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Does salicylic acid regulate antioxidant defense system, cell death, cadmium uptake and partitioning to acquire cadmium tolerance in rice?

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KEYWORDS
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Summary

Salicylic acid (SA) may accelerate the cell death of cadmium-stressed roots to avoid cadmium (Cd) uptake by plants or may play positive roles in protecting the stressed roots from Cd-induced damage. To test these hypotheses, we performed a series of split-root hydroponic experiments with one-half of rice (*Oryza sativa* L. cv. Jiahua 1) roots exposed to 50 μM Cd and the other half not exposed. The objectives were to elucidate the effects of SA pretreatment on the time-dependent changes of H_2O_2 levels in roots, antioxidant defense system in different organs, root cell death and the dynamic distribution of Cd in the plants. In the split-root system, a higher Cd uptake rate was observed in the Cd-stressed portions of roots compared with the treatment with the whole roots exposed to Cd. Furthermore, an appreciable amount of Cd was translocated from the Cd-exposed roots to the unexposed roots and trace amounts of Cd were released into the external solution. The split-root method also caused the two root portions to respond differently to Cd stress. The activities of major antioxidant enzymes (superoxide dismutase, SOD; peroxidase, POD; and catalase, CAT) were significantly suppressed in the Cd-treated roots, hence leading to H_2O_2 burst, lipid peroxidation, cell death and growth inhibition. By contrast, in

Abbreviations: CAT, catalase; GSH, glutathione; MDA, malondialdehyde; NPT, non-protein thiols; POD, peroxidase; SA, salicylic acid; SOD, superoxide dismutase.

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the non-Cd-treated roots, the activities of enzymes (SOD, CAT, and POD) and root growth were persistently stimulated during the experimental period. The H_2O_2 accumulation and lipid peroxidation were also induced in the non-Cd-treated roots, but they were significantly lower than those of the Cd-treated roots. The concentrations of glutathione (GSH) and non-protein thiols (NPT) in the Cd-treated roots were significantly higher than those of the untreated roots. SA pretreatment elevated enzymatic and non-enzymatic antioxidants, and the concentrations of GSH and NPT in roots and shoots, hence leading to alleviation of the oxidative damage as indicated by the lowered H_2O_2 and MDA levels. Furthermore, SA pretreatment mitigated the Cd-induced growth inhibition in both roots and shoots and increased transpiration compared with non-SA-pretreatment under Cd exposure. It is concluded that Cd can be partly transferred from the Cd-exposed roots to Cd-unexposed roots and that cell death can be accelerated in the Cd-stressed roots in response to Cd stress. The SA-enhanced Cd tolerance in rice can be attributed to SA-elevated enzymatic and non-enzymatic antioxidants and NPT, and to SA-regulated Cd uptake, transport and distribution in plant organs.

Introduction

Cadmium (Cd) is a ubiquitous element in the environment and is highly toxic to living organisms. In plants, Cd toxicity has been found to interfere with electron transport chains or block antioxidant enzymes structures, leading to accumulation of H_2O_2, and oxidative damage (e.g. lipid peroxidation), membrane leakage and finally cell death (Schützendübel et al., 2001; Schützendübel and Polle, 2002).

The H_2O_2-triggered cell death has been well-recognized and is an essential process to maintain tissue or organ homeostasis in concert with cell proliferation, growth, and differentiation (Greenberg, 1996; Mittler et al., 2004). Furthermore, under unfavorable conditions, cell death allows the plant to defend against biotic stress or to obtain more resources by reducing or even stopping growth of plant tissues. For example, H_2O_2-induced cell death has been best described during incompatible plant–pathogen interactions that form the basis for the hypersensitive response (HR) (Durner et al., 1997), and aerenchyma formation in root cortex for the toleration of low-oxygen soil environments (Drew et al., 2000). Studies with *Nicotiana tabacum* (TBY-2) (Fojtová and Kovařík, 2000) and Scots pine (*Pinus sylvestris*) (Schützendübel et al., 2001) have shown that Cd induced the morphology of cell death which was related to the H_2O_2 burst. However, to our knowledge, there have been no reports to show whether cell death in root tissues can build up a physical barrier to inhibit Cd uptake, and consequently benefit the whole plant through the avoidance of Cd toxicity as the mode of plant–pathogen interactions.

Salicylic acid (SA) acts as an important signaling element in plants, which has broad but divergent effects on damage development or stress acclimation of plants (Durner et al., 1997). Upon pathogen attack, SA accumulates to high levels at the site of pathogenic infection, binds and inhibits tobacco CAT activity *in vitro* and *in vivo*, thereby leading to an increase in the endogenous level of H_2O_2, which could then serve as a second messenger to induce cell death to create a physical barrier against pathogens. However, it is also reported that SA plays a key role in promoting plant resistance to various abiotic stresses. It has previously been reported that SA alleviated growth inhibition by Cd toxicity in barley (*Hordeum vulgare*) and soybean (*Glycine max*) (Metwally et al., 2003; Drazic and Mihailovic, 2005) and in rice (Guo et al., 2007a), although the underlying mechanism is not fully understood. Our more recent studies have shown that pretreatment of rice seeds with SA enhanced the antioxidant defense activities in Cd-stressed rice, thus alleviating Cd-induced oxidative damage and enhancing Cd tolerance. The possible mechanism involved was thought to be related to SA-induced H_2O_2 signaling in mediating Cd tolerance (Guo et al., 2007a). Thus, it is interesting to elucidate whether (1) SA has negative roles in accelerating partial root death to avoid Cd uptake, in an analog to the mode of action of SA-enhanced plant defense against pathogens through H_2O_2 bursts and consequent cell death, or (2) SA plays positive roles in protecting roots from damage in response to Cd stress. Therefore, we conducted a series of hydroponic experiments using a split-root system to investigate the time-dependent changes of H_2O_2 levels in roots, antioxidant defense

system in different organs and root cell death under Cd stress and their relationships with the dynamic distribution of Cd following pretreatment of SA.

