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REGULAR ARTICLE

Salicylic acid-mediated hydrogen peroxide accumulation and protection against Cd toxicity in rice leaves

Yun-Yang Chao · Chao-Yeh Chen · Wen-Dar Huang · Ching Huei Kao

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Abstract The role of H_2O_2 in salicylic acid (SA)-induced protection of rice leaves against subsequent Cd toxicity was investigated. SA pretreatment resulted in an increase in the contents of endogenous SA, as judged by the expression of *OsWRKY45* (a SA responsive gene), and H_2O_2 in rice leaves. Diphenyleneiodonium (DPI) and imidazole (IMD), inhibitors of NADPH oxidase, prevented SA-increased H_2O_2 production, suggesting that NADPH oxidase is a H_2O_2-generating enzyme in SA-pretreated rice leaves. DPI and IMD also inhibited SA-increased activities of superoxide dismutase (SOD), ascorbate peroixdase (APX), and glutathione reductase (GR) activities, but had no effect on SA-increased catalase (CAT) activity. Moreover, SA-induced protection against subsequent Cd toxicity could also be prevented by DPI and IMD. The inhibitory effect of DPI and IMD on SA-induced protection against subsequent Cd toxicity could be reversed by exogenous H_2O_2. All these results suggested that SA-induced protection against subsequent Cd toxicity is mediated through H_2O_2. This conclusion is supported further by the observations that exogenous H_2O_2 application resulted in an increase in SOD, APX, and GR activities, but not CAT activity and a protection against subsequent Cd toxicity of rice leaves.

Keywords Cadmium · Hydrogen peroxide · *Oryza sativa* L. · Oxidative stress · Salicylic acid

Abbreviations

APX	Ascorbate peroxidase
AsA	Ascorbate
CAT	Catalase
DAB	3,3-Diaminobenzidine
DPI	Diphenyleneiodonium
DW	Dry weight
FW	Initial fresh weight
GR	Glutathione reductase
IMD	Imidazole
IRT	Iron-regulated transporter
MDA	Malondialdehyde
ROS	Reactive oxygen species
SA	Salicylic acid
SOD	Superoxide dismutase

Introduction

Cadmium (Cd) is a widespread nonessential heavy metal, classified as a human carcinogen, and the uptake and accumulation of Cd in plants represent the main entry pathway into human and mammal food.

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Y.-Y. Chao · C.-Y. Chen · W.-D. Huang · C.H. Kao (✉)
Department of Agronomy, National Taiwan University, Taipei, Taiwan, Republic of China
e-mail: kaoch@ntu.edu.tw

Cd contamination is a vital concern because of its known toxicity to human health (Nawrot et al. 2006). In plants, Cd causes severe physiological and morphological effects such as chlorosis and growth reduction. Cd is a non-redox metal unable to participate in Fenton-type reactions, but it causes oxidative stress by generating reactive oxygen species (ROS) (Sanitá di Toppi and Gabbrielli 1999; Garnier et al. 2006). ROS react with lipids, proteins, pigments and nucleic acids and cause lipid peroxidation, membrane damage and inactivation of enzymes, thus resulting toxic effects. To minimize and /or to protect against the toxic effects of these damaging ROS, plants have evolved highly regulated enzymatic and non-enzymatic mechanisms to keep a balance between ROS production and destruction in order to maintain cellular redox homeostasis. Plants use enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reducatse (GR), and catalase (CAT) as well as low molecular weight antioxidants like ascorbate (AsA) and glutathione to scavenge ROS (Noctor and Foyer 1998).

Salicylic acid (SA) is an endogenous growth regulator of phenolic nature, which participates in the regulation of physiological processes in plants, such as growth, photosynthesis, nitrate metabolism, ethylene production, heat production and flowering (Hayat et al. 2007). SA is also known to be involved in abiotic stress signaling, including plant response to heavy metals. SA pretreatment alleviates Cd toxicity in barley (Metwally et al. 2003), soybean (Drazic and Mihailovic 2005), pea (Popova et al. 2009), maize (Krantev et al. 2008), and rice (Guo et al. 2007a, b, 2009). However, SA application has been shown to aggravate the symptoms of cadmium stress (Pál et al. 2002). Zawoznik et al. (2007) also demonstrated that endogenous SA may function in *Arabidopsis thaliana* as a signaling molecule necessary to potentiate Cd-induced oxidative damage. In a recent review, Horváth et al. (2007) claimed that the effect of exogenous SA depends on numerous factors such as the species and developmental stage of the plant, the mode of application, and the concentration of SA and its endogenous level in the given plant.

