynthesis. The activities of antioxidative enzymes, including catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APx), and superoxide dismutase (SOD), increased with ALA treatment. In particular, stimulation of CAT, GR, and APx activities by ALA was significant. The hydrogen peroxide (H_2O_2) content decreased after ALA treatment under NaCl-stress conditions. Peroxidation of membrane lipids, as measured by ethane evolution, also decreased after ALA treatment. These results suggest that ALA induces growth amelioration in NaCl-stressed rice by stimulating antioxidative enzyme activity, which resulted in decreased reactive oxygen generation and lipid peroxidation.

Keywords: δ-aminolevulinic acid; antioxidative enzyme; lipid peroxidation; *Oryza sativa*; salt stress; salt tolerance

Introduction

Salinity is one of the most serious environmental stresses inhibiting plant growth and development. The detrimental effects of high salinity on plants can be observed at the whole plant level as the death of plants or decrease in plant productivity.[1)] Improving salt tolerance in plants is essential to increase plant productivity. Several studies have examined chemical additives, such as proline and glycinebetaine,[2–4)] H_2O_2,[5,6)] silicon (Si),[7)] triadimefon,[8)] and paclobutrazol,[9)] to increase the salt tolerance of plants.

δ-Aminolevulinic acid (ALA) is the key precursor in the biosynthesis of porphyrins, such as chlorophylls and hemes. In plants, algae and some photosynthetic bacteria, ALA is synthesized from glutamate by the five-carbon pathway.[10)] It has been reported that exogenously applied ALA was herbicidal at high concentrations by increasing the content of chlorophyll intermediates, such as protoporphyrin IX and protochlorophyllide, in the dark.[11,12)] During the subsequent light period, these accumulated chlorophyll intermediates act as a photosensitizer to generate reactive oxygen species (ROS), including singlet oxygen, and this causes the death of plant cells by peroxidation of membrane lipids;[13)] however, with lower application rates, promoting effects of ALA have been reported. Growth and yield of kidney bean (*Phaseolus vulgalis* L. cv. Cyprus), barley (*Hordeum vulgar* L. cv. Kashimamugi), potato (*Solanum tuberosum* L. cv. Danshaku), and garlic (*Allium sativum* L. cv. Fukuchiwhite) increased 10–60% over the control when treating with ALA at concentrations lower than those eliciting herbicidal responses.[14)] Moreover, the amelioration of some environmental stresses by ALA has also been reported.[15–17)] The resistance to cold stress in rice seedlings[15)] and salt tolerance in cotton[16)] and spinach[17)] increased after ALA treatment. Seed germination of pakchoi (*Brassica camprestris* ssp. *Chinensis* var. *communis* Tsen et Lee) under salt stress conditions was promoted when treated with ALA in the range of 0.01 to 10 mg/L;[18)] however, the physiological mechanisms of ALA ameliorating plant growth under stress condi-

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Abbreviations: ALA: δ-aminolevulinic acid, APx: ascorbate peroxidase, CAT: catalase, DMSO: dimethyl sulfoxide, EDTA: ethylenediamine tetraacetic acid, FW: fresh weight, GR: glutathione reductase, GSH: glutathione, GSSG: oxidized glutathione, NADPH: nicotinamide-adenine dinucleotide phosphate, PVPP: polyvinyl polypyrrolidone, SOD: superoxide dismutase

tions are not yet thoroughly understood. Further studies are needed to understand the physiological functions of ALA before it can be used effectively in agricultural production.

The mechanism of salt stress is complex, but the imposition of water deficit leads to the formation of ROS.[1] Changes in antioxidative enzyme activities involved in the detoxification of ROS are often observed in plants under salt stress conditions. Increasing or maintaining enzyme activities is one mechanism that is related to increased salt tolerance in some plant species, such as purslane (*Portulaca oleracea* L.),[19] rice (*Oryza sativa* L.),[20,21] maize (*Zea mays*, L.),[22] *Sesbania rostrata*,[23] and tomato (*Lycopersicon esculentum* Mill cv. Pera).[24]

This study was designed to investigate the mechanism of salt tolerance improvement in rice by ALA treatment by determining growth, chlorophyll content, antioxidative enzyme activities, H_2O_2 content, and ethane production.

