

Title: Adaptive mechanism of *Echinochloa crus-galli* Beauv. var. *formosensis* Ohwi under salt stress: Effect of salicylic acid on salt sensitivity

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Abbreviations: APX, ascorbate peroxidase; CAT, catalase; GR, glutathione reductase; ROS, reactive oxygen species; SA, salicylic acid; SOD; superoxide dismutase; Φ_{PSII} , efficiency of photosystem II photochemistry

Abstract

Our previous study revealed that salicylic acid (SA) accumulates in salt-stressed rice (*Oryza sativa* L. cv. Nipponbare) seedlings, and we hypothesized that the accumulation of SA might potentiate oxidative injury in rice seedlings since the inhibition of SA synthesis alleviated the growth inhibition under high salinity. To further clarify the action of SA under salt stress, we investigated the changes in the SA content, the activities of the antioxidative enzymes, and the effects of exogenous SA on barnyardgrass (*Echinochloa crus-galli* Beauv. var. *formosensis* Ohwi), a gramineous weed which shows lower SA content and is more salt tolerant than rice. In *E. crus-galli* seedlings exposed to high salinity, neither free nor conjugated SA content showed any increase, while the fresh weight of the shoot and chlorophyll fluorescence (Φ_{PSII}) slightly decreased. When *E. crus-galli* seedlings were treated with salt after foliar application of SA, the absorbed SA resulted in the enhancement of the salt-induced growth inhibition and a striking reduction of the Φ_{PSII} value. Catalase (CAT) and superoxide dismutase (SOD) activities of *E. crus-galli* seedlings were induced by the salt treatment. However, SA pre-treatment suppressed such an induction of CAT activity and further promoted SOD activity, both of which led to the elevation of the leaf hydrogen peroxide (H_2O_2) level. The present results suggested that enlargement of the cellular SA pool facilitates the generation of H_2O_2 through the suppression of CAT activity and through a remarkable promotion of SOD activity, and thereby enhances the oxidative injury caused by salt stress.

Key words: Catalase; *Echinochloa crus-galli*; Hydrogen peroxide; Oxidative stress; Salicylic acid; Salt stress

1. Introduction

Salicylic acid (SA) is a signal molecule that induces the plant defense response to pathogen attacks. It develops systemic acquired resistance (SAR), which results in a long-lasting, nonspecific, systemic resistance to subsequent pathogen infections [1]. It has been shown that endogenous SA content increases dramatically not only due to pathogen infection, but also to environmental stressors such as ozone, low temperature, and salinity [2-4]. SA has been suggested to be physiologically important in stress tolerance since exogenous SA brought about plants' tolerance to various abiotic stresses including chilling, drought, and salt [5-8]. In contrast, high levels of SA are known to potentiate the oxidative damage caused by O₃ exposure and chilling temperature [4, 9]. It was also reported that pre-treatment with SA, which elevates the chilling tolerance in maize, causes an increased sensitivity to drought in maize and wheat [10]. Thus, under different abiotic stresses or in different plant species, SA has been reported to show apparently contrasting effects, i.e. either the reduction or promotion of stress tolerance.

Our previous study [11] using rice seedlings showed that the activity of benzoic acid 2-hydroxylase (BA2H), which catalyses SA biosynthesis, was induced by salinity, and increases in this activity paralleled the accumulation of free SA. Furthermore, we observed that the suppression of SA accumulation restored the seedlings' growth under salinity, suggesting that the accumulation of endogenous SA relates to salt stress-induced injury in rice seedlings [11]. The serious damage caused by salt stress is at least partially due to the generation of reactive oxygen species (ROS), such as superoxide (O₂^{·-}) and hydrogen peroxide (H₂O₂). SA could cause the inactivation of H₂O₂-removing enzymes [12, 13]. In salt-stressed rice, decreases in the activity of catalase (CAT), a major H₂O₂-scavenging enzyme, are frequently observed, while other antioxidative enzymes are induced [3, 14-16]. Recently, we found that a BA2H inhibitor,

which reduces SA accumulation, resulted in the restoration of CAT activity and alleviated oxidative injury in rice seedlings under salt stress [17]. Therefore, we hypothesized that the reduction of CAT activity by accumulated SA and the accompanying generation of H₂O₂ might be involved in the oxidative injury caused by salinity in rice seedlings.

