Role of salicylic acid in alleviating oxidative damage in rice roots (*Oryza sativa*) subjected to cadmium stress

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Pretreatment with SA enhanced the antioxidant defense activities in Cd-stressed rice, thus alleviating Cd-induced oxidative damage and enhancing Cd tolerance.

Abstract

Time-dependent changes in enzymatic and non-enzymatic antioxidants, and lipid peroxidation were investigated in roots of rice (*Oryza sativa*) grown hydroponically with Cd, with or without pretreatment of salicylic acid (SA). Exposure to 50 μM Cd significantly decreased root growth, and activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), but increased the concentrations of H_2O_2 , malondialdehyde (MDA), ascorbic acid (AsA), glutathione (GSH) and non-protein thiols (NPT). However, pretreatment with 10 μM SA enhanced the activities of antioxidant enzymes and the concentrations of non-enzymatic antioxidants, but lowered the concentrations of H_2O_2 and MDA in the Cd-stressed rice compared with the Cd treatment alone. Pretreatment with SA alleviated the Cd-induced inhibition of root growth. The results showed that pretreatment with SA enhanced the antioxidant defense activities in Cd-stressed rice, thus alleviating Cd-induced oxidative damage and enhancing Cd tolerance. The possible mechanism of SA-induced H_2O_2 signaling in mediating Cd tolerance was discussed.

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1. Introduction

Cadmium (Cd) is widespread in the environment and is highly toxic to living organisms. In plants, Cd interferes with many cellular processes mainly by formation of

complexes with functional proteins, displacement of essential metal ions from the plasma membrane, and the alteration of antioxidant system hence accumulating reactive oxygen species (ROS) and resulting in “oxidative stress” (see review by Schützendübel and Polle, 2002).

Accumulation of ROS, including H_2O_2 , causes oxidative damage in plants because they oxidize organic compounds and induce membrane lipid peroxidation in the cellular environment (Schützendübel and Polle, 2002). Due to their highly reactive nature, ROS are kept as low as possible in plants by an efficient ROS-scavenging system, including enzymatic antioxidants (e.g. superoxide dismutase, catalase, and peroxidase)

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and non-enzymatic metabolites (e.g. glutathione, ascorbic acid) (Dat et al., 2000; Mittler et al., 2004; Foyer and Noctor, 2005). Recently, ROS, particularly H_2O_2 , have been proposed as essential signals in both biotic and abiotic stress responses (Dat et al., 2000; Mittler et al., 2004; Foyer and Noctor, 2005). The homeostasis of ROS is required to control the subtle to balance between the important signaling roles in plant defense and the damaging effects of excessive ROS (Mittler et al., 2004; Foyer and Noctor, 2005). This is regulated by highly complex gene networks and signaling pathways (Mittler et al., 2004).

Salicylic acid (SA) has been proposed as an endogenous signal associated with regulating oxidant levels in response to biotic stress. Upon pathogen attack, SA accumulates to high levels at the site of pathogenic infection, binds and inhibits tobacco CAT activity both *in vitro* and *in vivo*, thereby leading to an increase in the endogenous level of H_2O_2 , which could then serve as a secondary messenger to induce the expression of pathogenesis-related proteins and initiate the development of systemic acquired resistance (Chen et al., 1993). Recent studies have shown that SA also plays an important role in provoking plant resistance to various abiotic stresses. For example, treating mustard (*Sinapis alba* L.) seedlings with exogenous SA improved their thermo-tolerance and heat acclimation, especially in young leaves (Dat et al., 1998). Spraying banana (*Musa acuminata* coll., cv.) seedlings with SA (Kang et al., 2003) and exposing seedling of maize (*Zea mays* L.), rice (*Oryza sativa* L.) and cucumber (*Cucumis sativus* L.) to SA significantly strengthened a tolerance to the subsequent chilling stress (Kang and Saltveit, 2002). SA is also involved in plants subject to salt (Borsani et al., 2001) and heavy metal stresses (Metwally et al., 2003; Yang et al., 2003; Drazic and Mihailovic, 2005).