Materials and methods

Plant materials and experimental design

Seeds of rice (*Oryza stativa* L. 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, 6 plants per pot) containing nutrient solution (full strength composition: 5 mM NH_4NO_3, 2 mM K_2SO_4, 4 mM $CaCl_2$, 1.6 mM $MgSO_4$, 1.2 mM KH_2PO_4, 50 μM Fe(II)-EDTA, 10 μM H_3BO_4, 1 μM $ZnSO_4$, 1 μM $CuSO_4$, 5 μM $MnSO_4$, 0.5 μM Na_2MoO_4, and 0.19 μM $CoSO_4$). The nutrient solution was adjusted to pH 5.5 daily using 0.1 M HCl or NaOH, and renewed twice a week. Forty days after the pre-culture, uniform individuals were selected and transferred to split-root pots (500 mL, PVC, 1 plant per pot), with a plastic divider embedded in the center. Approximately equal portions of roots were placed in each compartment. The experiment was conducted in a growth chamber with 14-h-photoperiod (8:00–22:00) daily, a photosynthetic photon flux density of 350 μmol m^{-2} s^{-1}, 25 °C/18 °C day/night temperatures, and 70% relative humidity.

After rice plants had been acclimated in the split-root system for 15 d, four treatments with four replicates each were established (Table 1): i.e. (1) neither SA nor Cd (CK), (2) both halves exposed to 50 μM Cd (W+Cd), (3) half exposed to Cd (+Cd) and the other half not exposed (−Cd), and (4) pretreatment of all roots with 10 μM SA for 72 h followed by exposing half of the roots to Cd (SA+Cd) and the other half not (SA−Cd). Cd was added as cadmium chloride ($CdCl_2$). Rice plants from the four replicates per treatment were harvested 0, 2, 5 or 10 d after Cd treatment. Two separate experiments were performed, one to measure biomass and Cd concentration, and the other to determine antioxidant enzyme activity and non-enzymatic antioxidants, and cell death. The experiments were duplicated to ensure that the data obtained were reliable.

Table 1. Details of each treatment in the split-root system

Treatment name	CK		W+Cd		±Cd		SA±Cd	
	R_L*	R_R	R_L	R_R	+Cd	−Cd	SA+Cd	SA−Cd
Cd treatment	−†	−	+	+	+	−	+	−
SA treatment	−	−	−	−	−	−	+	+

*R_L or R_R indicates root part in left or right compartment in the split root system, respectively.
†−, no Cd or SA treatment and +, Cd or SA treatment.

Determination of Cd contents in plant organs and nutrient solutions

After rice plants were sampled, 30 mL nutrient solution in each root compartment of four treatments was immediately taken out. The roots were immersed in a 5 mM $CdCl_2$ solution for 5 min to remove the apoplastic Cd, then washed thoroughly with deionized water and dried in an oven at 80 °C to a constant weight. Plant samples were weighted and ground to pass 0.5 mm and then digested with a mixture of HNO_3 and $HClO_4$ (10:1, v/v) at 160 °C for 30 h. The contents of Cd in nutrient solutions and digestions were determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (OPTIMA 2000, Perkin-Elmer, USA).

Assays of enzymatic antioxidants in rice roots

Fresh root samples (0.5 g) were ground in liquid N_2 and homogenized in an ice-bath in 10 mL homogenizing solution containing 50 mM potassium phosphate buffer and 1% (w/v) polyvinylpyrrolidone (pH 7.8). The homogenate was centrifuged at 8000 × *g* at 4 °C for 15 min. The supernatant was stored at 4 °C for the 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). One unit SOD activity was defined as the amount of enzyme required to result in a 50% inhibition of the rate of NBT (ρ-nitro blue tetrazolium chloride) reduction measured at 560 nm. The POD activity was assayed following the method of Beffa et al. (1990). 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 activity was assayed by monitoring the decrease in the absorbance at 240 nm as a consequence of H_2O_2 consumption. The enzyme activity was expressed as units (μM of H_2O_2 decomposed per minute) per mg of protein. Soluble protein concentration was analyzed according to Bradford (1976), using Coomassie Brilliant Blue G-250 (Sigma) as dye and albumin (Bovine V; Sigma) as a standard.

Assays of GSH concentrations in rice roots and shoots

Total glutathione (GSH) was determined according to Hissin and Hilf (1976). Fresh samples were ground with a homogenizing solution containing 25% H_3PO_3 and 0.1 M sodium phosphate–EDTA buffer (pH 8.0). The homogenate was centrifuged at 10,000 × *g* for 20 min. The final assay mixture contained supernatant, phosphate-EDTA buffer and *O*-phthalaldehyde (1 mg mL^{-1}). The solution was measured fluorimetrically at 420 nm after excitation at 350 nm.

Assay of concentration of NPT in rice roots and shoots

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, 0.5 g of fresh root segments were homogenized in an ice-bath in 5 mL of potassium phosphate buffer (pH 8.0), and the homogenate was centrifuged at 10,000 × *g* for 20 min. The supernatant was used for NPT assay using 5,5-dithio-2,2-dinitrobenzoic acid as a reagent.

Assay of concentration of MDA in rice roots

Root tissues (500 mg) were homogenized in 3 mL 0.1% trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 2500 × *g* for 10 min and the supernatant was assayed for malondialdehyde (MDA) concentration with thiobarbituic acid (TBA) test using the method given by Heath and Packer (1968).

Assay of concentration of H_2O_2 in rice roots

The concentration of H_2O_2 was determined using the method of Brennan and Frenkel (1977). Fresh roots (2 g) were homogenized in refrigerated acetone (10 mL) and centrifuged at 10,000 × *g* for 10 min. One milliliter of the supernatant was mixed with 0.1 mL 5% $Ti(SO_4)_2$ and 0.2 mL ammonia. After the precipitate was formed, the reaction mixture was centrifuged at 10,000 × *g* for 10 min. The resulting pellet was dissolved in 2 M H_2SO_4 and the absorbance was measured at 415 nm. The H_2O_2 level was calculated according to a standard curve of H_2O_2.