To further clarify the action of SA in plant tissue under salt stress, we investigated the effects of foliar-applied SA on the salt sensitivity, H₂O₂ generation, and antioxidative enzyme activities. To this end, barnyardgrass (*Echinochloa crus-galli* var. *formosensis* Ohwi), a gramineous weed which grows in paddy fields and therefore competes with rice for growth, was selected as a plant material because it is known to have lower SA contents and a higher salt tolerance than rice [18, 19]. The changes in the free and conjugated SA contents of this plant under salt treatment were also examined.

2. Materials and Methods

2.1 Plant Materials and Treatments

Seeds of barnyardgrass (*Echinochloa crus-galli* Beauv. var. *formsensis* Ohwi) were germinated in an incubator at 30°C for 48 h in the dark and then transferred to a growth chamber under a 12-h photoperiod (25/20°C; day/night, 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and 60% relative humidity. The seedlings were grown in a modified Kasugai's nutrient solution [20] for 3 weeks until the 3rd leaf stage. To impose salt stress, the seedlings were transferred to a nutrient solution containing 0, 80, or 120 mM NaCl for 3 days. For the SA treatment and salt stress, the seedlings were pre-treated by spraying 0.5 mM SA (Sigma, St. Louis, USA) solution (pH 6.8) one day before the salt stress with an atomizer onto the leaf blades until it ran off. Distilled water (pH 6.8) was applied to the leaves of control plants. The seedlings were then transferred to the nutrient solution

containing 120 mM NaCl for 3 days.

The 3rd leaves (the youngest fully expanded leaves) of the seedlings were harvested, frozen in liquid nitrogen, and then stored at -80°C for further analysis. The salt-stressed responses were clearer in the 3rd leaves rather than the growing 4th leaves (data not shown).

2.2 Chlorophyll Fluorescence Measurement

Chlorophyll fluorescence was measured on the 3rd leaf blades of the above intact seedlings using a chlorophyll fluorometer (MINI-PAM, WALZ, Effeltrich, Germany) with light-emitting diodes at a peak wavelength of 655 nm and an $18,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ saturation pulse. The efficiency of photosystem II photochemistry (Φ_{PSII}) was calculated according to Gentry et al. [21].

2.3 Antioxidative Enzyme Extraction and Activity Assays

Leaf samples (0.5 g fresh weight; FW) were ground into a fine powder in liquid nitrogen with a chilled mortar and pestle and transferred into a potassium phosphate buffer (25mM, pH7.8) containing 0.4mM ethylenediamine tetra-acetic acid (EDTA-4H), 1mM ascorbate and 2% (w/v) polyvinylpolypyrrolidone. The mixture was homogenized and then centrifuged at $15,000 \times g$ for 20 min at 4°C . The supernatant, filtered through 1 layer of Myracloth[®] (Calbiochem, San Diego, USA), was used as the crude enzyme solution. The filtrate was then used as an enzyme extract for CAT (EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), and glutathione reductase (GR; EC 1.6.4.2) assays. For the assay of superoxide dismutase (SOD; EC 1.15.1.1), the enzyme extract was dialyzed against Seamless Cellulose Tubing[®] (Wako, Osaka, Japan) overnight with 10 mM potassium phosphate buffer (pH 7.8) at 4°C . CAT activity was determined by

the method of Aebi [22]. The 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 H₂O₂) and 0.05 mL of enzyme extract. Subsequent decomposition of H₂O₂ was observed at 240 nm.

APX activity was determined by the method of Nakano and Asada [23]. The activity was assayed 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 H₂O₂, 0.19 mL distilled water, and 0.05 mL of enzyme extract. A subsequent decrease in ascorbic acid was observed at 290 nm.

GR activity was determined by the method of Halliwell and Foyer [24]. The activity was assayed 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, 0.12 mL of 1mM NADPH, 0.48 mL of distilled water, and 0.1 mL of enzyme extract. A resultant decrease in NADPH was observed at 340 nm.

SOD activity was determined by the method of Asada [25]. SOD activity was assayed in a 1 mL reaction mixture containing 0.1 mL 500 mM potassium phosphate buffer (pH 7.8, containing 0.1 mM EDTA-4H), 0.1 mL of 1mM cytochrome *c* from horse heart, 0.1 mL of 1 mM xanthine dissolved in 10 mM NaOH, 0.02 mL of xanthine oxidase, 0.66 mL of distilled water, and 0.02 mL of dialyzed enzyme extract. The reduction rate of cytochrome *c* was measured in terms of the initial rate of increase in absorbance at 550 nm.

The protein concentration of the crude enzyme extracted from *E. crus-galli* leaves was measured according to Bradford [26].