Since the mode of SA signaling pathway is associated with increased H_2O_2 levels (Chen et al., 1993), it might be expected that SA pretreatment would accentuate the oxidative stress caused by Cd toxicity. However, several studies have shown that pretreatment of SA enhanced the resistance of plants to Cd-induced toxicity (Drazic and Mihailovic, 2005), including oxidative damage (Metwally et al., 2003). Metwally et al. (2003) concluded that SA alleviated Cd toxicity to barley roots not at the level of antioxidant defense but by affecting other mechanisms of Cd detoxification. H_2O_2 has been considered as an essential signal involved in plant defense against abiotic stress (Dat et al., 2000; Mittler et al., 2004; Foyer and Noctor, 2005). It is hypothesized that the increased H_2O_2 induced by SA may act as an important secondary messenger to improve plant defense against the subsequent Cd stress. Hence, this study focused on the effects of pretreatment of exogenous SA on time-dependent changes in the activities of SOD, POD, CAT, and the concentrations of AsA, GSH and H_2O_2 , and non-protein thiols (NPT) and lipid peroxidation in rice roots exposed to Cd. This study therefore aimed to explore the mechanisms of (1) the positive effect of SA on Cd tolerance through the regulation of the antioxidant system; and (2) the possible mechanisms of SA-induced H_2O_2 signaling pathways in mediating Cd tolerance in rice roots.

2. Materials and methods

2.1. Plant material and experimental design

Seeds of rice (*O. sativa* cv Jiahua 1) were surface sterilized with H_2O_2 (10%) for 10 min, rinsed thoroughly with distilled water, and sown in trays. When the second leaf emerged, seedlings of uniform size were transferred to hydroponics pots (1 L, PVC, six plants per pot) in a growth chamber. Each pot contained 1 L of the kimura B nutrient solution (full strength composition: 0.37 mM $(\text{NH}_4)_2\text{SO}_4$, 0.55 mM MgSO_4 , 0.18 mM KNO_3 , 0.18 mM KH_2PO_4 , 0.37 mM $\text{Ca}(\text{NO}_3)_2$, 0.09 mM K_2SO_4 , 50 μM Fe(II)-EDTA, 1 μM ZnSO_4 , 1 μM CuSO_4 , 5 μM MnSO_4 , 10 μM H_3BO_3 , 0.5 μM Na_2MoO_4 , 100 μM NaCl, 0.2 μM CoSO_4). The nutrient solution was renewed twice a week. The pots were randomly arranged daily during the growth period. Daily photoperiod was 14 h (8:00–22:00) with controlled temperature (25 °C/18 °C, day/night), 70% relative humidity all day, and photosynthetic photon flux density (PPFD) in the range of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Forty-five days after germination (17 days for emergence of second leaf and 28 days for acclimation of hydroponic condition and growth), uniform individuals were selected for the experiments. After preliminary experiments on the effects of a range of concentration of Cd and SA on root growth had been performed, the following four treatments with four replicates each were established, including control (neither addition of Cd nor SA), 50 μM Cd (CdCl_2), pretreatment of 10 μM SA alone and pretreatment of 10 μM SA followed by 50 μM Cd. Rice roots were exposed to 10 μM SA in nutrient solution for 24 h before Cd treatment. Then, roots from four replicates per treatment were harvested on 2, 4 and 6 days after Cd treatment, and stored at –80 °C immediately after fresh weight determination. The nutrient solution was renewed immediately after sampling. Three independent experiments were performed, one to study root antioxidant enzyme activities, one to determine non-enzymatic antioxidants, and the other to measure root growth (fresh weight) and concentrations of H_2O_2 and MDA. Each experiment was duplicated to ensure that the data obtained were reliable.

2.2. Assays of enzymatic antioxidants in rice roots

Fresh root samples (0.5 g) were ground in lipid N_2 using a mortar and pestle and the ground samples were homogenized on ice bath in 10 mL homogenizing solution containing 50 mM potassium phosphate buffer and 1% (w/v) polyvinylpyrrolidone (pH 7.8), and extracted at 4 °C. The homogenate was centrifuged at 8000 $\times g$ at 4 °C for 15 min. The supernatant was stored at 4 °C for analysis of the SOD, CAT and POD activities and soluble protein concentration.