Because H_2O_2 is relatively stable and diffusible through membranes (in contrast with superoxide), it is now considered as an alarm signal that triggers acclamatory/defense mechanisms in plant cells (Chen et al. 1993; Prasad et al. 1994a, b; Cruz de Carvalho 2008). H_2O_2 treatment has been shown to enhance tolerance against oxidative stress generated by chilling, heat salinity, drought, and high light intensities (Prasad et al. 1994a, b; Dat et al. 1998; Lopez-Delgado et al. 1998; Gong et al. 2001; Gechev et al. 2002; Uchida et al. 2002; Yu et al. 2002, 2003; Azevedo Neto et al. 2005; Wahid et al. 2007). Recent work also demonstrated that H_2O_2 pretreatment protects against the subsequent Cd stress of rice seedlings (Hsu and Kao 2007). Several lines of evidence indicate that SA pretreatment results in an accumulation of H_2O_2 (Chen et al. 1993; Kauss and Jeblick 1995; Rao et al. 1997; Dat et al. 1998; Ganesan and Thomas 2001; Agarwal et al. 2005; Harfouche et al. 2008). It has been shown that thermotolerance obtained by exogenous application of SA can be achieved by an early increase in H_2O_2 in mustard seedlings (Dat et al. 1998) and potato (Lopez-Delgado et al. 1998).

In the present study, we showed that a SA pretreatment could induce Cd stress tolerance in rice leaves and investigated the possible involvement of H_2O_2 in Cd stress tolerance.

Materials and methods

Plant material

Rice (*Oryza sativa* L., cv. Taichung Native 1) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively seeds with distilled water. These seeds were then germinated in Petri dishes with wetted filter paper at 37°C under dark conditions. After 48 h incubation, uniformly germinated seeds were selected and cultivated in a 500 ml beaker containing half-strength Kimura B solution as described previously (Hsu and Kao 2003). The hydroponically cultivated seedlings were grown for 12 days in a Phytotron (Agricultural Experimental Station, National Taiwan University, Taipei, Taiwan) with natural sunlight at 30°C day/25°C night and 90% relative humidity.

SA pretreatment and Cd stress treatment

The apical 3-cm of the third leaves of 12-day-old seedlings was used for all experiments. Ten leaf segments were floated in a Petri dish containing 10 ml

SA containing 0.01% Triton X-100. Control leaf segments were floated in 0.01% Triton X-100 alone. Control and SA-pretreated rice leaves were then treated with or without $CdCl_2$ (5 mM) for 24 h. All pretreatments and treatments were carried out at 27°C and irradiance of 40 μmol m^{-2} s^{-1}.

Measurement of H_2O_2, malondialdehyde (MDA), and Cd

H_2O_2 was visually detected in the leaves by using 3,3-diaminobenzidine (DAB) as substrate (Orózco-Cárdenas and Ryan 1999). Rice leaves were supplied through the cut ends with DAB (1 mg ml^{-1}) solution for 24 h under light at 27°C. Rice leaves were then decolorized in boiling ethanol (95%) for 0.5 h. This treatment decolorized the leaves except for the brown polymerization product produced by DAB with H_2O_2. After cooling, the leaves were extracted at room temperature with fresh ethanol to visualize the brown spots. The H_2O_2 staining was repeated four times with similar results. In some experiments, the H_2O_2 content was measured spectrophotometrically after reaction with $TiCl_4$ (Tsai et al. 2004). The reaction mixture consisted of 2 ml of 50 mM phosphate buffer (pH 6.8) leaf extract supernatant and 1 ml reagent [0.1% (*v*/*v*) $TiCl_4$ in 20% (*v*/*v*) H_2SO_4]. The blank probe consisted of 50 mM phosphate buffer in the absence of leaf extract. The absorbance was measured at 410 nm. The amount of H_2O_2 was calculated by use of a standard curve prepared with known concentration of H_2O_2. The H_2O_2 content was expressed on the basis of initial fresh weight (FW).

Malondialdehyde (MDA), routinely used as an indicator of lipid peroxidation, was extracted with 5% (*w*/*v*) trichloroacetic acid and determined by the thiobarbituric acid reaction as described by Heath and Packer (1968). MDA content was expressed on the basis of FW.

For determination of Cd, leaves were dried at 65°C for 48 h. Dried material was ashed at 550°C for 20 h. The ash residue was incubated with 31% HNO_3 and 17.5% H_2O_2 at 72°C for 2 h, and dissolved in distilled water. Cd concentration was then quantified using an atomic absorption spectrophotometer (Model AA-6800, Shimadzu, Kyoto, Japan). Amount of Cd is expressed on the basis of dry weight (DW).