Materials and Methods

1. Plant materials

Seeds of rice (*Oryza sativa* L. cv. Nipponbare) were soaked in deionized water at 30°C for 2 days in the dark and sown in plastic boxes containing commercially obtained compost mixture (Golden Soil; Iris Ohyama Co., Miyagi, Japan) moistened sufficiently with deionized water. The plants were grown in a growth chamber at 25/20°C (day/night) with 12 h of light (450 μmol m^{-2} s^{-1}) to the 1.5–2.0 leaf stage. Water was added to the soil daily. Just before ALA treatment, the roots were washed carefully to remove the soil.

2. ALA treatment and NaCl treatment

The roots of rice seedlings were immersed in 0, 0.1, or 1 μM ALA (ALA hydrochloride; Sigma Chemical Co., St. Louis, MO, USA) at 25°C for 12 h with 450 μmol m^{-2} s^{-1} light intensity. After ALA treatment, rice seedlings were transplanted into Kasugai nutrient solution[25] and returned to the growth chamber. Five days after ALA treatment, NaCl (final concentration: 50 mM) was added to the nutrient solution. The nutrient solution containing 50 mM NaCl was renewed every 4 days. The fresh weights of whole plants were measured after 20 days of salt treatment.

3. Chlorophyll content

Chlorophyll content was determined in accordance with Chappelle *et al*.[26] The shoot parts of rice were excised and soaked in DMSO (100 ml g FW^{-1}) in a glass vial. The vial was sealed tightly and incubated at 30°C for 48 h in darkness. The concentration of the extracted pigments (chlorophyll *a*, chlorophyll *b*) was calculated based on their absorbance values at 664 and 648 nm.

4. Enzyme extraction and activity assays

4.1. Enzyme extraction

Five hundred milligrams of excised leaves were homogenized in 5 ml of 25 mM potassium phosphate buffer (pH 7.8) containing 0.4 mM EDTA-4H, 1 mM ascorbic acid, and 2% polyvinyl polypyrrolidone (PVPP). The homogenate was centrifuged at 15,000×*g* for 20 min at 4°C and the supernatant was filtered through one layer of Miracloth® (Calbiochem, San Diego, USA). The filtrate was then used as an enzyme extract for the assays of catalase (CAT; EC 1.11.1.6), ascorbic acid peroxidase (APx; EC 1.11.1.11), and glutathione reductase (GR; EC 1.6.4.2) activity. For the superoxide dismutase (SOD; EC 1.15.1.1) assay, the enzyme extract was dialyzed against Seamless Cellulose Tubing® (Wako Pure Chemical Industries) overnight with 10 mM potassium phosphate buffer (pH 7.8) at 4°C. The dialyzed extract was centrifuged at 15,000×*g* for 20 min at 4°C. The supernatant was then filtered through Miracloth® and the filtrate was used for SOD assay.

4.2. Enzyme assays

CAT activity was assayed at 30°C 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 enzyme extract. Activity was determined spectrophotometrically at 240 nm by measuring the decomposition of H_2O_2 (E=0.0394 mM^{-1} cm^{-1}).[27]

GR activity was assayed at 30°C by the method of Halliwell and Foyer[28] in a 1 ml reaction mixture containing 0.25 ml of 100 mM potassium phosphate buffer (pH 7.8), 0.05 ml of 10 mM oxidized glutathione (GSSG), 0.12 ml of 1 mM NADPH, 0.48 ml distilled water, and 0.1 ml enzyme extract. Activity was determined at 340 nm by measuring the decrease of NADPH absorbance (E=6.1 mM^{-1} cm^{-1}).

APx activity was assayed at 30°C by the method of Nakano and Asada[29] in a 1 ml reaction mixture containing 0.25 ml of 100 mM potassium phosphate buffer (pH 7.0), 0.25 ml of 1 mM ascorbic acid, 0.25 ml of 0.4 mM EDTA-4H, 0.01 ml of 10 mM H_2O_2, 0.19 ml distilled water, and 0.05 ml enzyme extract. Activity was determined at 290 nm by measuring the decrease of ascorbic acid (E=2.8 mM^{-1} cm^{-1}).