The SOD activity was measured by the photochemical method described by Giannopolitis and Ries (1977). The reaction mixture (3.0 mL) consisted of 2.95 mL 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM nitroblue tetrazolium, 0.1 mM EDTA, 2 mM riboflavin and 0.05 mL of enzyme extract. The reaction mixture, which was not exposed to light, did not develop color and served as control. One unit SOD activity was defined as the amount of enzyme required for a 50% inhibition of the rate of NBT (p-nitro blue tetrazolium chloride) reduction measured at 560 nm.

The POD activity was assayed following the method of Beffa et al. (1990). Briefly, the assay mixture contained 2.95 mL 100 mM NaH_2PO_4 – Na_2HPO_4 buffer (pH 6.0), 2 mM H_2O_2 , 9 mM guaiacol and 0.05 mL enzyme extract in a total volume of 3.0 mL. Changes in the absorbance of the brown guaiacol at 460 nm in the presence of H_2O_2 were recorded for calculating POD activity.

The CAT activity was assayed using the method described by Aebi (1984). The assay mixture in a total volume of 3.0 mL contained 2.8 mL 50 mM NaH_2PO_4 – Na_2HPO_4 buffer (pH 7.0), 19 mM H_2O_2 and 0.2 mL enzyme extract. The activity was assayed by monitoring the decrease in the absorbance at 240 nm as a consequence of H_2O_2 consumption. Activity was expressed as units (μM of H_2O_2 decomposed per minute) per mg of protein.

Soluble protein concentration was measured following the method described by Bradford (1976) using bovine serum albumin as standard. The assay is based on the stable dye–albumin complex, which can be quantified spectrophotometrically at 590 nm. The protein-dye reagent consisted of 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid.

2.3. Assays of AsA and GSH concentrations in rice roots

The concentration of ascorbic acid (AsA) was measured according to Law et al. (1983). Fresh tissues (0.5 g) were homogenized in 3 mL 5% TCA (trichloroacetic acid) solution. The assay mixture (5.0 mL) contained 1.0 mL 5% TCA, 1.0 mL 100% ethanol, 0.5 mL 0.4% H_3PO_4 –ethanol, 1.0 mL 0.5% phenanthroline–ethanol, 0.5 mL 0.03% FeCl_3 –ethanol and 1.0 mL enzyme extract. The assay was based on the reduction of Fe^{3+} to Fe^{2+} by AsA; Fe^{2+} was quantified spectrophotometrically at 534 nm for 90 min at 30 °C.

Glutathione (GSH) was quantified fluorimetrically according to Hissin and Hilf (1976). Fresh roots (0.5 g) were ground in a mixture of 1 mL of 25% H_3PO_3 and 3 mL of 0.1 M sodium phosphate–EDTA buffer (pH 8.0). The homogenate was centrifuged at $10,000\times g$ for 20 min, and the supernatant was further diluted five folds with sodium phosphate–EDTA buffer (pH 8.0). The final assay mixture (2.0 mL) contained 100 μL of the diluted supernatant, 1.8 mL of phosphate–EDTA buffer and 100 μL of *O*-phthalaldehyde (1 mg mL^{-1}). After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette and the fluorescence at 420 nm was measured after excitation at 350 nm.

2.4. Concentration of NPT in rice roots

The concentration of non-protein thiols (NPT) was determined by measuring the absorbance at 412 nm following the method of Metwally et al. (2003). For this, fresh segments weighing 0.5 g were homogenized in an ice bath in 5 mL of potassium phosphate buffer (pH 8.0), and the homogenate was centrifuged at $10,000\times g$ for 20 min. The supernatant was used for NPT assay using 5,5-dithio-2,2-dinitrobenzoic acid as a reagent.

2.5. Concentration of MDA in rice roots

The TBA (thiobarbituric acid) test was used to measure lipid peroxidation in roots. The method determines the concentration of MDA (malondialdehyde) as an end product of lipid peroxidation. For this, fresh tissues (0.5 g) were homogenized in 3 mL 10% TCA (trichloroacetic acid) solution. The homogenate was centrifuged at $2500\times g$ for 10 min and the supernatant was assayed for MDA concentration using an extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as nmol g^{-1} fresh weight, following the method of Heath and Packer (1968).