Measurement of cell death

Cell death was measured according to the method described by Schützendübel et al. (2001). After Cd or SA treatment, three root tips (2 cm) were incubated in Evans blue solution (0.025% (w/v) Evans blue in water) for 30 min, followed by washing with water for 15 min. The trapped Evans blue was released from the roots by homogenizing root tips with a microhomogenizer in 1.6 mL of a measuring solution (50% (v/v) MeOH and 1% (w/v) SDS). The homogenate was incubated for 15 min in a water bath at 50 °C and centrifuged at 10,000 × *g* for 15 min. The optical density of the supernatant was determined at 600 nm and expressed on the basis of fresh weight.

Measurement of transpiration

Whole-shoot transpiration in different treatments was calculated by measuring the heights of water levels at the beginning and the end of the experiments. The volumes of water loss were calculated by using the heights of water levels and the bottom area of the cylinder pots. In all cases, evaporation from the pots was minimized by covering the exposed surface with a layer of PVC.

Statistical analysis

SPSS software (SPSS Inc., 2000) was used for data analysis. All data were expressed as means ± S.D. and the means were subjected to test of statistical significance using Duncan's method at the 5% probability level.

Results

Effects of SA and Cd on plant growth and Cd distribution in rice in the split-root system

On Day 2, plant biomass did not differ among treatments (Figure 1). On Day 5, Cd addition significantly decreased the root dry weight in the +Cd compartment, while pretreatment with SA produced no effect ($P < 0.05$) (Figure 1). On Day 10, root dry weight of the +Cd compartment was 35.3% less than that of the W+Cd compartment (Figure 1). By contrast, root dry weight of the −Cd compartment was 25.0% higher than the control. SA pretreatment with Cd exposure (SA+Cd) significantly increased root dry weight by 36.4% compared to the +Cd compartment alone. Shoot growth was decreased in the W+Cd and −Cd treatments on Day 5 and Day 10. SA treatment significantly alleviated the inhibitory effect of Cd on shoot growth compared with the Cd treatment alone (Figure 1).

Cd concentration in roots of the +Cd compartment was significantly higher than that of the W+Cd compartment on Day 5 and Day 10, whereas the reverse was true for shoots on Day 10 (Figure 1). The time-dependent increase in Cd was also found in the −Cd compartment, although this portion of the roots was not exposed to Cd directly. For example, Cd concentration in roots of the −Cd compartment was 1.5-fold higher on Day 10 than on Day 2 (Figure 1). By contrast, no time-dependent change in Cd concentration was observed in the +Cd compartment. The Cd concentration was significantly higher in the SA−Cd compartment than in the −Cd compartment. For example, Cd concentration in the SA−Cd compartment increased by 25% and 28% on Day 5 and Day 10, respectively, compared with the −Cd compartment (Figure 1). Specific Cd uptake expressed as total amounts of Cd in whole plant on a basis of dry weights of roots exposed to Cd in the +Cd and SA+Cd compartments was about two-fold higher than in the W+Cd compartment from Day 5 (Figure 2), showing higher Cd uptake by roots in the +Cd compartment than in the W+Cd compartment.