SOD activity was assayed at 30°C by the method of Asada[30] in a 1 ml reaction mixture containing 0.1 ml of 500 mM potassium phosphate buffer (pH 7.8, containing 0.1mM EDTA-4H), 0.1 ml of 0.1 mM cytochrome *c* (from horse heart), 0.1 ml of 1 mM xanthine dissolved in 10 mM NaOH, 0.02 ml xanthine oxidase, 0.66 ml distilled water, and 0.02 ml enzyme extract. Activity was determined at 550 nm by measuring the reduction of cytochrome *c*.

5. H_2O_2 content

H_2O_2 levels were assayed according to Velikova *et al*.[31] Five hundred milligrams of leaf tissue were homogenized in an ice bath with 5 ml 0.1% (w/v) TCA. The homogenate was centrifuged at 12,000×*g* for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. The absorbance of the super-

natant was measured at 390 nm and the content of H_2O_2 was determined with a standard curve.

6. Ethane production

Peroxidation of membrane lipids was detected through the analysis of ethane production by the method of Sunohara and Matsumoto.[32] Leaves of rice seedlings were excised, cut into 1 cm lengths, and 20 segments were transferred to a 5 ml glass vial containing 1 ml distilled water. The glass vials were sealed tightly with a rubber plug and an aluminum stopper using a crimper and placed in the growth chamber for 48 h. The ethane content in the vial was quantified using a gas chromatograph (Shimadzu GC-17A) equipped with a glass column packed with Unipak S (GL Sciences Co.) by injecting 1 ml gas sample taken from each glass vial with a syringe. The temperatures of the oven, injector, and detector were maintained at 60, 120, and 120°C, respectively. N_2 carrier gas was applied at 50 ml min^{-1}, and H_2 gas and air pressure were 60 and 50 kPa, respectively. The retention time of ethane was 0.87–0.90 min under these conditions. The concentration of ethane was calculated from the calibration curve using standard ethane (GL Sciences. Tokyo, Japan).

7. Statistical analyses

All results were presented as the means±standard error (S.E.) of at least three replicates, and all experiments were repeated at least twice. For all statistical analyses, the relationships were considered to be significant when $P<0.05$. The effects of ALA on growth, chlorophyll content, antioxidative enzyme activity, H_2O_2 content, and ethane production in NaCl-stressed rice were analyzed using Dunnett's test.

Results and Discussion

1. Effects of ALA pretreatment on seedling growth in NaCl-stressed rice

The growth was determined by the fresh weight of whole plants 20 days after 0 or 50 mM NaCl treatment. ALA itself had no promoting effect on the growth of rice plants when it was applied through the roots with 0.1 and 1 μM (Fig. 1A). The growth of NaCl-treated seedlings was inhibited by 50 mM NaCl (Fig. 1B). The growth levels of ALA-pretreated rice with 0.1 and 1 μM ALA were 0.826±0.022 and 0.845±0.023 g FW $plant^{-1}$ (Fig. 1B). The results indicated that ALA has no promoting effect on the seedling growth of non-treated rice, but ameliorates the growth of NaCl-stressed rice.

The salinity of soil and water is most commonly caused by high Na^+ and Cl^- concentration, which leads to a reduction in initial growth and productivity.[1] Application of some chemicals has been reported to mitigate the adverse effects of salinity on the growth of intact plants and cultured cells.[2–9] Supplementation of KNO_3 and proline significantly ameliorated the adverse effects of salinity on vegetative growth, fruit yield, and physiological parameters, such as relative water