2.6. Concentration of H_2O_2 in rice roots

The concentration of H_2O_2 was determined according to Mukherjee and Choudhuri (1983). Briefly, 1.0 g of fresh segments was homogenized in refrigerated acetone (10 mL) and was centrifuged at $10,000\times g$ for 10 min. One milliliter of the supernatant was mixed with 0.1 mL 5% $\text{Ti}(\text{SO}_4)_2$ and 0.2 mL 19% ammonia. After the precipitate was formed, the reaction mixture was centrifuged at $10,000\times g$ for 10 min. The resulting pellet was dissolved in 2 M H_2SO_4 and the absorbance was read at 415 nm. The H_2O_2 level was calculated according to a standard curve of H_2O_2 .

2.7. Statistical analysis

All data in the tables were subject to analysis of variance and expressed as means $\pm$ standard errors of four replicates. Statistical significance of the means was compared by Duncan's New Multiple Range Test at the 5% probability level using SPSS software.

3. Results

Fresh weight of plants in the control treatment increased with time ($r = 0.9995$, $n = 3$, $P < 0.01$), whereas Cd exposure did not increase plant growth linearly with time during the experimental period ($r = 0.7206$, $n = 3$, $P > 0.05$) (Table 1).

Table 1

Fresh weight (means $\pm$ S.E. expressed in g, $n = 4$) of rice roots treated with 50 μM Cd or pretreated with 10 μM SA

Day (d) after treatment	2	4	6
Treatment			
Control	1.59 ± 0.12 a	1.85 ± 0.12 a	2.14 ± 0.15 a
50 μM Cd	1.54 ± 0.08 a	1.62 ± 0.17 a	1.60 ± 0.10 c
10 μM SA	1.57 ± 0.08 a	1.86 ± 0.17 a	2.11 ± 0.22 a
10 μM SA + 50 μM Cd	1.51 ± 0.05 a	1.69 ± 0.03 a	1.85 ± 0.06 b

Means followed by different letters within the same column are significantly different at $P < 0.05$ according to Duncan's New Multiple Range Test at the 5% probability level.

Pretreatment with SA did not affect the root growth compared with the control. However, pretreatment with SA significantly mitigated the growth inhibition by Cd stress on day 6 (by 16%) compared with Cd treatment alone.

Addition of Cd significantly increased H_2O_2 concentration in rice roots, by 2-, 3- and 3.5-fold compared with control on days 2, 4 and 6, respectively (Table 2). SA pretreatment alone also significantly increased H_2O_2 concentrations throughout the experimental period, but the effect (42–100% increase) was smaller than that of Cd stress (Table 2). Under Cd stress, SA pretreatment decreased H_2O_2 concentrations progressively throughout the 6-day experimental period, and the significant difference was observed on day 6.

Table 3 shows the effects of SA pretreatment and Cd exposure on the concentration of MDA in rice roots. MDA levels in Cd-stressed plants were significantly higher than control throughout the experimental period. SA pretreatment significantly reduced MDA concentrations on days 4 and 6 compared with Cd treatment alone (Table 3). There was no significant difference in MDA between the SA pretreatment and control throughout the experimental period (Table 3).

Addition of Cd significantly decreased SOD activity in rice roots on day 2 compared with control (Table 4), followed by a gradual decrease with increasing Cd exposure time. Under Cd stress, SA pretreatment significantly increased SOD activity compared with corresponding Cd alone treatment (by 15.3% on day 2, 20% on day 4, and 23% on day 6, respectively, Table 4). SA pretreatment alone did not affect SOD activity.

POD activity in rice roots was significantly decreased by Cd stress compared with control throughout the experimental period (Table 5). Pretreatment with SA in Cd-stressed roots

Table 2

H_2O_2 concentration (means $\pm$ S.E. expressed in nmol g^{-1} FW, $n = 4$) in rice roots treated with 50 μM Cd or pretreated with 10 μM SA

Day (d) after treatment	2	4	6
Treatment			
Control	223 ± 39 c	201 ± 46 c	234 ± 30 d
50 μM Cd	461 ± 32 a	601 ± 121 a	819 ± 55 a
10 μM SA	322 ± 32 b	402 ± 57 b	333 ± 43 c
10 μM SA + 50 μM Cd	413 ± 54 a	505 ± 45 ab	684 ± 43 b

Means followed by different letters within the same column are significantly different at $P < 0.05$ according to Duncan's New Multiple Range Test at the 5% probability level.