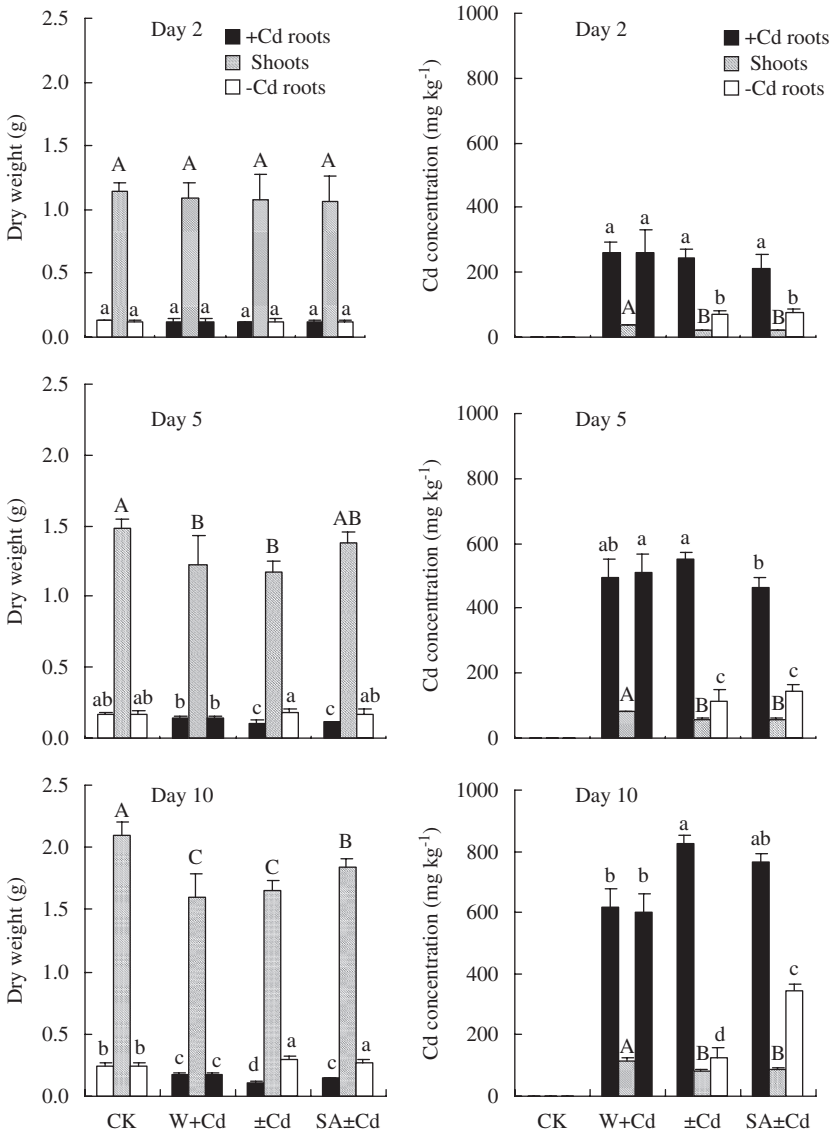

Figure 1. Shoot and root dry weight (g) and Cd concentration of rice (expressed on a dry weight basis) pretreated with 10 μM SA or exposed to 50 μM Cd in a split-root system. Means of $n = 4 \pm$S.D. Different letters above bars mean significance of differences in shoots (capitals) and roots (lower case) between any two treatments ($P < 0.05$, ANOVA; according to Duncan's multiple range test). CK: neither SA pretreatment nor Cd treatment; W+Cd: both portions of the root system exposed to 50 μM cadmium; ±Cd: half of the roots exposed to cadmium (+Cd) and the other half not exposed (−Cd); SA±Cd: half of the roots exposed to cadmium (SA+Cd) and the other half not exposed (SA−Cd) following pretreatment of all roots with 10 μM SA for 72 h.

Water loss from the nutrient solutions, used as a measure of transpiration, is shown in Figure 3A. Depletion of Cd in the nutrient solution was more pronounced in the +Cd and SA+Cd compartments than in the W+Cd compartment, showing higher Cd uptake by roots in the +Cd compartment than in the W+Cd compartment (Figure 3B). The fact that Cd was detectable in the solutions of the −Cd and SA−Cd compartments (Figure 3B) showed that Cd was released into the solutions from the roots grown in the non-Cd-amended compartments.

Effect of SA and Cd on H_2O_2 levels and antioxidant system of rice root in the split-root system

Cd exposure increased H_2O_2 levels in roots from Day 2 to Day 5, and H_2O_2 tended to decline on Day 10 in all +Cd treatments (Figure 4A). The addition

of Cd to the +Cd compartment induced the highest H_2O_2 concentration in the Cd-exposed roots during the experimental period. The increments in H_2O_2 levels were also observed in the roots grown in the −Cd compartment, but were significantly lower than those in the +Cd compartment. Pretreatment with SA prior to Cd addition increased H_2O_2 concentrations in roots compared with the control. However, after Cd exposure, SA pretreatment decreased the H_2O_2 accumulation in the roots grown in the SA+Cd compartment compared with the +Cd compartment.

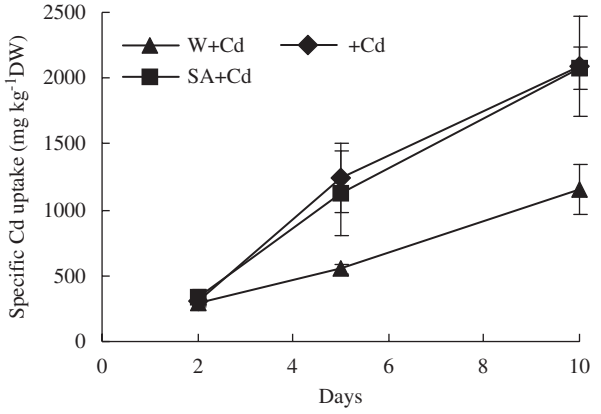

Figure 2. Effect of SA and split-root mode on specific Cd uptake by rice plants in hydroponic solutions. Specific Cd uptake was calculated as total amounts of Cd in whole rice divided by dry weights of roots exposed to Cd. Data are expressed as means ± S.D. ($n = 4$). W+Cd: both portions of the root system exposed to 50 μM cadmium; +Cd: half of the roots exposed to cadmium; SA+Cd: half of the roots exposed to cadmium following pretreatment of all roots with 10 μM SA for 72 h.

Cd significantly increased the MDA levels in roots compared with the control in the order of +Cd > W+Cd > SA+Cd compartment during the experimental period (Figure 4B). Although the increments in MDA concentration in the roots grown in the −Cd and SA−Cd compartment were also found during the Cd-exposure period, they were all significantly lower compared with the +Cd compartment.

Cd initially increased SOD and POD activities in roots grown in the W+Cd, +Cd and SA+Cd compartments on Day 2 (Figure 5A and B). However, this response diminished on Day 5, and was significantly lower than those in the control on Day 10, except for POD in the SA+Cd compartment. In the −Cd and SA−Cd compartments, root SOD activity increased initially but fell at the later stage, whereas POD activity increased consistently during the whole experimental duration.

Cd exposure decreased CAT activities of roots grown in the W+Cd and +Cd compartments during the experimental period (Figure 5C). Pretreatment with SA initially decreased the activity by 47% prior to Cd treatment. Exposure to Cd increased CAT activity by 61% in the SA+Cd on Day 2, compared with the control. The activity of CAT in the roots grown in the SA+Cd compartment declined rapidly and dropped below the level of control on Day 10. In the −Cd and SA−Cd compartments, the enzyme activity was always higher than the control throughout the Cd-exposure period.

SA pretreatment initially increased GSH concentration in roots by 57% prior to Cd treatment (Figure 6A). By contrast, Cd exposure strongly decreased GSH concentration on Day 2, compared with the control (Figure 6A). A recovery of GSH level occurred in the W+Cd, +Cd and SA+Cd

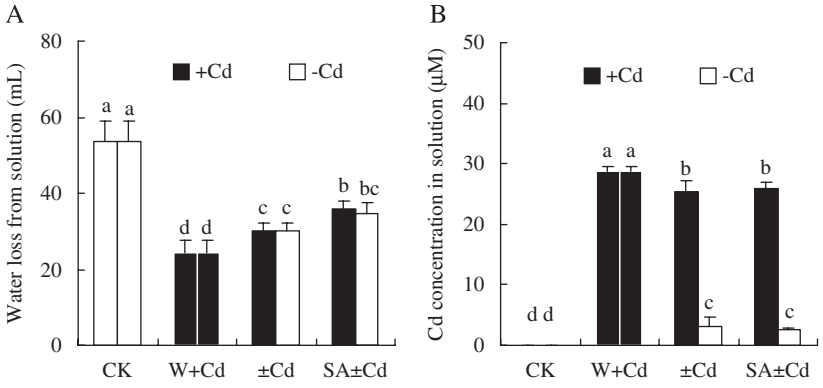

Figure 3. Transpiration measured as water loss in solution (A) and Cd concentration (B) in the two compartments after 48-h-exposure of 50 μM Cd in different treatments. Data are expressed as means ± S.D. ($n = 4$). Different letters above the bars mean significance of difference between any two treatments ($P < 0.05$, ANOVA; according to Duncan's multiple range test). CK: neither SA pretreatment nor Cd treatment in the split-root system; W+Cd: both portions of root system exposed to 50 μM cadmium; ±Cd: half of the roots exposed to cadmium (+Cd) and the other half not exposed (−Cd); SA±Cd: half of the roots exposed to cadmium (SA+Cd) and the other half not exposed (SA−Cd) following pretreatment of all roots with 10 μM SA for 72 h.