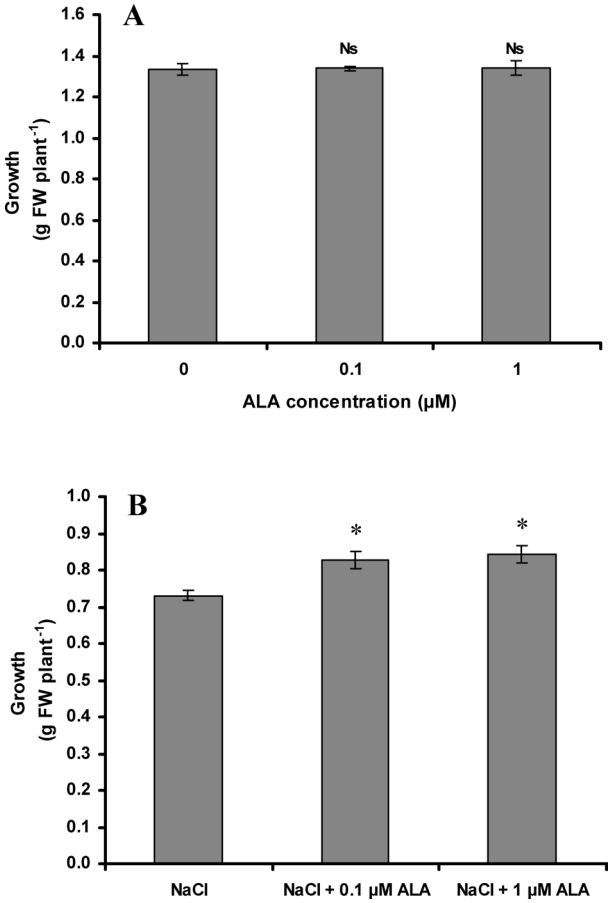

Fig. 1. Effects of ALA pretreatment on seedling growth of rice: (A) non-treated and (B) NaCl-treated rice. Growth was determined by g FW of the whole plant. Rice seedlings were grown hydroponically (A) without or (B) with 50 mM NaCl for 20 days. Bars indicate±S.E. Ns: not significant, * $P<0.05$ (A, control vs. ALA treatments; B, NaCl vs. NaCl+ALA treatments).

content, chlorophyll content, and membrane permeability, in melon (*Cucumis melo* L.).[33] The growth of NaCl-stressed wheat (*Triticum durum* cv. Gediz-75 and *Triticum aestivum* cv. Izmir-85)[7] and cucumber (*Cucumis sativus* L.)[34] was improved by the addition of Si to the nutrient solution. Moreover, several studies showed that ALA had the potential to restore plant growth levels under salt stress conditions when it was applied at low concentrations.[16,17,35]

The herbicidal action of ALA at high concentrations was reported to be due to the abnormal accumulation of chlorophyll intermediates in the dark.[11,12] These accumulated intermediates led to the formation of highly reactive singlet oxygen ($^1O_2$) when plants were transferred to the light and caused the death of plant cells by peroxidation of membrane lipids.[13] To prevent this phenomenon, rice plants were treated with ALA in the light in the present study. The results from rice corresponded with previous reports showing an ameliorative effect of ALA on the growth of cotton[16] and potato (*Solanum tuberosum* L.)[35] under salt stress conditions.

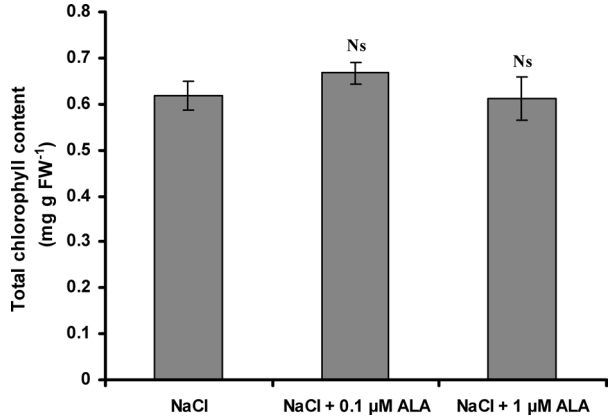

Fig. 2. Total chlorophyll contents in the shoots of ALA-pretreated rice. Rice seedlings were grown hydroponically with 50 mM NaCl for 20 days. Bars indicate±S.E. Ns: not significant (NaCl vs. NaCl+ALA treatments).