Table 3

MDA concentration (means $\pm$ S.E. expressed in nmol g^{-1} FW, $n = 4$) in rice roots treated with 50 μM Cd or pretreated with 10 μM SA

Day (d) after treatment	2	4	6
Treatment			
Control	29.8 $\pm$ 6.4 c	34.4 $\pm$ 4.5 b	27.5 $\pm$ 2.7 c
50 μM Cd	44.5 $\pm$ 5.1 a	48.2 $\pm$ 7.6 a	46.9 $\pm$ 0.7 a
10 μM SA	34.9 $\pm$ 4.9 bc	39.0 $\pm$ 3.4 b	31.0 $\pm$ 2.4 c
10 μM SA + 50 μM Cd	43.2 $\pm$ 6.4 ab	35.8 $\pm$ 4.0 b	38.1 $\pm$ 4.9 b

Means followed by different letters within the same column are significantly different at $P < 0.05$ according to Duncan's New Multiple Range Test at the 5% probability level.

significantly alleviated the reduction in POD activity on day 2, but this effect diminished on days 4 and 6. Pretreatment of SA alone did not alter POD activity significantly compared with control (Table 5).

Exposure of Cd decreased CAT activity in rice roots by approximately 50% throughout the 6-day experimental period compared with control (Table 6). SA pretreatment alone also decreased CAT activity, and the effect was similar to Cd treatment alone. However, SA pretreatment significantly increased CAT activities in Cd-stressed rice roots compared with that of Cd alone treatment throughout the whole experiment (Table 6).

Effects of Cd and SA on levels of GSH and NPT in rice roots are shown in Table 7. Cd exposure significantly increased the concentration of GSH, by 151% on day 4 and 120% on day 6 compared with control (Table 7). The largest response in the GSH concentration occurred in SA pretreatment followed by Cd exposure; the GSH concentration increased by 2.2–3.6-fold compared with the control.

There were no significant differences in non-protein thiols' (NPT) concentrations among Cd treatment, SA pretreatment and control on day 2 (Table 7). SA pretreatment and Cd alone treatment significantly increased NPT concentration compared with control on days 4 and 6. Cd stress with SA pretreatment produced a larger effect on NPT concentration than Cd alone treatment.

On day 2, Cd stress induced an increase in the AsA concentrations in rice roots (Table 8). AsA concentrations were increased by 161% in Cd alone treatment, and 145% in SA pretreatment under Cd stress on day 4, respectively. Significant differences were observed on day 6 among the four treatments. For example, AsA concentrations increased by 82% in Cd alone

Table 4

Effect of SA on SOD activity (means $\pm$ S.E. expressed in U mg^{-1} protein, $n = 4$) in rice roots with 50 μM Cd or pretreated with 10 μM SA

Day (d) after treatment	2	4	6
Treatment			
Control	104 $\pm$ 8 a	107 $\pm$ 9 a	116 $\pm$ 3 a
50 μM Cd	85 $\pm$ 7 b	70 $\pm$ 9 c	65 $\pm$ 5 c
10 μM SA	98 $\pm$ 6 a	118 $\pm$ 3 a	103 $\pm$ 10 a
10 μM SA + 50 μM Cd	93 $\pm$ 7 a	84 $\pm$ 11 b	80 $\pm$ 9 b

Means followed by different letters within the same column are significantly different at $P < 0.05$ according to Duncan's New Multiple Range Test at the 5% probability level.

Table 5

Effect of SA on POD activity (means $\pm$ S.E. expressed in U mg^{-1} protein, $n = 4$) in rice roots treated with 50 μM Cd or pretreated with 10 μM SA

Day (d) after treatment	2	4	6
Treatment			
Control	9.3 $\pm$ 1.5 a	7.4 $\pm$ 0.3 a	8.9 $\pm$ 0.5 a
50 μM Cd	6.9 $\pm$ 0.6 b	4.6 $\pm$ 0.4 b	4.1 $\pm$ 0.7 b
10 μM SA	10.5 $\pm$ 0.3 a	7.1 $\pm$ 0.5 a	7.0 $\pm$ 1.7 a
10 μM SA + 50 μM Cd	10.2 $\pm$ 2.2 a	5.0 $\pm$ 0.8 b	4.1 $\pm$ 0.1 b

Means followed by different letters within the same column are significantly different at $P < 0.05$ according to Duncan's New Multiple Range Test at the 5% probability level.

treatment, 169% in SA pretreatment and 229% in SA pretreatment under Cd stress, respectively, compared with control.