compartments on Day 5. However, GSH concentration of roots grown in the +Cd compartment dropped again to 25% lower than the control on Day 10. In shoots, time-dependent increases in GSH concentration were found in all the Cd treatments regardless of SA pretreatment throughout the experimental period (Figure 6B). The addition of Cd consistently increased NPT levels in roots in all treatments except the control (Figure 6C). NPT level in roots was initially stimulated by the SA pretreatment, and then further enhanced by the subsequent Cd exposure, which was 1.9- and 2.1-fold higher on Day 5 and Day 10, respectively, compared with the control. NPT concentration was highest in the shoots grown in the SA$\pm$Cd treatment, followed by the W+Cd and $\pm$Cd treatments throughout the experimental period (Figure 6D).

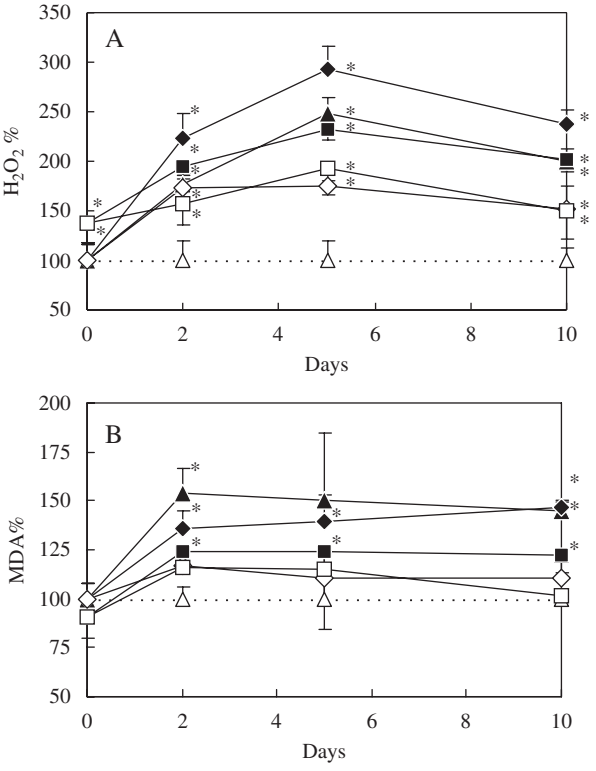

Figure 4. H_2O_2 (A) and MDA (B) concentrations in roots pretreated with 10 μM SA or treated with 50 μM Cd. The concentrations were expressed relative to the concentration in control plants (= 100%, dashed line). Each value is the mean of four individual replicates ($\pm$S.D.). Asterisks indicate values that differ significantly from the control at $P<0.05$ (ANOVA; according to Duncan's multiple range test). Root portions harvested were: neither SA pretreatment nor Cd treatment in the split-root system (Δ, CK); both portions exposed to 50 μM cadmium (▲, W+Cd); half of the roots exposed to cadmium (◆, +Cd) and the other half not exposed (◇, −Cd); half of the roots exposed to cadmium (■, SA+Cd) and the other half not exposed (□, SA−Cd) following pretreatment of all roots with 10 μM SA for 72 h.