2. Effects of ALA pretreatment on total chlorophyll contents in shoots of NaCl-stressed rice

The total chlorophyll content in non-stressed plants was 0.951 ± 0.031 mg g FW^{-1} (data not shown) and decreased to 0.618 ± 0.031 mg g FW^{-1} with 50 mM NaCl (Fig. 2); however, pretreatment with ALA did not change the chlorophyll content under NaCl-stressed conditions (Fig. 2). Previous studies demonstrated that salt stress decreased the chlorophyll content in cotton and sunflower;[36,37] therefore, the increase in chlorophyll content is a possible factor in growth amelioration. Externally applied chemicals, such as Si,[7] proline and KNO_3,[33] were reported to increase the chlorophyll content in plants under salt stress conditions. Santos[37] reported that the decrease in chlorophyll content in NaCl-stressed sunflower (*Helianthus annuus* L. cv. SH222) leaves was mainly because of the decrease in ALA synthesis. Exogenously applied ALA may cause the accumulation of chlorophyll, leading to a higher rate of photosynthesis and plant growth. Hotta *et al.*[14] showed that pothos (*Epipremnum aureus*) treated with ALA induced the accumulation of chlorophyll. The phycocyanin and chlorophyll contents of *Spirulina platensis* cells increased when ALA was added to the culture medium,[38] and the chlorophyll content in *Vigna catjung*, *V. mungo*, and *V. radiata* also increased when ALA was applied.[39] Watanabe *et al.*[40] reported that the chlorophyll contents of tomato treated with NaCl (10000 mg L^{-1}) were +6% and −21% of non-treated controls with and without ALA at 100 mg L^{-1}, respectively; however, the present results suggest that the increase in seedling growth of rice plants by ALA under salt stress was not due to increased chlorophyll levels.

3. Effects of ALA pretreatment on antioxidative enzyme activities in shoots of NaCl-stressed rice

ROS such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$), and singlet oxygen ($^1O_2$), are produced in both unstressed and stressed plant cells.[41,42] Generally, plants possess a number of antioxidative enzymes, such as SOD, CAT, GR, and different classes of peroxidases[20,43] that protect them against damaging effects of ROS; however, under NaCl-stressed conditions, the balance between the production of ROS and the quenching activity of the enzymes is disturbed,[1] which disrupts normal metabolism through oxidative damage to lipids and proteins.[44,45] Plants containing higher constitutive levels of antioxidative enzymes showed greater tolerance to the oxidative damage caused by ROS.[19,22,34,45–50] Vaidyanathan *et al.*[21] reported that salt-tolerant rice (*O. sativa* L. cv. Pokkali) showed higher activity of CAT and higher levels of the antioxidants ascorbate and glutathione than a salt-sensitive rice variety (*O. sativa* L. cv. Pusa Basmati 1). Moreover, increases in the activities of SOD, APx, GR, and guaiacol peroxidase (GPX) were induced in salt-tolerant maize (*Zea mays* L.) genotypes.[22] Hence, the effect of ALA on antioxidative enzyme activity was surveyed.

SOD activity in rice shoots tended to increase with ALA pretreatment; however, it was not statistically significant (Fig. 3A). SOD is a ubiquitous enzyme in aerobic organisms[22] and constitutes the first line of defense against ROS[42] by catalyzing the dismutation of O_2^- into O_2 and H_2O_2.[43] The induction of SOD could increase the scavenging ability of O_2^- radicals that can interact with H_2O_2 to form highly reactive $^{\cdot}OH$, which is thought to be primarily responsible for oxygen toxicity.[51] SOD activity in Indian mustard (*Brassica juncea* L. cv. RH-30) treated with putrescine increased in response to salt stress.[48] Similar induction has also been observed in NaCl-stressed barley[52] and cucumber[34] treated with Si.