Enzyme extraction and assays

For extraction of enzymes, leaf tissues were homogenized with 0.1 M sodium phosphate buffer (pH 6.8) in a chilled pestle and mortar. For analysis of ascorbate peroxidase (APX) activity, 2 mM ascorbate (AsA) was added to the extraction buffer. The homogenate was centrifuged at 12,000 *g* for 20 min and the resulting supernatant was used for determination of enzyme activity and protein content. The whole extraction procedure was carried out at 4°C. Superoxide dismutase (SOD) was determined according to Paoletti et al. (1986). The reaction mixture (2.73 ml) contained 100 mM triethanoamine-diethanolamine buffer (pH 7.4), 7.5 mM NADH, EDTA/$MnCl_2$ (100 mM/50 mM, pH 7.0), 10 mM 2-mercaptoethanol, and enzyme extract (0.2 ml). The reaction was started by the addition of NADH. The reaction was allowed to proceed for 10 min. The absorbance was measured at 340 nm. One unit of SOD was defined as the amount of enzyme that inhibits by 50% the rate of NADH oxidation observed in blank sample. Catalase (CAT) activity was assayed according to Kato and Shimizu (1987). The decrease in H_2O_2 was followed as the decline in the absorbance at 240 nm, and the activity was calculated using the extinction coefficient (40 mM^{-1} cm^{-1} at 240 nm) for H_2O_2. One unit of CAT was defined as the amount of enzyme which degraded 1 μmol H_2O_2 per min. APX activity was determined according to Nakano and Asada (1981). The decrease in AsA concentration was followed as a decline in the absorbance at 290 nm and activity was calculated using the extinction coefficient (2.8 mM^{-1} cm^{-1} at 290 nm) for AsA. One unit of activity for APX was defined as the amount of enzyme that degraded 1 μmol of AsA per min. Glutathione reductase (GR) was determined by the method of Foster and Hess (1980). One unit of GR was defined as the amount of enzyme that decreased 1 absorbance min^{-1} at 340 nm. The enzyme extracts were used for the determination of protein by the method of Bradford (1976).

Quantitative RT-PCR analysis of *OsWRKY 45* gene

Total RNA was isolated from rice leaves treated with or without SA using the TRIZOL reagent (Invitrogen, CA, USA), accroding to supplier's recommendations. To remove contaiminating genomic DNA, RNA was

Fig. 1 Histochemical detection of H_2O_2 with DAB staining in rice leaves treated with SA, AsA, and SA + AsA. The concentrations of SA and AsA were 3 mM and 2 mM, respectively

treated with Turbo DNase I (Ambio, TX, USA) for 30 min at 37°C before the cDNA systhesis system for RT-PCR (Invitrogen, CA, USA). cDNA was amplified by PCR in 7500 Real-Time PCR System (Applied Biosystems) with SYBR Advantage qPCR Premix (Clontech, USA) with 1 cycle of 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 1 min.

The gene-specific primers were designed from the 3′-UTR of the rice *WRKY* gene. *OsWRKY45* (AY870611) forward primer (ACG ATCGAA AGAAGATGGAT) and reverse primer (TCGTGTTGTTACTTGCT AGCATG). The mean value of the three replicates was normalized using *OsUbiquitin* as the internal control. *OsUbiqutin* (D12629.1) forward primer (CGCAAGTACAACCAGGACAA) and reverse primer (TGGTTGCTGTGACCACACTT). The primer sets were tested by dissocitation curve analysis and verified for the absence of nonspecific amplification. The relative expression level was then normalized to the treatment without SA.

Statistical analysis

Statistical differences between measurements (n=4) for different treatments or different times were analyzed following Student's t-test or Duncan's multiple range test. A $P<0.05$ was considered statistically significant.

Results

H_2O_2 content and antioxidant enzyme activities in response to SA

In the present study, DAB staining was used to examine whether SA induces H_2O_2 production in rice leaves. To verify the specificity of brown polymerization product produced by DAB with H_2O_2, some leaves were immersed for 1 h in solution containing 2 mM AsA, a scavenger of H_2O_2. It was observed that the development of DAB-H_2O_2 reaction product by SA in rice leaves could be reduced by AsA (Fig. 1), indicating that the DAB staining method for H_2O_2 is specific. Little DAB staining was observed in control leaves (Figs. 1 and 2a). In our preliminary work, we observed that increasing concentration of SA from 1 to 3 mM for 3 h progressively increased DAB staining in rice leaves and no further increase was observed at 5 mM SA. Thus, in the present study, 3 mM SA was used for all experiments. The increase in DAB-H_2O_2 reaction product was evident 1 h after SA treatment (Fig. 2a). SA-responsive rice gene *WRKY45* has been reported (Ryu et al. 2006; Shimono et al. 2007). To examine the effect of SA on the expression of *OsWRKY45*, rice leaves were treated with 3 mM SA for 1, 2, and 3 h. The expression of *OsWRKY45* was notably increased by SA 1 h after treatment (Fig. 2b).