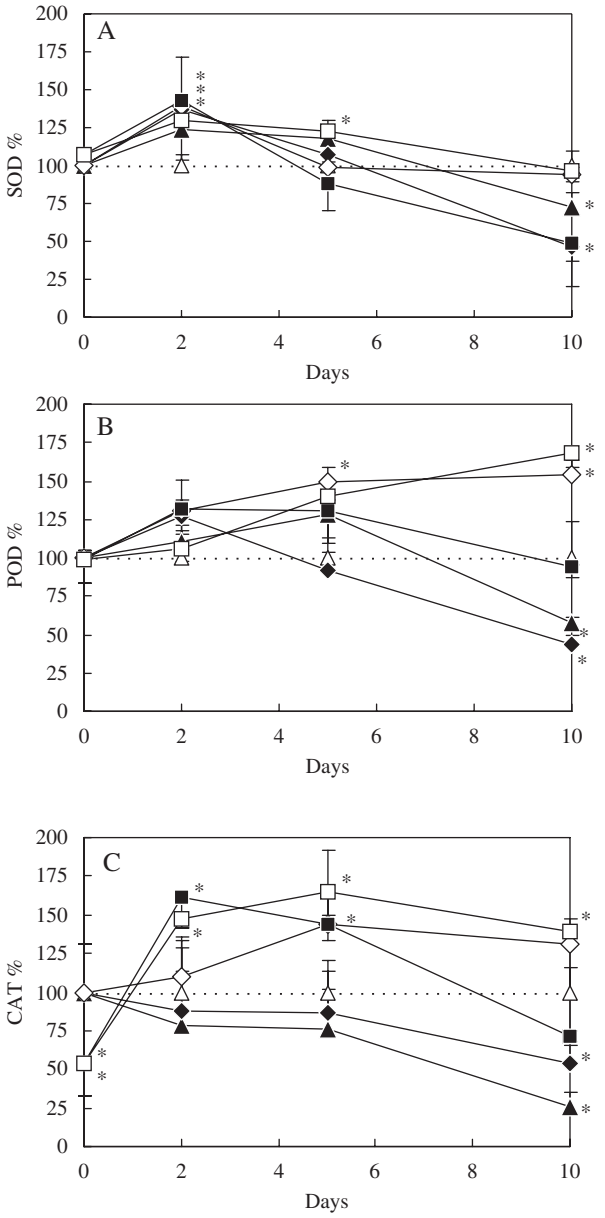

Figure 5. SOD (A), POD (B) and CAT (C) activities in roots pretreated with 10 μM SA or treated with 50 μM Cd. The activities were expressed relative to the activities in control plants (= 100%, dashed line). Each value is the mean of four individual replicates ($\pm$S.D.). Asterisks indicate values that differ significantly from the control at $P<0.05$ (ANOVA; according to Duncan's multiple range test). Root portions harvested were: neither SA pretreatment nor Cd treatment in the split-root system (Δ, CK); both portions of the roots exposed to 50 μM cadmium (▲, W+Cd); half of the roots exposed to cadmium (◆, +Cd) and the other half not exposed (◇, −Cd); half of the roots exposed to cadmium (■, SA+Cd) and the other half not exposed (□, SA−Cd) following pretreatment of all roots with 10 μM SA for 72 h.

Effect of SA and Cd on cell death of roots in the split-root system

Cell death in root parts was induced upon Cd exposure during the experimental period (Figure 7A and B). The percentage of uptake of Evans dye reached a plateau after 2 d. There were no significant differences in cell death within 2, 5 and 10 d for +Cd and SA+Cd treatments. Cell death caused by Cd was more pronounced in the +Cd and SA+Cd compartments compared with the W+Cd compartment on Day 2 ($P<0.05$). SA pretreatment had no significant effect on cell death under partial Cd stress.

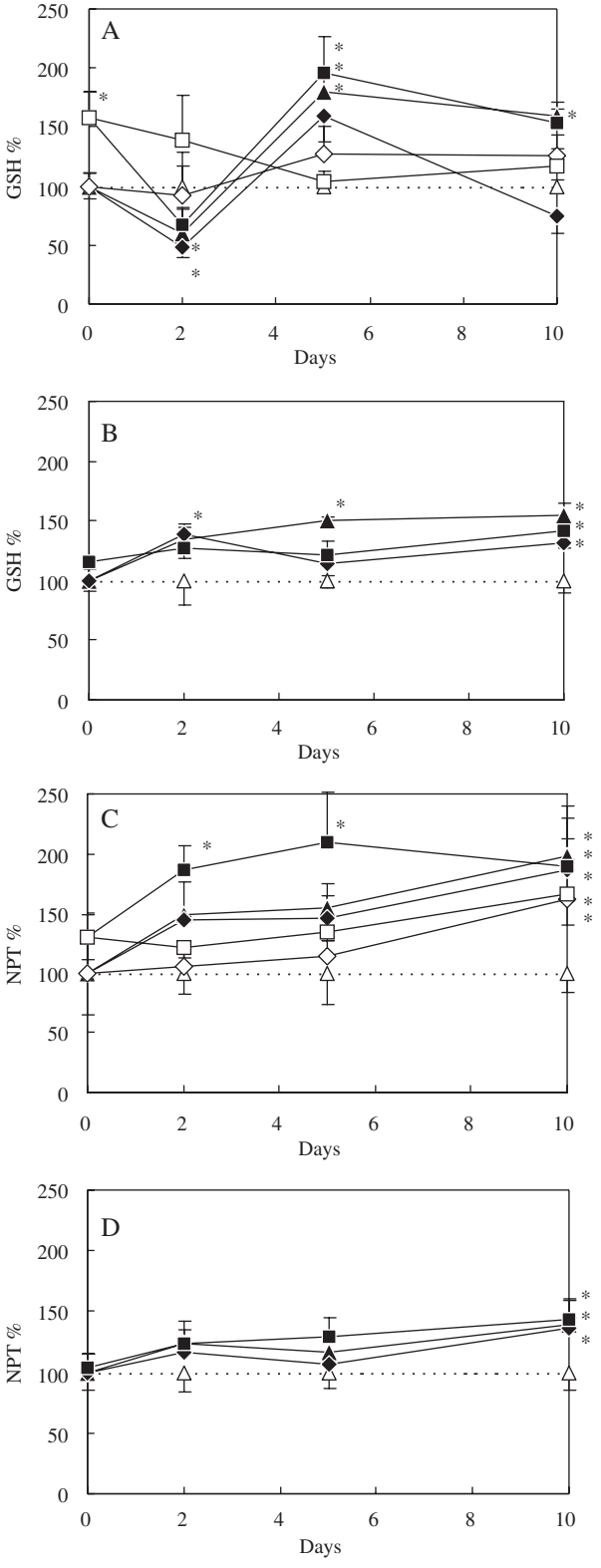

Figure 6. GSH (A and B) and NPT (C and D) concentrations in roots and shoots pretreated with 10 μM SA or treated with 50 μM Cd. The concentrations were expressed relative to the concentration in control plants (= 100%, dashed line). Each value is the mean of four individual replicates (±S.D.). Asterisks indicate values that differ significantly from the control at $P<0.05$ (ANOVA; according to Duncan's multiple range test). Root portions harvested were: neither SA pretreatment nor Cd treatment in the split-root system (Δ, CK); both portions of the roots exposed to 50 μM cadmium (▲, W+Cd); half of the roots exposed to cadmium (◆, +Cd) and the other half not exposed (◇, −Cd); half of the roots exposed to cadmium (■, SA+Cd) and the other half not exposed (□, SA−Cd) following pretreatment of all roots with 10 μM SA for 72 h.

Discussion

Cell death, plant growth and Cd transference in the split-root system subjected to Cd stress

More growth inhibition (Figure 1) and root cell death (Figure 7B) were observed in the +Cd compartment than in the W+Cd compartment under the same Cd stress (50 μM) in the split-root system ($P<0.05$). This suggests that there may be an adaptation mechanism by which the plant roots grown in the Cd-stressed compartment were self-sacrificed, hence protecting the whole rice plant from avoiding excessive Cd uptake and toxicity. Surprisingly, Cd concentration (Figure 1), Cd specific uptake (Figure 2) and Cd depletion in the nutrient solution (Figure 3A) were higher in the +Cd compartment than in the W+Cd compartment ($P<0.05$).