4. Discussion

4.1. Inhibition of antioxidant enzymes: a possible cause of the Cd-induced oxidative stress

Cd is known to induce oxidative damage to higher plants (Schützendübel and Polle, 2002). In the present study, progressive enhancement of H_2O_2 and MDA concentrations in rice roots with increasing Cd exposure time (Tables 2 and 3) showed that the oxidative stress and the peroxidation of membrane lipids were induced by Cd treatment. Activities of the major antioxidant enzymes (SOD, CAT and POD) in rice roots were significantly decreased by Cd stress during the experimental period (Tables 4–6). Generally, a decrease in SOD activity results in a decrease in H_2O_2 levels, since SOD detoxifies superoxide anion free radicals accompanying the formation of H_2O_2 . However, the levels of ROS and the extent of oxidative damage depend largely upon the whole antioxidant defense system and the co-operation or coordination among the ROS-scavenging enzymes (Liang et al., 2003). It was also reported that SOD activity was decreased along with enhancement of H_2O_2 levels in *Arabidopsis thaliana* exposed to 300 or 500 μM Cd for a longer time (21 days) and such accumulation of the H_2O_2 might rather be a result of lowered activity of CAT responsible for scavenging H_2O_2 (Cho and Seo, 2005). This decreased CAT activity along with the decreased SOD activity might contribute to Cd-induced phytotoxicity (Cho and Seo, 2005). Similar results were reported in *Populus $\times$ canescens* roots exposed to 50 μM Cd for 50 h

Table 6

Effect of SA on CAT activity (means $\pm$ S.E. expressed in U mg^{-1} protein min^{-1} , $n = 4$) in rice roots treated with 50 μM Cd or pretreated with 10 μM SA

Day (d) after treatment	2	4	6
Treatment			
Control	20 $\pm$ 4 a	17 $\pm$ 2 a	20 $\pm$ 5 a
50 μM Cd	9 $\pm$ 1 b	9 $\pm$ 3 b	11 $\pm$ 2 b
10 μM SA	11 $\pm$ 5 b	8 $\pm$ 1 b	7 $\pm$ 1 b
10 μM SA + 50 μM Cd	20 $\pm$ 4 a	21 $\pm$ 4 a	21 $\pm$ 3 a

Means followed by different letters within the same column are significantly different at $P < 0.05$ according to Duncan's New Multiple Range Test at the 5% probability level.

Table 7
GSH and NPT concentrations (means $\pm$ S.E. expressed in ng g^{-1} FW, $n = 4$) in rice roots treated with 50 μM Cd or pretreated with 10 μM SA

Day (d) after treatment	2	4	6
Treatment			
GSH			
Control	6.1 $\pm$ 1.2 c	5.7 $\pm$ 1.3 c	6.0 $\pm$ 1.4 c
50 μM Cd	9.1 $\pm$ 1.9 b	14.3 $\pm$ 2.3 b	12.9 $\pm$ 1.5 b
10 μM SA	7.6 $\pm$ 1.3 bc	5.7 $\pm$ 0.6 c	7.8 $\pm$ 0.7 c
10 μM SA + 50 μM Cd	13.2 $\pm$ 1.5 a	19.4 $\pm$ 2.3 a	21.8 $\pm$ 1.6 a
NPT			
Control	55 $\pm$ 6 b	55 $\pm$ 5 c	54 $\pm$ 9 c
50 μM Cd	62 $\pm$ 3 b	70 $\pm$ 6 b	74 $\pm$ 3 b
10 μM SA	53 $\pm$ 5 b	65 $\pm$ 5 b	74 $\pm$ 3 b
10 μM SA + 50 μM Cd	76 $\pm$ 8 a	124 $\pm$ 4 a	101 $\pm$ 11 a

Means followed by different letters within the same column are significantly different at $P < 0.05$ according to Duncan's New Multiple Range Test at the 5% probability level.

(Schützendübel et al., 2002). It seems to suggest that the decrease in capability of scavenging H_2O_2 enzymes, such as POD and CAT (Tables 5 and 6) might be the reason for the accumulation of H_2O_2 (Table 2).