SOD, APX, GR, and CAT activities in SA-treated rice leaves were higher than their respective control leaves (Fig. 3a, b, c, d). The increase in SOD activity was evident 1 h after SA treatment (Fig. 3a). However,

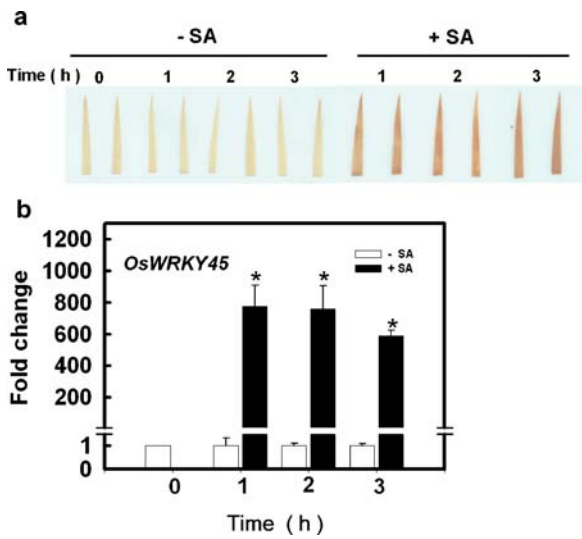

Fig. 2 Histochemical detection of H_2O_2 with DAB staining (**a**) and change in mRNA level of *OsWRKY45* (**b**) in rice leaves treated with or without SA. Rice leaves were incubated in 3 mM SA in 0.01% Triton X-100 for 1, 2, and 3 h in the light. Control leaves (−SA) were incubated in Triton X-100 alone. *Bars* indicate standard error (n=4). *Asterisk* represents values that are significantly different between −SA and +SA treatments at $P<0.05$

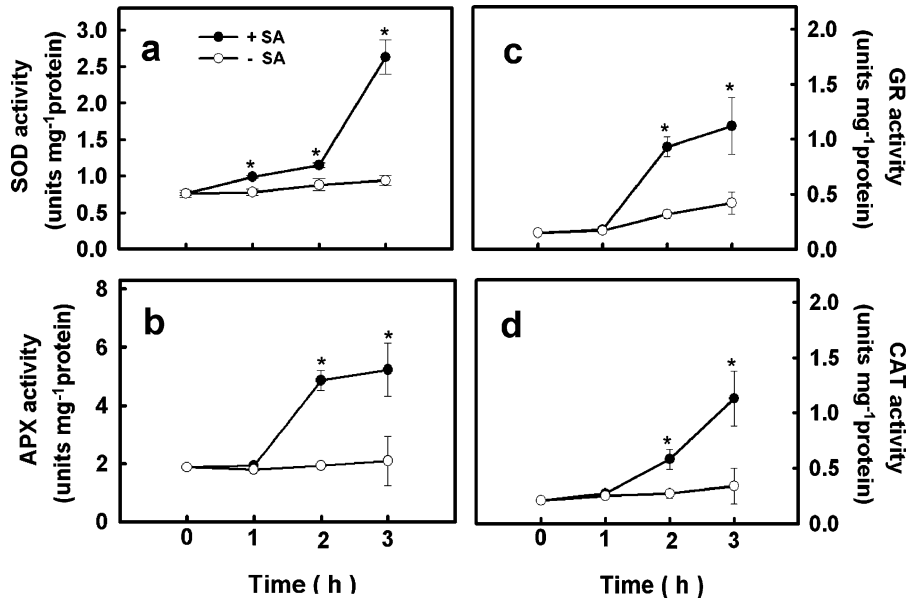

Fig. 3 Changes in the activities of SOD (**a**), APX (**b**), GR (**c**), and CAT (**d**) in rice leaves in response to SA. Rice leaves were incubated in 3 mM SA in 0.01% Triton X-100 for 1, 2, and 3 h in the light. Control leaves (−SA) were incubated in Triton X-100 alone. *Bars* indicate standard error (n=4). *Asterisk* represents values that are significantly different between −SA and +SA treatments at $P<0.05$

the increase in APX, GR, and CAT activities occurred 2 h after SA treatment (Fig. 3b, c, d).

Effect of SA pretreatment on subsequent Cd toxicity of rice leaves

In the present study, Cd toxicity in rice leaves caused by Cd was assessed by an increase in MDA content. Increasing concentration of Cd from 0.1 to 5 mM progressively increased MDA content in rice leaves in the light and no further increase was observed at 10 mM $CdCl_2$ (data not shown). Thus, 5 mM $CdCl_2$ was used in the present study. To test if SA pretreatment would affect the subsequent Cd-induced toxicity, rice leaves were pretreated with 3 mM SA for 3 h in the light and then treated with 5 mM $CdCl_2$ for 24 h in the light. It was observed that a 3-h SA pretreatment exhibited a significant reduction of Cd-increased MDA content (Fig. 4a).