Cd-induced cell death with rigid lignification in root cell wall has been reported in Scots pine (Schützendübel et al., 2001) and rice (Guo et al., 2007a). Immobilization of Cd in cell wall is one of important mechanisms of Cd resistance in plants, as our previous study with rice roots showed that Cd concentration in cell wall was 5.4-fold higher than

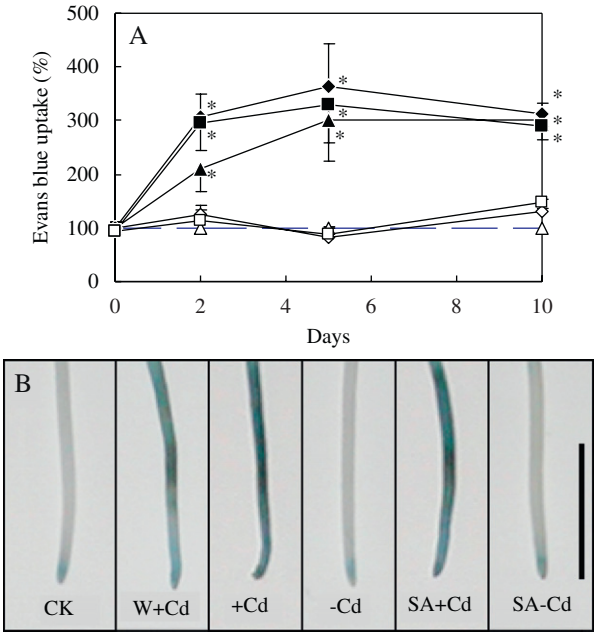

Figure 7. Cell death indicated as Evans blue staining of rice root tips (A) and the picture taken on Day 5 (B); bar = 1 cm. Sections (1.5 cm) of rice roots were subjected to Evans blue solution (0.025% (w/v) Evans blue in water) to detect cell death (visualized as blue colour). Asterisks indicate values that differ significantly from the control at $P<0.05$ (ANOVA; according to Duncan's multiple range test). All root sections received identical treatment before photography, and all are represented at identical magnification. Root portions harvested were: neither SA pretreatment nor Cd treatment in the split-root system (Δ, CK); both portions of roots exposed to 50 μM cadmium (▲, W+Cd); half of the roots exposed to cadmium (◆, +Cd) and the other half not exposed (◇, −Cd); half of the roots exposed to cadmium (■, SA+Cd) and the other half not exposed (□, SA−Cd) following pretreatment of all roots with 10 μM SA for 72 h. Each value is the mean of three individual replicates (±S.D.) and was expressed relative to controls.

in the soluble fraction of cells (Guo et al., 2007b). In the present study, more root cell death in +Cd compartment (Figure. 7) might be due to higher Cd retention in this root part than in the W+Cd compartment (see Figure 1, Day 5 and Day 10). However, in fact, the Cd-immobilization in rice root may be a defense mechanism that plays a limiting role in Cd tolerance. As the Cd stress experiment continued, more Cd entered into the rice roots and was further transferred into the shoots due to its "saturation effect" (Figure 1). A further question then arises as to why Cd specific uptake (Figure 2) and Cd depletion in the nutrient solution (Figure 3A) were higher in the +Cd compartment than in the W+Cd compartment ($P<0.05$). A previous study with Indian mustard showed that Cd accumulation in shoots was strongly influenced by transpiration (Salt et al., 1995). Generally, transpiration is the most important mechanism for the uptake of nutrient elements (Novák and Vidovič, 2003; Tani and Barrington, 2005; Liao et al., 2006) by its gradient of water potential, which can drive them to move into roots and upward to shoots. This mechanism could be applicable to the Cd uptake pattern in the present study where more water loss from solution (Figure 3A) along with lower Cd concentration in the solution (Figure 3B) was observed in the +Cd compartment than in the W+Cd compartment during the 36-h-Cd exposure period. Since Cd has been shown to strongly inhibit transpiration of plants (Perfus-Barbeoch et al., 2002), the higher transpiration in the +Cd treatment (Figure 3A) might be accounted for by less Cd accumulation in the shoots (Figure 1) compared with the W+Cd compartment.

An important result of the present study was that considerable amounts of Cd were re-translocated downward from shoots to the Cd-unexposed roots via phloem (Figure 1), which was also previously reported in wheat (Welch et al., 1999; Page and Feller, 2005). There are two possible mechanisms for this redistribution: (1) Cd is loaded from the xylem sap into phloem sap during the acropetal Cd transportation and (2) Cd stored in shoots is re-translocated into roots together with photosynthate, as with some micronutrients (such as Cu and Zn) (Marschner, 1995). Studies with Indian mustard suggested that Cd transport in the xylem sap was coordinated predominantly with oxygen or nitrogen ligands (Salt et al., 1995). The phloem sap contains various types of ligands, such as metal-binding proteins, nicotianamine and citrate, facilitating the transport of micronutrient cations from source to sink organs (Welch 1995). However, to our knowledge, the process of Cd loading from xylem sap into phloem sap and the components with Cd in phloem have not yet been determined and need further investigating.

Interestingly, some Cd in plants was released from the roots grown in the −Cd compartment into the external solution (Figure 3B). One possible explanation for the Cd secretion might be ascribed to the exudation by the cells themselves as has been reported in *Lupinus albus* (Costa and Morel, 1983). There was some evidence that such exudation in *Thlaspi caerulescens* was mediated by *TcHMA4*, a *Thlaspi* heavy metal ATPase, which is involved in active efflux of a number of heavy metals (Papoyan and Kochian, 2004). Another hypothesis about Cd release is that the Cd is

derived from the decomposed root epidermis of plants, as some evidence has shown that Cd was principally stored in epidermal cells (Chardonnens et al., 1999; Cosio et al., 2005).