CAT activity increased significantly in ALA-pretreated rice. The mean activities were 1.12 ± 0.01 and 1.08 ± 0.01 mmol H_2O_2 decomposed g FW^{-1} min^{-1} in 0.1 and 1.0 μM ALA-pretreated rice, respectively, while that of NaCl-stressed control plants was 0.86 ± 0.01 mmol H_2O_2 (Fig. 3C). APx activity was also stimulated by ALA pretreatment under NaCl stress conditions. The APx activities of 0.1 and 1.0 μM ALA-treated rice were 13.90 ± 0.18 and 13.32 ± 0.04 μmol AsA-decomposed g FW^{-1} min^{-1}, respectively, and that of NaCl-stressed control plants was 12.67 ± 0.14 μmol (Fig. 3B). CAT and APx play a central role in scavenging H_2O_2 to form water and oxygen to protect plants against oxidative stress,[21,53,54] although APx uses ascorbate as an electron donor.[19,55] Nishihara *et al.*[17] reported that APx and CAT activities in spinach (*Spinacea oleracea* L. cv. Virginia) increased temporarily when they were treated with ALA. The present study also showed that ALA enhanced these two enzyme activities in rice. The increased CAT and APx activities in response to ALA treatment might potentiate salt tolerance in rice. These increased activities may be related to heme synthesis because APx is a heme-containing protein[56] and one family of CAT uses heme as a cofactor.[57] These enzyme activities might increase if elevated ALA levels lead to increased heme content, although heme determination was not attempted in this study.