The effect of NADPH oxidase inhibitors

The role of NADPH oxidase in the SA-induced H_2O_2 production was investigated by using NADPH oxidase inhibitors such as diphenyleneiodonium (DPI) and imidazole (IMD). When rice leaves were treated with a solution of DPI (10 μM) or IMD (100 μM), SA-induced accumulation of H_2O_2 was reduced by DPI or IMD (Fig. 5a). DPI or IMD also inhibited SA-increased activities of SOD, APX, GR (Fig. 5 b, c, d). However, DPI or IMD had no effect

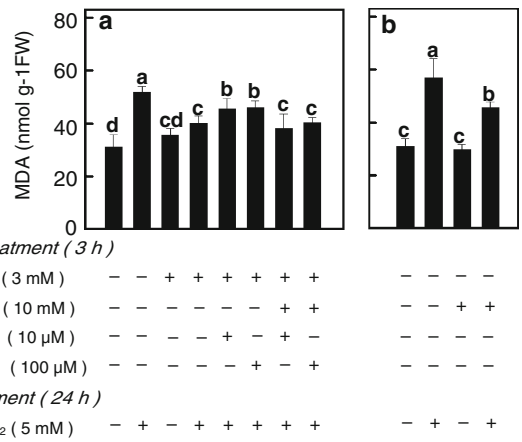

Fig. 4 Effect of $CdCl_2$ on MDA content in rice leaves pretreated with SA, SA + DPI, SA + IMD, SA + DPI + H_2O_2, or SA + IMD + H_2O_2 (**a**) or pretreated with or without H_2O_2 (**b**) for 3 h in the light. The concentrations of $CdCl_2$, SA, DPI, IMD, and H_2O_2 were 5 mM, 3 mM, 10 μM, 100 μM, and 10 mM, respectively. *MDA* was determined 24 h after $CdCl_2$ treatment. *Bars* indicate standard error (n=4). Values with the same letter are not significantly different at $P<0.05$

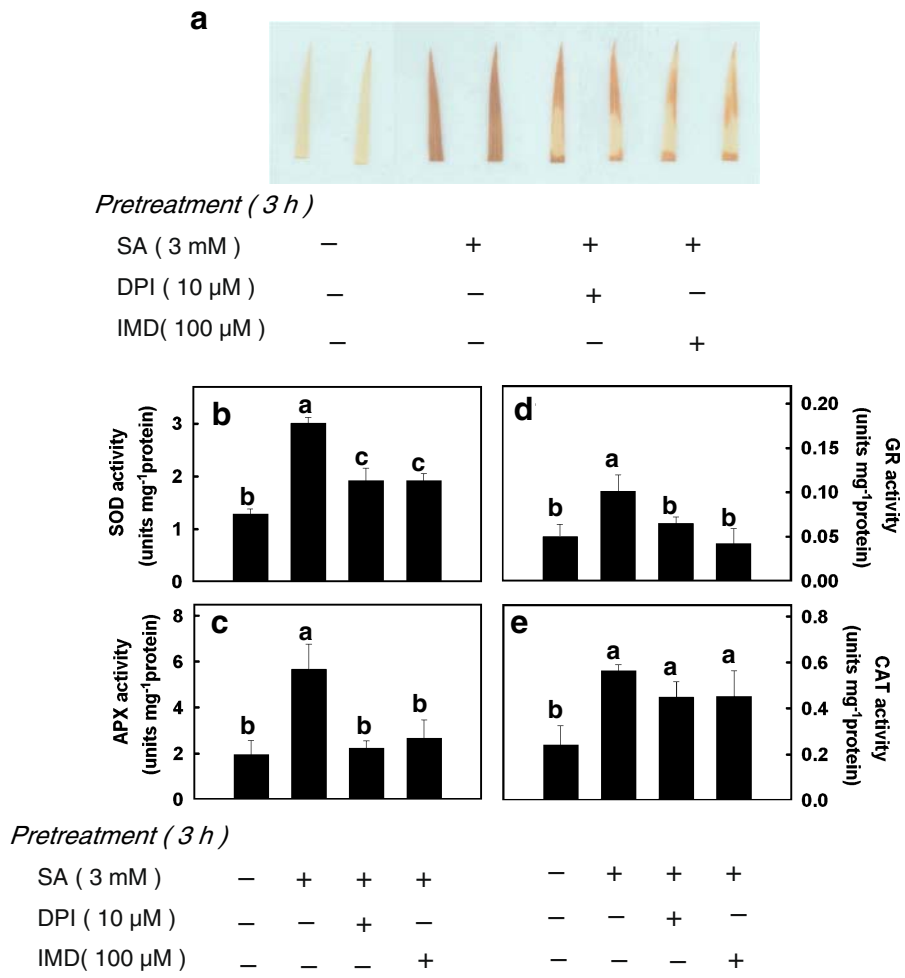

Fig. 5 Effect of DPI and IMD on SA-induced H_2O_2-DAB reaction product (**a**) activities of SOD (**b**), APX (**c**), GR (**d**), and CAT (**e**) in rice leaves. Rice leaves were incubated in SA, SA + DPI, and SA + IMD in 0.01% Triton X-100 for 1, 2, and 3 h in the light. Control leaves (−SA) were incubated in Triton X-100 alone. Concentrations of SA, DPI, and IMD were 3 mM, 10 μM, and 100 μM, respectively. *Bars* indicate standard error (n=4). Values with the same letter are not significantly different at P<0.05