4.2. Increased non-enzymatic molecular antioxidants under Cd stress

Significant increases in GSH, NPT concentrations were observed in Cd-stressed rice roots (Table 7). NPT are mainly phytochelatins (Metwally et al., 2003), which play an essential role in Cd detoxification. Increase in GSH and NPT in plants might be an important defense response to Cd toxicity, as has been found in Cd-stressed *Phragmites australis* and *Arabidopsis* (Lee et al., 2003; Pietrini et al., 2003). Generally, Cd initially depletes GSH in a matter of hours because of an enhanced demand of GSH for Cd detoxification (Schützendübel et al., 2001). Upon prolonged exposure, GSH level tends to recover, or even exceed the control level (Schützendübel et al., 2001). In the present study, 2-day-Cd-exposure was probably too long to detect this initial change in GSH.

In the present study, increase in AsA under Cd stress (Table 8) concurred with the effect of Cd treatment on *P. australis* (Iannelli et al., 2002) and pine (Schützendübel et al., 2001). The enhancement of AsA levels might help the plant to cope with the Cd-induced oxidative damage. In wheat, increasing

levels of endogenous AsA induced by L-Galactono-1,4-lactone (GalL) increased Cd tolerance (Zhao et al., 2005). Accumulation of AsA under Cd stress might be due to the enhancement of monodehydroascorbate reductase (MDHAR) activity hence accelerating AsA cycling (Schützendübel et al., 2001).

Clearly, the oxidative response of plants to Cd stress is complex with the extent of oxidative stress being dependent upon the whole antioxidant defense system. Cd stress initially depletes GSH and inhibits CAT, APX (Schützendübel et al., 2001), and glutathione reductase (GR). This causes an accumulation of H_2O_2 acting as a signaling molecule in the activation of cellular defenses including CAT and APX (Prasad et al., 1994) and induces the synthesis of ASA and GSH (Schützendübel et al., 2001), which involves APX, GR and MDHAR/DHAR. Unfortunately, the activity of APX, GR and MDHAR/DHAR was not monitored in the present study considering the extent of analysis. Thus, further studies are needed aiming at examining the whole picture of the antioxidant defense system including antioxidant enzymes and non-enzymatic antioxidants in response to Cd exposure.

4.3. SA-elevated enzymatic and non-enzymatic antioxidants contributed to alleviation of Cd toxicity in rice roots

In the present study, pretreatment with SA significantly alleviated growth inhibition by Cd stress (Table 1) and also mitigated declines in activities of antioxidant enzymes (SOD, CAT and POD), and further increased levels of GSH, NPT and AsA in Cd-stressed rice roots (Tables 4–8). The increase in GSH might be ascribed to SA-activation of serine acetyltransferase (SAT), an enzyme closely related to the synthesis of GSH (Freeman et al., 2005). Elevated AsA levels might be due to SA-enhanced activities of dehydroascorbate reductase (DHAR) and MDHAR (Dat et al., 1998). As a result, SA pretreatment increased Cd tolerance by alleviation of growth inhibition (Table 1) and of Cd-induced oxidative stress as evidenced by the decrease in MDA concentrations (Table 3). The results presented here are in disagreement with the report by Metwally et al. (2003), who showed that the SA effect on alleviating Cd toxicity in barley seedlings was not related to the antioxidant defense system since SA treatment strongly or completely suppressed the Cd-induced up-regulation of the antioxidant enzyme activities. This discrepancy might be caused by the different research methods and plant species used. In the experiment with barley (Metwally et al., 2003), seeds were soaked for 6 h in 0.5 mM SA, whereas in the present study, rice seedlings were exposed to 10 μM SA for 24 h before Cd treatment (see Section 2).