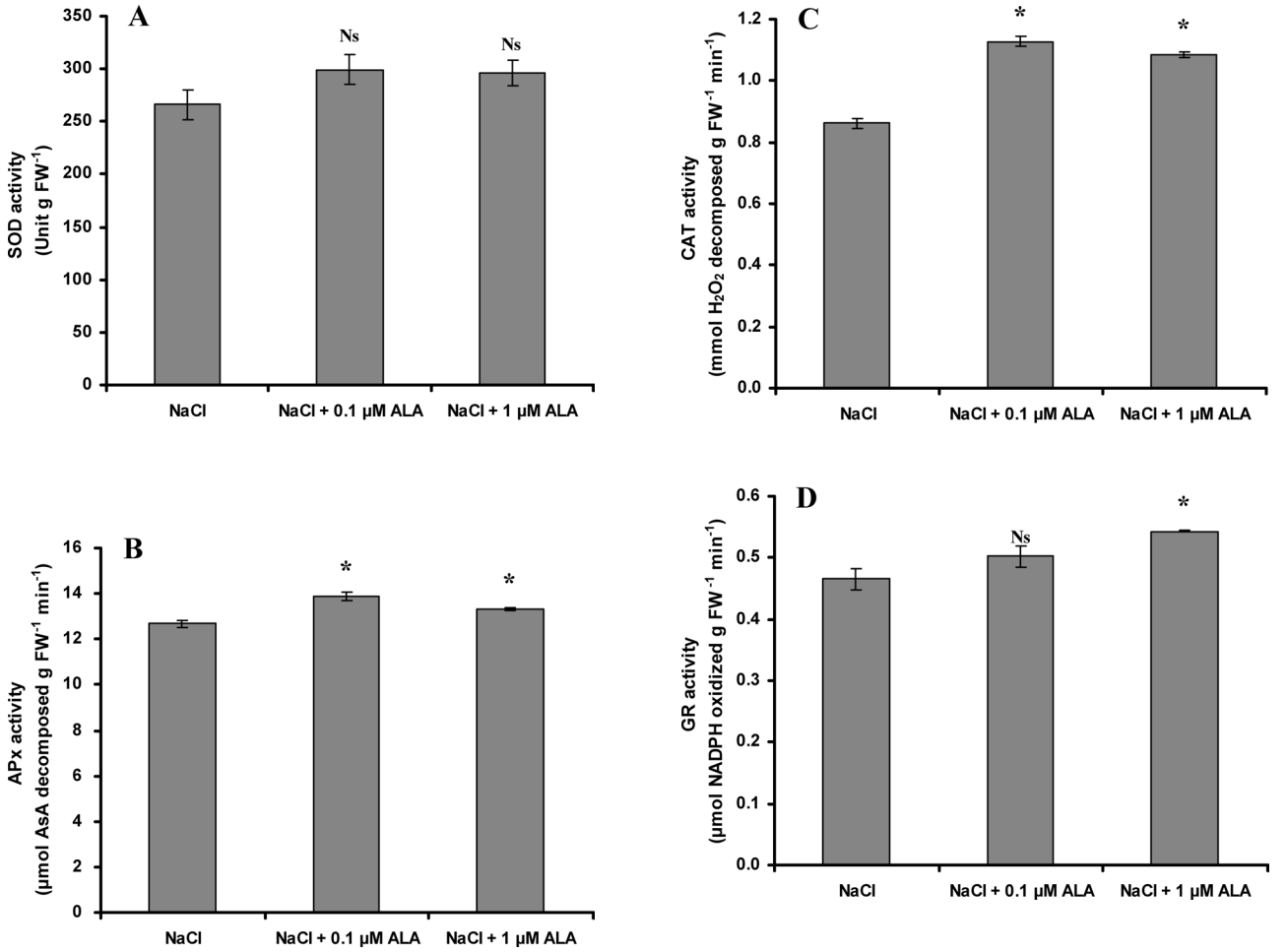

Fig. 3. Effects of ALA pretreatment on the activities of antioxidative enzymes (A) SOD, (B) APx, (C) CAT, and (D) GR in the shoots of rice, which were grown hydroponically with 50 mM NaCl for 20 days. Bars indicate±S.E. Ns: not significant, * $P<0.05$ (NaCl vs. NaCl+ALA treatments).

Similar patterns of CAT and APx response have been observed in *Vigna unguiculata* treated with paclobutrazol,[9] triadimefon-treated *Withania somnifera*,[8] and putrescine-treated Indian mustard.[48]

After ALA treatment, GR activity in leaves of NaCl-stressed rice was stimulated (Fig. 3D). The highest activity of GR (0.542 ± 0.001 μmol NADPH oxidized g $FW^{-1}min^{-1}$) was observed when the rice seedling was pretreated with 1 μM ALA. Salt-tolerant plants have been reported to show higher GR activity.[36,44,48,58] The GR activity in spinach (*Spinacia oleracea* L. cv. Virginia) under salt stress also increased after treatment with ALA.[17] GR plays a key role in the protective system by converting oxidized glutathione (GSSG) to its reduced form (GSH).[43] This activity increases the GSH/GSSG ratio, which is required for ascorbate regeneration.[5] Increased GR activity also increases the ratio of $NADP^+$/NADPH, thereby ensuring the availability of $NADP^+$ to accept electrons from the photosynthetic electron transport chain[50] resulting in less leakage of electrons to O_2 for the generation of O_2^-.[48] In addition, elevated levels of GSH could be associated with increased tolerance to oxidative stress.[36]

4. Effects of ALA pretreatment on H_2O_2 contents in shoots of NaCl-stressed rice

The H_2O_2 content in ALA-pretreated rice was determined to confirm the effectiveness of ALA treatment in scavenging H_2O_2. The amounts of H_2O_2 decreased in the shoots of ALA-pretreated rice under salt stress conditions and the decrease was more distinct when plants were pretreated with ALA at 1 μM (Fig. 4). Reduction in H_2O_2 content might be caused by well-organized functioning of the APx, CAT, and GR systems, which are stimulated by ALA treatment; therefore, it was considered that the greater scavenging activity in 1 μM ALA-treated rice was also related to GR activity. Decreased amounts of H_2O_2 because of the enzyme activities of these defense systems and the amelioration of growth in NaCl-stressed plants were observed in nitric oxide-treated cucumber (*Cucumis sativus* L. cv. Jinchun 5)[51] and putrescine-treated Indian mustard (*Brassica juncea* L. cv. RH-30).[48]