on SA-induced CAT activity (Fig. 5e). DPI or IMD alone had no effect on MDA content in rice leaves (Table 1). Content of MDA in Cd-exposed DPI-pretreated rice leaves was similar to that in the Cd-treated control, whereas that in Cd-exposed IMD-pretreated rice lee leaves was higher than in Cd-treated controls (Table 1). SA-induced protection against subsequent increase in MDA content by Cd was reduced by DPI or IMD (Fig. 4a). The inhibitory effect of DPI or IMD on SA-induced protection against subsequent increase in MDA content by Cd could be reversed by exogenous H_2O_2 (10 mM) (Fig. 4a).

Effect of H_2O_2 pretreatment

To test if exogenous H_2O_2 acts similarly as SA on the activities of antioxidant enzymes, rice leaves were pretreated with 10 mM H_2O_2 for 3 h. Figure 6a shows that H_2O_2 pretreatment resulted in an increase in endogenous H_2O_2 content in rice leaves. Furthermore, rice leaves pretreated with H_2O_2 showed an enhancement in SOD, APX, and GR activities (Fig. 6b, c, d) but had no effect on CAT activity in rice leaves (Fig. 6e). When H_2O_2-pretreated rice leaves were then transferred to solution with or without $CdCl_2$ for 24 h. It was observed that pretreatment of rice leaves with H_2O_2 greatly improved tolerance of rice leaves exposed to Cd stress (Fig. 4b).

Effect of SA or H_2O_2 pretreatment on Cd concentration in rice leaves

To test if SA- or H_2O_2 pretreatment affects subsequent Cd uptake, rice leaves were first treated with SA or H_2O_2 for 3 h and then transferred to solution with or without 5 mM $CdCl_2$. It was

Table 1 Effect of DPI and IMD on MDA content in rice leaves treated with or without $CdCl_2$ for 3 h in the light

Pretreatment (3 h)	Treatment (24 h)	MDA (nmol g^{-1}FW)
H_2O	H_2O	28.4±4.9[c]
DPI	H_2O	31.2±4.0[c]
IMD	H_2O	24.8±4.3[c]
H_2O	$CdCl_2$	57.4±1.4[b]
DPI	$CdCl_2$	54.6±7.5[b]
IMD	$CdCl_2$	70.0±4.3[a]

The concentrations of $CdCl_2$, DPI, and IMD were 5 mM, 10 μM, and 100 μM respectively. MDA was determined 24 h after $CdCl_2$ treatment. *Bars* indicate standard error (n=4). Values with the same letter are not significantly different at $P<0.05$

observed that rice leaves pretreated with 3 mM SA had higher Cd concentration than those pretreated without SA (Fig. 7a). Interestingly, pretreatment of rice leaves with H_2O_2 also increased Cd accumulation (Fig. 7b).

Discussion

The expression of *OsWRKY45* is known to increase in rice leaves treated with SA (Ryu et al. 2006; Shimono et al. 2007). Thus, the level of SA reported in this study was judged by the transcript level of *OsWRKY45*. The expression of *OsWRKY45* was enhanced in rice leaves by 3 mM SA (Fig. 2b), indicating that exogenous SA treatment indeed resulted in an increase in SA content in rice leaves.

In previous work, it has been demonstrated that Cd can induce oxidative stress in rice leaves, characterized by an increase in the content of MDA (an indicator of lipid peroxidation) (Kuo and Kao 2004; Hsu and Kao 2005). Thus, in the present study, Cd toxicity was evaluated by the increase in MDA content of rice leaves. On the basis of this criterion, we demonstrated that rice leaves pretreated with SA protected against subsequent Cd stress (Fig. 4a). The protective effect of SA against subsequent Cd toxicity has also been described previously (Metwally et al. 2003;