2.4 Free and Conjugated Salicylic Acid Measurement

The extraction and determination of free salicylic acid were conducted according to the method of Malamy et al. [27]. Leaf samples (0.3 g FW) were ground with 7 mL of 90% methanol and centrifuged at $7,000 \times g$ 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 *in vacuo* at 35°C to remove the methanol. Distilled water (5 mL) was added to resuspend the extracts at 80°C 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 to 2.0 using HCl. To quantify glucose-conjugated SA, the remaining half of the extract was incubated overnight at 37°C with an equal volume of 0.2 M sodium acetate (pH 4.5) containing 10 U β -glucosidase (EC 3.2.1.21; from almond; Oriental Yeast, Tokyo, Japan.). After hydrolysis, the samples were acidified to pH 1.5 to 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 vacuo* at 35°C to be fully dried. 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 (μ Bondasphere 5 μ C18-100A, 3.9 \times 150 mm; Waters, Milford, USA) at 35°C and a 1.0 mL/min flow rate of solvent (23% (v/v) methanol/20 mM sodium acetate buffer; pH 5.0). Free and conjugated SAs were analyzed by using a spectrofluorescence detector (RF-10AXL; Shimadzu, Kyoto, Japan) at the excitation wavelength of 313 nm and an emission wavelength of 405 nm.

2.5 H_2O_2 Content Measurement

Hydrogen peroxide levels were determined according to Velikova et al. [28]. Leaf tissues (0.5 g FW) were homogenized in an ice bath with 5 ml 0.1% (w/v) TCA. The

homogenate was centrifuged at $12,000 \times g$ for 15 min, and 0.5 ml of the supernatant was added to 0.5 ml 10 mM potassium phosphate buffer (pH 7.0) and 1ml 1 M KI. The absorbancy of the supernatant was read at 390 nm. The content of H₂O₂ was given on a standard curve.

2.6 Statistics

All the data presented here were based on two independent experiments with three replications. In each experiment three different pots were used for the same treatment. SPSS (Ver. 11.0J) software was used for the statistical analysis. To assess the statistical significance of the treatment differences, a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or *t*-test (with *P* set at 0.05) was employed.

3. Results

3.1 Effects of NaCl Treatment on the Shoot Growth, Chlorophyll Fluorescence, and Salicylic Acid Content in *E. crus-galli*

E. crus-galli seedlings showed no serious damage even at 120 mM NaCl (Fig. 1A) compared with rice seedlings in which the growth was inhibited depending on the NaCl concentration, and the first and second leaves exhibited wilting at 120 mM NaCl (Fig. 1 B). The shoot growth of *E. crus-galli*, especially the fourth leaf, was repressed by 80 and 120 mM NaCl treatment (Fig. 1A). However, the fresh weight of the whole shoot decreased only to 88 and 79% of the control seedlings at three days after the 80 and 120 mM NaCl treatments, respectively (Fig. 2A). Similarly, the chlorophyll fluorescence (Φ_{PSII}) of the leaf blade was not significantly affected by the NaCl treatment both at 80 mM and at 120 mM over three days (Fig. 2B), although prolonged treatment (six days)

with 120 mM NaCl resulted in a 27% decrease in Φ_{PSII} value (data not shown).

In *E. crus-galli* seedlings exposed to 80 or 120 mM NaCl, neither free SA nor conjugated SA showed any significant increase over three days (Figs. 2C and D).

3.2 Effects of SA Pre-Treatment on Shoot Growth, Chlorophyll Fluorescence, and H_2O_2 Content in *E. crus-galli* under Salt Stress

In the seedlings treated with 0.5 mM SA by foliar spraying, the free and conjugated SA contents in the 3rd leaves increased up to 160% and 165%, respectively, of those of the control seedlings after one day (Table I).

In the absence of salinity, such foliar treatment with exogenous SA did not affect either the shoot growth or Φ_{PSII} in *E. crus-galli* seedlings (Figs. 3A and B). However, in the presence of salinity, the growth inhibitory effect of salt was reinforced with SA pre-treatment; the increase of the shoot fresh weight over three days was completely blocked when *E. crus-galli* seedlings were treated with 120 mM NaCl after foliar treatment with SA (Fig. 3A). Similarly, SA pre-treatment caused a remarkable decrease in Φ_{PSII} in *E. crus-galli* seedlings three days after the NaCl treatment, while the Φ_{PSII} value hardly changed in the seedlings treated with NaCl alone (Fig. 3B). The H_2O_2 content of the leaves did not statistically change as a result of treatment with either 0.5 mM SA or 120 mM NaCl alone (Fig. 4). However, a significant increase in the H_2O_2 content of 46% above the level in the control seedlings could be observed in the seedlings treated with NaCl after foliar treatment with SA (Fig. 4).