4.4. Possible signaling role of SA and SA-induced H_2O_2 in plant defense against Cd-induced oxidative stress

SA is an important signal molecule in plant defense. The mode of SA was proposed to be inhibition of CAT activity and increase in H_2O_2 level during plant–pathogen interactions (Chen et al., 1993). In the present study, pretreatment with SA

Table 8
AsA concentration (means $\pm$ S.E. expressed in mg g^{-1} FW, $n = 4$) in rice roots treated with 50 μM Cd or pretreated with 10 μM SA

Day (d) after treatment	2	4	6
Treatment			
Control	1.39 $\pm$ 0.35 b	0.78 $\pm$ 0.23 b	1.27 $\pm$ 0.20 d
50 μM Cd	1.94 $\pm$ 0.27 a	2.04 $\pm$ 0.22 a	2.32 $\pm$ 0.43 c
10 μM SA	1.46 $\pm$ 0.15 b	1.06 $\pm$ 0.16 b	3.42 $\pm$ 0.28 b
10 μM SA + 50 μM Cd	1.49 $\pm$ 0.08 b	1.91 $\pm$ 0.35 a	4.19 $\pm$ 0.29 a

Means followed by different letters within the same column are significantly different at $P < 0.05$ according to Duncan's New Multiple Range Test at the 5% probability level.

alone specifically inhibited CAT activity (Table 6) and increased H_2O_2 level (Table 2) in rice roots. H_2O_2 accumulation affected by SA has also been observed in other plant species (Chen et al., 1993; Chen et al., 1997; Rao et al., 1997). It has been proposed that SA acts as a one-electron donor that siphons the extremely fast CAT cycle into the relatively slow peroxidative cycle (~ 1000 times slower) (Durner and Klessig, 1996). Besides inhibition of CAT, SA-blocked electron flow from the substrate dehydrogenises to the ubiquinone pool in mitochondria might also be an important mechanism of triggering H_2O_2 generation (Norman et al., 2004).

Interestingly, recent data show that H_2O_2 acts as an essential messenger inducing gene expression in response to environmental stresses (Dat et al., 2000; Mittler et al., 2004; Foyer and Noctor, 2005). In the process of programmed cell death, the specific induction of the genes coding for glutathione S-transferase (GST) and glutathione peroxidases (GPX) in soybean confirmed the signaling properties of H_2O_2 in plants (Levine et al., 1994). Generally, sensing H_2O_2 signaling in plant is mediated by the antioxidant buffers, such as NPT (including GSH) and AsA (Foyer and Noctor, 2005), and the latter in turn plays a key role in defense against various environmental stresses (Foyer and Noctor, 2005). For example, GSH biosynthesis is directly linked to stress defense gene expression in *Arabidopsis*, such as *APX2* (Ball et al., 2004). The *vtc1*, an AsA resistant *Arabidopsis* mutant, is very sensitive to various abiotic stresses (e.g. ozone, freezing and UV-B irradiation), which suggests that low AsA decreased the threshold for sensing stress (Conklin et al., 1996). Thus, in the present study, SA-induced H_2O_2 (Table 2) along with an increase in production of NPT and AsA (Tables 7 and 8) in rice roots might “set up” the rice plant to respond to Cd more effectively. This hypothesis was not only supported by the alleviated growth inhibition (Table 1) and decreased H_2O_2 and MDA concentrations (Tables 2 and 3) in Cd-treated rice plants, but also confirmed by several previous studies on the signaling role of H_2O_2 with respect to plant stress acclimation. Maize seedlings injected with H_2O_2 and menadione (a H_2O_2 -generating compound) became more tolerant against chilling stress (Prasad et al., 1994; Prasad, 1996). Nodal potato explants sub-cultured from H_2O_2 -treated micro-plants were resistant to heat shock for 15 h even after 4 weeks of treatment (Lopez-Delgado et al., 1998). Heat shock response can be completely inhibited by removal of H_2O_2 because the expression of genes such as those encoding *HSF21* and *HSF5* and cytosolic *APX1* is modulated by H_2O_2 signals (Davletova et al., 2005).

Taken together, evidence from this study showed that SA enhanced the enzymatic and non-enzymatic antioxidants in rice roots subject to Cd stress, thus suppressing Cd-induced oxidative damage and enhancing Cd tolerance. SA not only specifically regulated the CAT activity, but also played roles in enhancing AsA, GSH, and NPT levels in plants subject to Cd stress. In addition, SA influenced the other signaling pathways in plant defense, such as H_2O_2 . The current limitation of the complete description of SA signal transduction pathway in plants means that future studies are needed on the dissection of

the complex network of SA and its involvement in plant defense against biotic and abiotic stresses using genetic, genomic and biochemical approaches.

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