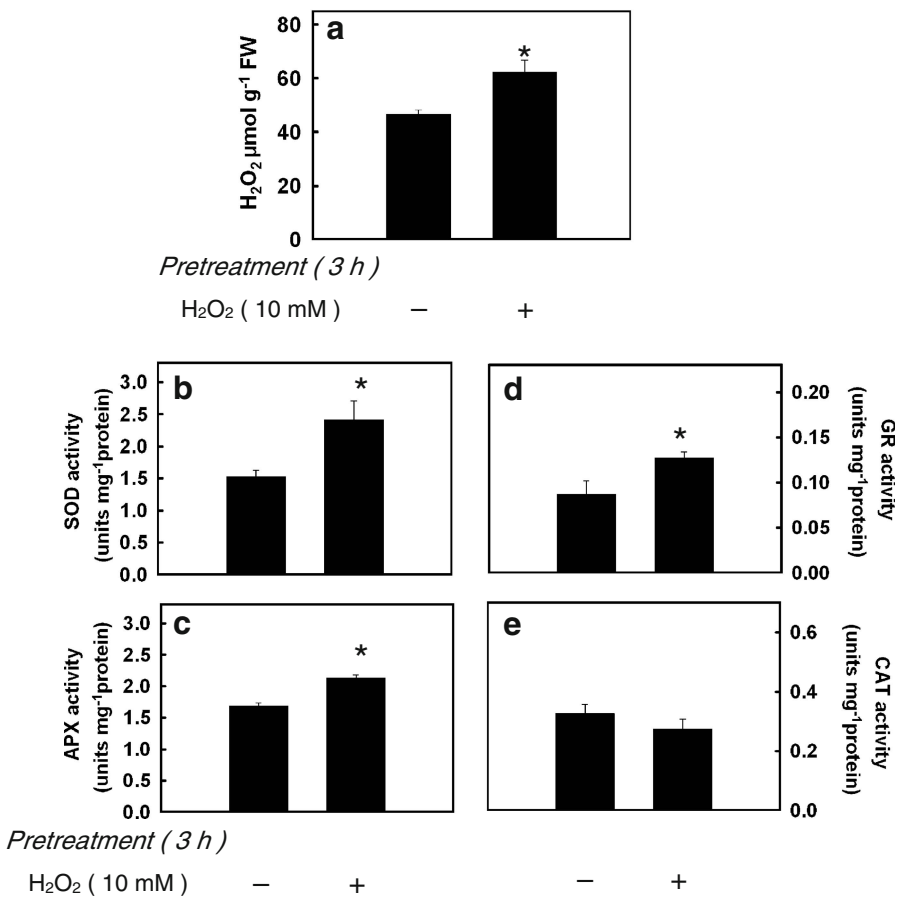

Fig. 6 Effect of exogenous H_2O_2 on the content of H_2O_2 (**a**) and the activities of SOD (**b**), APX (**c**), GR (**d**), and CAT (**e**) in rice leaves. The concentration of H_2O_2 was 10 mM. *Bars* indicate standard error (n=4). *Asterisk* represents values that are significantly different between $-H_2O_2$ and $+H_2O_2$ treatments at $P<0.05$

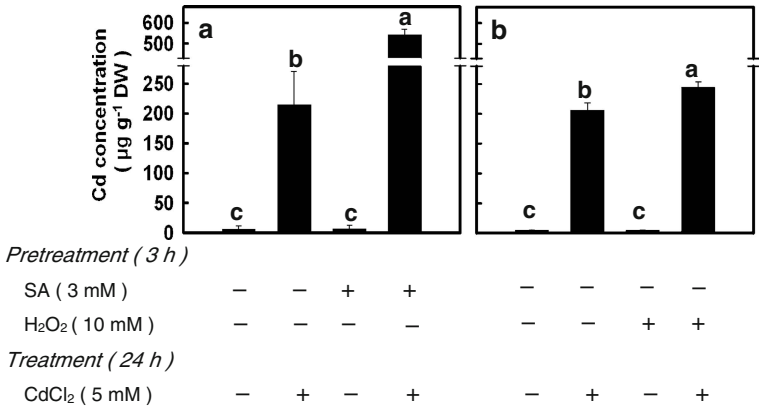

Fig. 7 Effect of $CdCl_2$ on Cd concentration in rice leaves pretreated with or without SA (**a**) or pretreated with or without H_2O_2 (**b**) for 3 h in the light. The concentration of $CdCl_2$, SA and H_2O_2 were 5 mM, 3 mM, and 10 mM, respectively. Cd was determined 24 h after $CdCl_2$ treatment. *Bars* indicate standard error (n=4). Values with the same letter are not significantly different at P<0.05

Drazic and Mihailovic 2005; Guo et al. 2007a, b, 2009; Krantev et al. 2008; Popova et al. 2009).

Several lines of evidence indicate that SA pretreatment results in an accumulation in H_2O_2 content (Chen et al. 1993; Kauss and Jeblick 1995; Rao et al. 1997; Dat et al. 1998; Ganesan and Thomas 2001; Agarwal et al. 2005; Harfouche et al. 2008). Using DAB staining method, we have shown that SA induced the accumulation H_2O_2 in rice leaves (Fig. 2a). Figure 2b shows that *OsWRKY45* expression was increased in rice leaves treated with SA. The negligible H_2O_2 detected in control leaves suggested that H_2O_2 observed in SA-treated rice leaves is indeed caused by SA applied.

SA-induced accumulation of H_2O_2 has been suggested to be due to SA-inhibited CAT and APX activities (Chen et al. 1993; Sánchez-Casas and Klessig 1994; Klessig et al. 2000; Horváth et al. 2007). This does not seem to be the case in rice leaves, because SA increased APX and CAT activities (Fig. 3b, d). Agarwal et al. (2005) reported that SA-induced H_2O_2 production in wheat seedlings is mediated through a plasma membrane NADPH oxidase activity. The fact that SA-induced H_2O_2 production in rice leaves can be inhibited by DPI and IMD, inhibitors of NADPH oxidase (Fig. 5a), suggests that SA-dependent H_2O_2 generation in rice leaves originated, at least in part, from plasma membrane NADPH oxidase.