3.3 Effects of SA Pre-Treatment on the Activities of Antioxidative Enzymes in *E. crus-galli* under Salt Stress

CAT activity was significantly induced after one day of salt treatment in the leaves of

E. crus-galli seedlings (Fig. 5A). Such an induction of CAT activity was strongly repressed with SA pre-treatment: The CAT activity was 30% lower than that of the seedlings treated with NaCl alone and decreased to the level of the control seedlings after three days.

APX activity was also induced by the salt treatment, while GR activity was slightly inhibited (Figs. 5B and C). SA pre-treatment did not affect either the APX or GR activities of the seedlings exposed to salinity.

SOD activity was significantly induced one day after the NaCl treatment, although the SOD activity transiently increased only with SA pre-treatment prior to NaCl treatment (Fig. 5D). In contrast with the CAT activity, however, the increase of the SOD activity under salinity was further enhanced with SA pre-treatment: Foliar application of SA brought about an over five-fold increase in the SOD activity after three days (Fig. 5D).

4. Discussion

Salinity caused growth inhibition and decreased efficiency in photosynthetic energy conversion (Φ_{PSII}) in *E. crus-galli* seedlings (Figs. 2A and B). However, the growth inhibition and damage symptoms in *E. crus-galli* were not as severe as those in rice seedlings (Fig. 1). The preliminary experiment using rice seedlings showed that 120 mM of NaCl caused a striking decrease in the leaf blade Φ_{PSII} value (a 37% and 64% decrease after three and six days, respectively). The present results supported the results of an earlier report showing that the gramineous weed *E. crus-galli* is more tolerant of salt stress than rice [19]. The decrease in Φ_{PSII} accounts for the occurrence of the disorder in photosynthetic electron transport, which leads to the generation of ROS such as $\text{O}_2^{\cdot -}$ and H_2O_2 [29]. Thus, it appeared that the difference in salt tolerance between

rice and *E. crus-galli* might be associated with the difference in their ability to scavenge cytotoxic ROS.

CAT is known as a major H₂O₂ scavenging enzyme in higher plants and is generally induced by various oxidative stresses in stress-tolerant plants [30, 31]. It has been reported that the accumulation of H₂O₂ due to a decrease in CAT activity or CAT deficiency is involved in susceptibility to environmental stresses [32, 33]. In *E. crus-galli* seedlings, the CAT activity increased immediately after NaCl treatment (Fig. 5A), while CAT activity in rice seedlings decreased under salt stress [3, 14], indicating an apparent difference between those plants in terms of their H₂O₂ scavenging ability.

The free and conjugated SA contents in the leaves of *E. crus-galli* were 0.5 and 3.4 µg g⁻¹FW, respectively, which were 1/10 and 1/20 lower than those in rice leaves [11]. Our previous study [11] showed that SA biosynthetic enzyme (BA2H) in rice seedlings was induced immediately after salt stress and was accompanied by a remarkable increase in free SA (a two-fold increase one day after 80 mM NaCl treatment). In *E. crus-galli* seedlings, however, neither free nor conjugated SA contents showed any increase resulting from the treatment with 80 mM or 120 mM NaCl (Figs. 2C and D). The two pathways of SA biosynthesis have been so far revealed: One is the pathway via benzoic acid from phenylalanine and the other via isochorismate [34]. Ogawa et al. [35] showed that transcription levels of phenylalanine ammonia-lyase genes were increased to a greater extent in the O₃-sensitive tobacco (Bel W3) than in the O₃-tolerant tobacco (Bel B) and Pasqualini et al. [36] found that Bel W3 accumulated higher levels of SA than did Bel B after O₃ exposure. In contrast, SA was reported to be synthesized by isochorismate synthase in *Arabidopsis* after exposure of O₃ [35]. Although the synthetic route or metabolic pathway of SA in *E. crus-galli* is unknown at present, our results suggested that the expression of the genes involved in SA synthesis was not induced

with salt stress in *E. crus-galli*.

Mohase and van der Westhuizen [37] found that the pattern and extent of CAT inhibition largely coincided with those of SA accumulation. It was reported that specific binding of SA to plant CAT resulted in the reduction of CAT activity and in elevated H₂O₂ [13, 38]. Kim et al. [39] showed a reduction of *CatA* and *CatC* expression at the mRNA level in rice seedlings subjected to salt stress. It was also reported that *CatC* gene was down regulated in rice leaves treated with SA [40]. These data, together with our finding that, unlike the salt-sensitive rice seedlings, salt-tolerant *E. crus-galli* seedlings induced CAT activity but not SA synthesis under high salinity, strongly suggest that the accumulation of SA depresses the stress tolerance of plants through the inhibition of CAT.