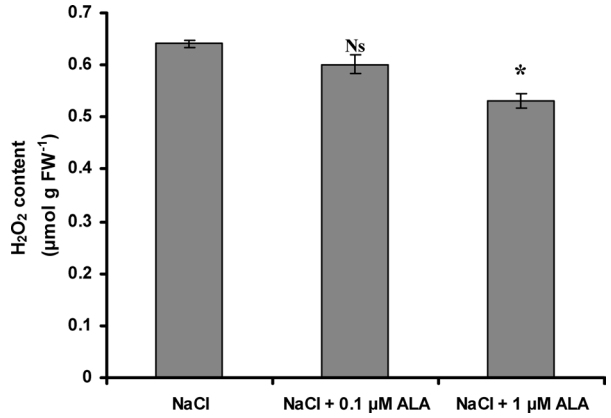

Fig. 4. Effects of ALA pretreatment on H_2O_2 content in shoots of rice grown with 50 mM NaCl for 20 days. Bars indicate ± S.E. Ns: not significant, * $P<0.05$ (NaCl vs. NaCl + ALA treatments).

5. Effects of ALA pretreatment on ethane production in shoots of NaCl-stressed rice

In the present study, lipid peroxidation was estimated by measuring the formation of ethane, which occurs by decomposition of the 16-hydroperoxide form of linolenic acid.[59] In NaCl-stressed rice, the amount of ethane evolution decreased significantly when treated with 0.1 and 1.0 μM ALA (Fig. 5). Salt stress is known to induce ROS, which causes extensive lipid peroxidation in plant cells.[22] Several reports suggested that salt-tolerant plants were better protected from oxidative damage under salt stress conditions.[22,36,44,48,58,60] Demiral and Türkan[44] reported no increase in lipid peroxide formation in the roots of a salt-tolerant rice cultivar (*O. sativa* L. cv. Pokkali) but increased formation in a salt-sensitive cultivar (*O. sativa* L. cv. IR-28). In the leaves of NaCl-stressed maize (*Zea mays* L.), lipid peroxidation in the tolerant genotype BR5033 was lower than in a salt-sensitive genotype, BR5011.[22] The lower level of lipid peroxidation was related to the induction of antioxidative enzyme activities and the concentration of non-enzymatic antioxidants in plants.[36,58]

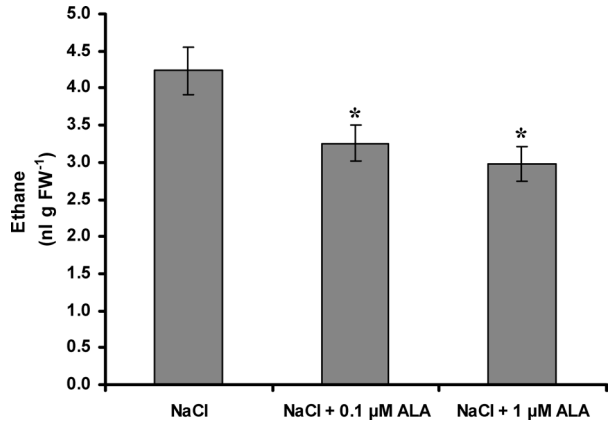

Fig. 5. Effects of ALA pretreatment on ethane production in rice seedlings grown with 50 mM NaCl for 20 days. Bars indicate ± S.E. Ns: not significant, * $P<0.05$ (NaCl vs. NaCl + ALA treatments)

The present result indicated that lipid peroxidation was reduced in ALA-treated rice. This reduction might be due to the higher antioxidative ability to scavenge ROS. A negative correlation between lipid peroxidation and antioxidative enzyme activity was also reported in several plant species. In putrescine-treated Indian mustard (*B. juncea* L. cv. RH-30), the activated enzymes suppressed free radical generation and prevented membrane peroxidation under high salinity conditions.[48] Higher activities of antioxidant enzymes and lower lipid peroxidation were also observed in a salt-tolerant barley cultivar (*H. vulgare* L. cv. Jian 4).[58]

The results presented above strongly suggest that ALA ameliorates the growth of rice seedlings grown under NaCl-stress conditions by enhancing antioxidative enzyme activities. In particular, ALA-pretreatment enhanced CAT, GR, and APx activities, which scavenge ROS, and this may result in less damage to membrane lipids in NaCl-stressed rice seedlings.

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