The present study indicated that H_2O_2 was involved in SA-induced protection against subsequent Cd stress of rice leaves. This conclusion was based on the observations that (1) endogenous H_2O_2 was increased in rice leaves pretreated with SA (Fig. 2a); (2) SA-induced H_2O_2 production (Fig. 5a) and protection against subsequent Cd stress (Fig. 4a) could be counteracted by DPI or IMD, inhibitor of NADPH oxidase; (3) the effect of DPI or IMD on SA-induced protection against subsequent Cd stress could be reversed by exogenous application of H_2O_2 (Fig. 4a), and (4) pretreatment of rice leaves with exogenous H_2O_2 greatly improved tolerance of rice leaves to Cd (Fig. 4b).

It has been shown that various abiotic stresses induce oxidative stress and improvement of stress tolerance is often related to the increase in the activities of antioxidant enzymes (Noctor and Foyer 1998). Cho and Seo (2005) reported that seedlings of Cd-resistant *Arabidopsis* have higher activities of SOD, APX, and GR and experienced lower oxidative stress from Cd exposure. In the present study, we show that SA pretreatment resulted in higher activities of SOD, APX, and GR activities (Fig. 3a, b, c) and protection against of subsequent Cd stress in rice leaves (Fig. 4a). H_2O_2 is now considered as a signal molecule that induces gene expression and activities of antioxidant enzyme (Foyer et al. 1997; Morita et al. 1999; Prasad et al. 1994a, b; Hong et al. 2009). The fact that DPI or IMD treatment was effective in reducing SA-increased SOD, APX, and GR activities (Fig. 5b, c, d) but not CAT activity (Fig. 5e) in rice leaves suggests that H_2O_2 can act as a signal molecule in SA- increased SOD, APX, and GR activities. This suggestion was supported further by the observations that exogenous H_2O_2 treatment increased SOD, APX and GR activities (Fig. 6b, c, d) but not CAT activity (Fig. 6e).

Earlier work by Metwally et al. (2003) demonstrated that pretreatment of barley seeds with SA protects Cd toxicity to roots. However, this protection

was not due to upregulation of antioxidant activity. In soybean seedlings, the influence of SA on the alleviation of toxic effect of Cd is possibly mediated through the regulation of K and Mg distribution (Drazic and Mihailovic 2005). Guo et al. (2007a, 2009) concluded that the SA-elevated enzymatic and non-enzymatic antioxidants contribute to alleviation of Cd toxicity in rice roots. Using detached rice leaves, we showed that early accumulation of H_2O_2 during SA pretreatment signals the increase in SOD, APX, and GR activities, which in turn prevents rice leaves from oxidative damage by Cd. It appears that the mechanism of SA on the alleviation of Cd toxicity depends on the methods of SA application and plant species examined.

SA-pretreatment increased Cd accumulation in rice leaves (Fig. 7a). Thus, the protective effect of SA against subsequent Cd toxicity of rice leaves is unlikely due to inhibition of Cd uptake. The increase in the Cd uptake by SA has also been described previously (Drazic et al. 2006). However, there are other reports showing that exogenous SA application reduced the uptake of Cd (Pál et al. 2002; Krantev et al. 2008: Popova et al. 2009). Of particular interest is the finding that H_2O_2 pretreatment resulted in an increase in Cd concentration (Fig. 7b). The possibility that H_2O_2 acts as a signal molecule for SA-enhanced Cd uptake remained to be seen.

In the present study, the total leaf Cd concentration was measured. It is not known whether SA-pretreatment alters Cd distribution between the vacuolar compartment and the rest of the cell. In future studies, it will be important to determine the effect of SA on Cd concentration in different cellular compartments. SA pretreatment increased endogenous SA content, as judged by the expression of *OsWRKY45* (Fig. 2b). Thus, the possibility that formation of stable SA-Cd complexes, which might reduce Cd toxicity after SA pretreatment, cannot be excluded.

It has been suggested that SA-enhanced Cd uptake is mediated through activation of some divalent cation transporters (Drazic et al. 2006). Iron-regulated transporter 1 (IRT1) is a major Fe transporter (Eide et al. 1996). A role for OsIRT1 in Cd transport has been previously investigated in yeast (Nakanishi et al. 2006). Recent experiment using transgenic rice plants over-expressing *OsIRT1* confirmed the conclusion from those earlier yeast experiments that OsIRT1 indeed transports Cd (Lee and An 2009). It is not known whether SA or H_2O_2 pretreatment activates the expression of *OsIRT1*. Future research on the effect of SA or H_2O_2 on the expression of *OsIRT1* is likely to be highly rewarding.

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