To ascertain whether the elevation of the tissue SA level results in a reduction of CAT activity, an increase in H₂O₂ and an alteration of stress tolerance, the effects of exogenous SA on *E. crus-galli* seedlings under high salinity were examined. Foliar application of SA significantly increased not only free SA but also conjugated SA in the leaves (Table I). Absorbed SA itself affected neither the shoot growth nor the Φ_{PSII} value of *E. crus-galli* (Fig. 3). However, 120 mM NaCl treatment after foliar spraying of SA enhanced both salt stress-induced growth inhibition and the decrease in the Φ_{PSII} value. Preliminary experiments showed that pre-treatments with 0.01 to 1 mM SA reinforced the growth inhibitory effect of salt stress, and the damage was more severe with higher SA concentrations. Furthermore, such exogenous SA treatment resulted in the striking suppression of CAT activity (Fig. 5A) and significant increases in H₂O₂ content in the seedlings under high salinity (Fig. 4). The increase rate of H₂O₂ was much higher than that in rice seedlings exposed to the same salt concentration (data not shown).

A remarkable elevation of SOD activity under SA pre-treatment was also observed in

the present experiment (Fig. 5D). SA and ROS have been proposed to be on a positive feedback loop [41]. Kawano et al. [42] reported that the addition of SA to tobacco suspension culture induces $O_2^{\cdot -}$ generation through the extracellular peroxidase-catalyzed oxidation of SA. SOD is an enzyme that catalyzes a dismutation reaction which converts $O_2^{\cdot -}$ to H_2O_2 [25]. Therefore, the promotion of SOD activity by exogenous SA is also considered to be involved in the observed elevation of leaf H_2O_2 under salt stress.

The present study demonstrated that the enlargement of the cellular SA pool potentiates oxidative injury under salt stress, possibly due to the elevation of H_2O_2 caused by the decreased CAT and increased SOD activities. The repression of free SA formation under high salinity observed in *E. crus-galli* (Fig. 2C) could be one of the strategies of this highly adaptive weed for coping with various environmental stresses. It was reported that SA-deficient *Arabidopsis thaliana* expressing SA hydroxylase (*NahG*), which catalyzes SA breakdown, was better able to resist the oxidative damage generated by salt and osmotic stress than the wild-type plants [43]. Recently, we also observed that the suppression of SA accumulation by BA2H inhibitor is effective in alleviating salt stress injury in rice seedlings [11, 17]. The genes related to the metabolic regulation of SA might be promising targets in rice breeding for improving the stress tolerance of this important gramineous crop.

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FIGURE CAPTIONS AND LEGENDS

Fig. 1. *Echinochloa crus-galli* (A) and rice (B) seedlings subjected to NaCl treatment for three days. *E. crus-galli* and rice seedlings were grown hydroponically for three weeks.

Fig. 2. Effects of NaCl treatment on the fresh weight of shoots and the chlorophyll fluorescence and SA content in the 3rd leaves of *Echinochloa crus-galli* seedlings. (A) Fresh weight. (B) Chlorophyll fluorescence (Φ_{PSII}). (C) Free SA content. (D) Conjugated SA content. Asterisks indicate significant differences at $P < 0.05$ compared with their controls according to Tukey's multiple comparison test. Bars indicate \pm S.E. ($n = 3$).

Fig. 3. Effects of SA pre-treatment on the fresh weight of shoots and the chlorophyll fluorescence (Φ_{PSII}) in *Echinochloa crus-galli* seedlings. SA solution (0.5 mM, pH 6.8) was sprayed on the seedlings one day before 120 mM NaCl treatment. (A) Fresh weight. (B) Chlorophyll fluorescence (Φ_{PSII}). Asterisks indicate significant differences at $P < 0.05$ between the NaCl treatment and the SA + NaCl treatment according to the t -test. Bars indicate \pm S.E. ($n = 3$).

Fig. 4. Effect of SA pre-treatment on H_2O_2 content in the 3rd leaves of *Echinochloa crus-galli* seedlings with or without NaCl treatment. 120 mM NaCl was treated for three days after foliar application of distilled water (DW) or 0.5 mM SA. Asterisk indicates significant difference at $P < 0.05$ compared with their controls according to the t -test. Bars indicate \pm S.E. ($n = 3$).