The root growth in the −Cd compartment was significantly stimulated (Figure 1). The mechanism of stimulating plant growth by low, sub-lethal concentrations of heavy metals has not been well-elucidated (Schützendübel et al., 2002). It is hypothesized that the H_2O_2 accumulation in the roots grown in the −Cd compartment might act as a signal in regulating root growth (Figure 1A) by involving cellular redox control and by mediating direct cross-linking of functional groups in the cell walls (Brisson et al., 1994; Foreman et al., 2003).

Different organs in rice plants responded to Cd-induced oxidative stress differently in the split-root system

Cadmium is known to induce oxidative damage to higher plants (Schützendübel and Polle, 2002). In the present study, the split-root method caused the roots to respond to Cd stress differently in terms of H_2O_2 accumulation (Figure 4A), lipid-peroxidation (Figure 4B) and cell death (Figure 7A and B) between the plants with or without Cd treatment. In the +Cd treatments, the initially increased activities of SOD and POD (Figure 5A and B) cooperatively controlled the Cd-induced H_2O_2 at high homeostatic levels (Figure 4A) during the 2-day-Cd-exposure. However, sustained Cd stress resulted in decreased activities of antioxidant enzymes (Figure 5A, B and C), possibly due to the binding of Cd to the thiol groups of enzyme proteins. Thus, the high homeostatic balance of H_2O_2 was broken eventually, causing a H_2O_2 burst on about Day 5 (Figure 4A). The lower H_2O_2 levels at the later stage might be due to more cell death of these roots. However, H_2O_2 levels in the −Cd treatments also increased during the experimental period but much less than those in the +Cd treatments (Figure 4A), which might be induced by the Cd translocated from the shoots via phloem (Figure 1). Persistent enhancement of activities of SOD, POD and CAT showed that these root portions maintained the antioxidant defense activity to control Cd-induced H_2O_2 level during the experimental period.

Both GSH and NPT are important antioxidant molecules for Cd detoxification by forming Cd-bindings with their high affinity for SH groups (Lee et al., 2003; Pietrini et al., 2003). Cd initially depleted root GSH levels in the +Cd compartment for the first 2 d (Figure 6A), which is a common response to Cd stress in plants as a result of the synthesis of phytochelatins (Schützendübel and Polle, 2002). As the experiment continued, the GSH levels in the +Cd compartment recovered (Figure 6A), while in the −Cd treatments, GSH maintained higher levels and exceeded the control levels throughout the experiment. NPT was higher in the +Cd compartment than in the −Cd compartment with increasing Cd-exposure time (Figure 6C and D).

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

Pretreatment with SA significantly alleviated the growth inhibition (Figure 1) and transpiration suppression (Figure 3A) caused by Cd stress. Such SA-mitigated Cd toxicity has been shown in other plant species, including barley (Metwally et al., 2003), soybean (Drazic and Mihailovic, 2005) and rice (Guo et al., 2007a), although the underlying mechanism is not fully understood. SA acts as an important signal involved in strengthening cell walls during the process of cell death in plant–pathogen interactions (Durner et al., 1997). However, the increase in Cd influx in the present study (Figure 1) ruled out the mechanism of immobilization of Cd by SA-induced reinforcement of cell wall. Furthermore, SA pretreatment did not further induce cell death of Cd-stressed roots (Figure 7).

In the present study, the effect of increase in transpiration by SA pretreatment under Cd stress is consistent with previous studies on SA-induced reversal of inhibited transpiration rate in zucchini yellow mosaic virus-infected leaves (Radwan et al., 2006) and on reversal of ABA-induced stomatal closure by phenolic compounds including SA (Rai et al., 1986), which might be accounted for by the SA-reversed stomatal closure (Rai et al., 1986). Furthermore, SA mitigated the Cd-induced decline in activities of antioxidant enzymes (CAT and POD) of roots (Figure 5B and C), and further increased the levels of GSH and NPT both in roots and in shoots (Figure 6A–D). Hence, SA pretreatment alleviated Cd-induced oxidative stress as evidenced by decrease in concentrations of MDA, an end product of lipid peroxidation (Figure 4B).

It has been recognized that SA acts as a signaling molecule in regulating H_2O_2 levels associated with H_2O_2-metabolizing enzymes (Chen et al., 1993). In the present study, pretreatment with SA specifically inhibited CAT activity (Figure 5C) and increased H_2O_2 level (Figure 4A) in the roots before Cd exposure as the mode during plant–pathogen interactions (Chen et al., 1993). Although high concentrations of H_2O_2

can cause irreversible damage and cell death, it can also influence signaling and gene expression in response to environmental stresses, such as chilling (Prasad et al., 1994; Prasad, 1996) and heat shock (Davletova et al., 2005). In the present study, it seems to suggest that SA-induced H_2O_2 "set up" the rice plant to respond to Cd-induced oxidative damage more effectively. For example, the SA-induced H_2O_2 was associated with the stimulation of levels of GSH and NPT in rice roots (Figure 6A and C) before Cd exposure, which prepared the rice plant better in the detoxification of Cd by chelation. Hence, pretreatment with SA suppressed the Cd-induced oxidative damage and strengthened the Cd tolerance of rice. The SA-increased GSH was also found in *Thlaspi goesingense* by activation of serine acetyltransferase (SAT), an enzyme related closely to the synthesis of GSH, and enhanced Ni tolerance in this species (Freeman et al., 2005).

In conclusion, the results from this study using a split-root system show that cadmium can be partly transferred from Cd-exposed roots to Cd-unexposed roots and that cell death can be accelerated in the Cd-stressed roots in response to Cd stress. The SA-enhanced Cd tolerance in rice can be attributed to SA-elevated enzymatic and non-enzymatic antioxidants, NPT and SA-regulated Cd uptake, transport and distribution in plant organs. SA might influence H_2O_2 signaling pathways in plant defense against various forms of abiotic stress, which should be further investigated to dissect the complicated network of SA and its involvement in plant defense at a molecular level.

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