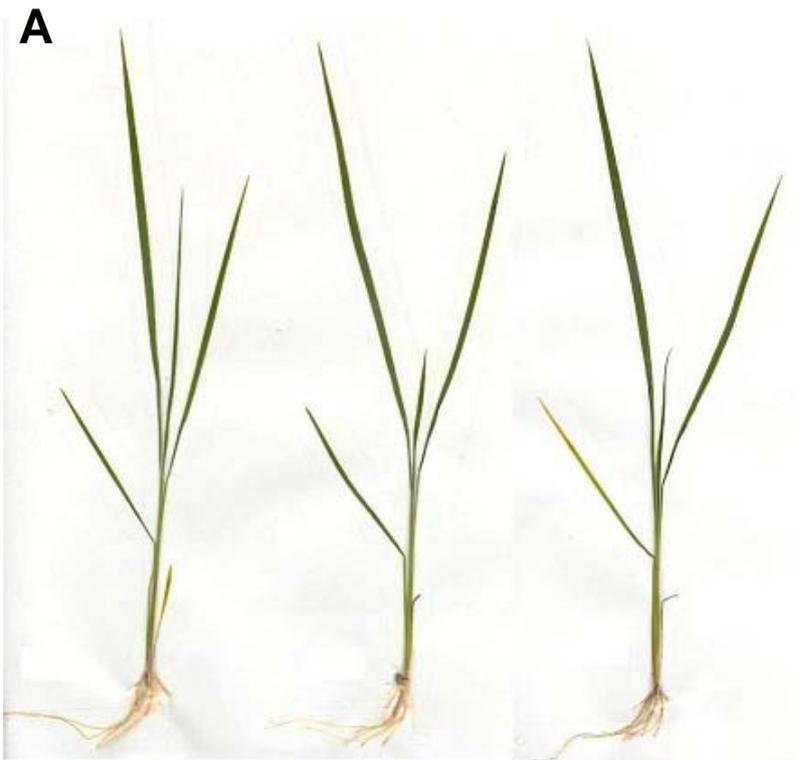
Fig. 5. Effects of SA pre-treatment on NaCl-induced change in the activities of antioxidative enzymes in *Echinochroa crus-galli* seedlings. SA solution (0.5 mM, pH 6.8) was sprayed on the seedlings one day before 120 mM NaCl treatment. (A) Catalase (CAT). (B) Ascorbate peroxidase (APX). (C) Glutathione reductase (GR). (D) Superoxide dismutase (SOD). Asterisks indicate significant differences at $P < 0.05$ between the NaCl treatment and the SA + NaCl treatment according to the *t*-test. Bars indicate \pm S.E. (n = 3).

Table 1. SA content in the 3rd leaves of *Echinochloa crus-galli* seedlings one day after foliar application of SA.

	Control	SA
Free SA ($\mu\text{g g}^{-1}$ FW)	0.72 \pm 0.09	1.15 \pm 0.12 (160%) *
Conjugated SA ($\mu\text{g g}^{-1}$ FW)	2.92 \pm 0.04	4.81 \pm 0.39 (165%) *

Values are mean \pm S.E. (n = 3). Values in parentheses represent a percentage of the control value. SA solution (0.5 mM, pH6.8) was sprayed on the seedlings with an atomizer.

* Significant at the $P < 0.05$ level compared to control.



0 mM

80 mM

120 mM NaCl



0 mM

80 mM

120 mM NaCl

Fig. 1

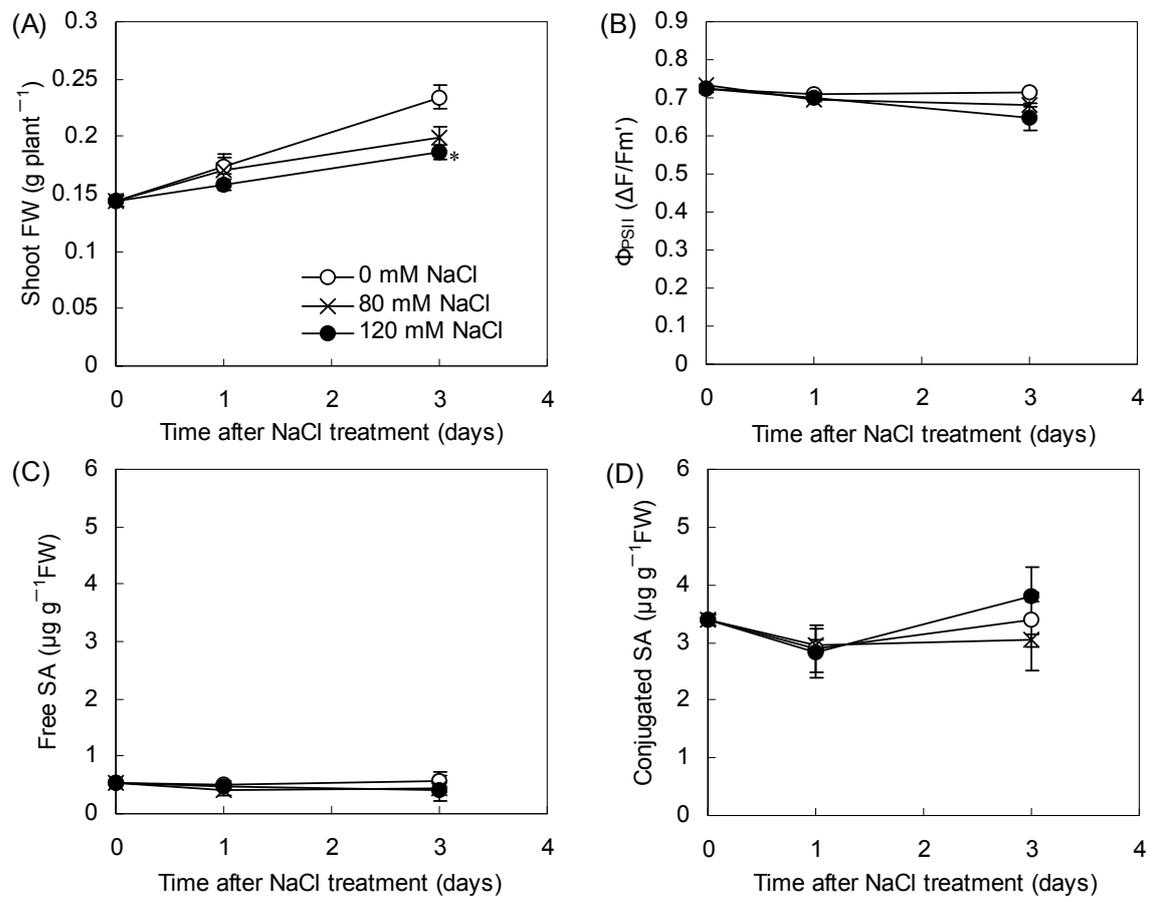


Fig. 2

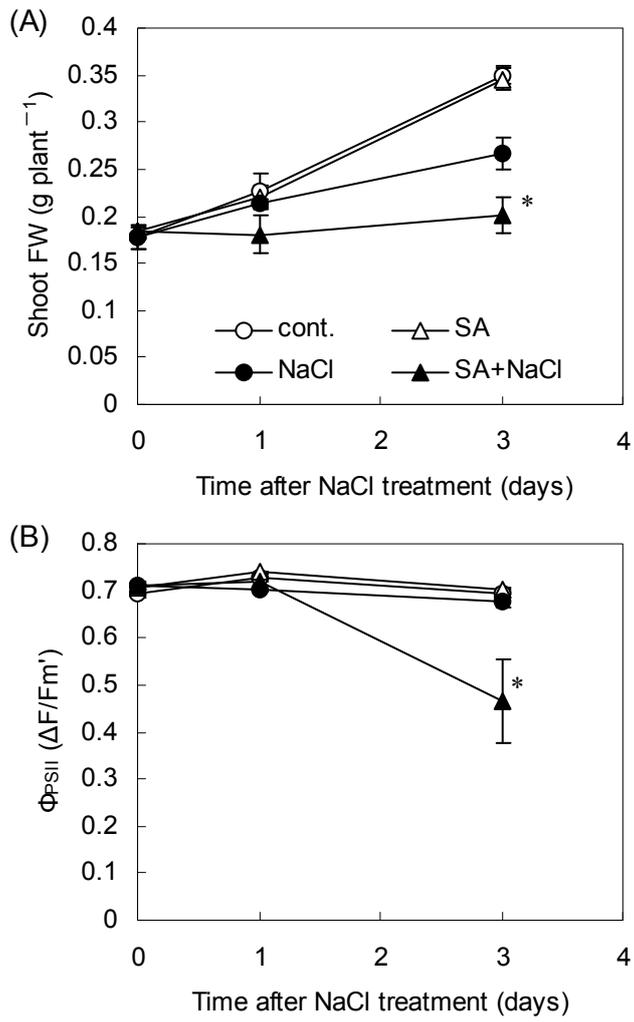


Fig. 3

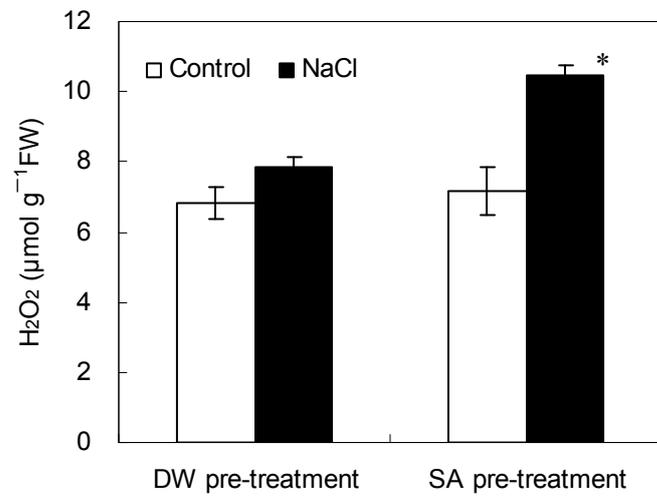


Fig. 4

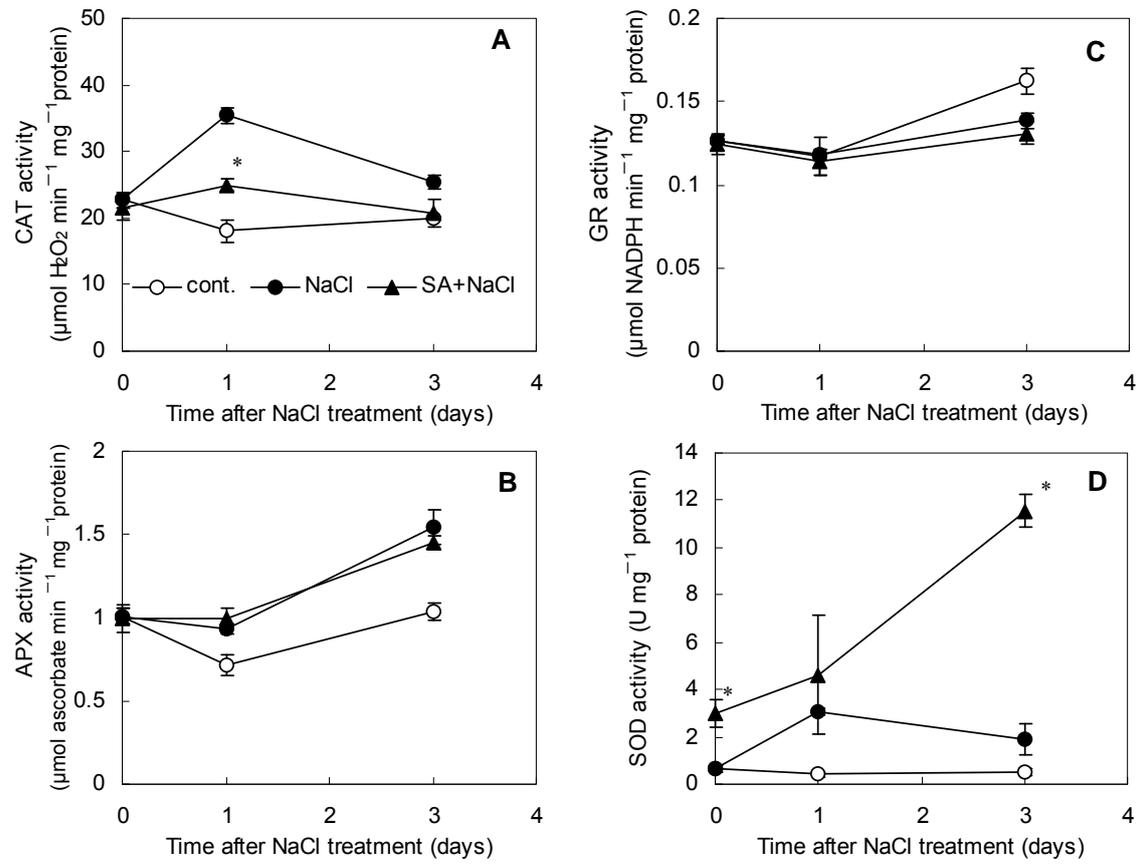


Fig. 5