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REGULAR ARTICLE

Heat shock-mediated H_2O_2 accumulation and protection against Cd toxicity in rice seedlings

Yi Ting Hsu · Ching Huei Kao

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Abstract Rice (*Oryza sativa* L.) seedlings stressed with $CdCl_2$ (0.5 mM or 50 μM) showed typical Cd toxicity (leaf chlorosis, decrease in chlorophyll content, or increase in H_2O_2 and malondialdehyde contents). Rice seedlings pretreated with heat shock at 45°C (HS) for 2 or 3 h were protected against subsequent Cd stress. Rice seedlings pretreated with HS had similar Cd concentration in leaves caused by $CdCl_2$ as those non-HS. The content of H_2O_2 increased in leaves 1 h after HS exposure. However, APX and GR activities were higher in HS-treated leaves than their respective control, and it occurred after 2 h of HS treatment. Pretreatment of rice seedlings with H_2O_2 under non-HS conditions resulted in an increase in APX, GR, and CAT activities and protected rice seedlings from subsequent Cd stress. HS-induced H_2O_2 production and protection against subsequent Cd stress can be counteracted by imidazole, an inhibitor of NADPH oxidase complex. Results of the present study suggest that early accumulation of H_2O_2 during HS signals the increase in APX and GR activities, which in turn prevents rice seedlings from Cd-caused oxidative damage.

Keywords Cadmium · Heat shock · Hydrogen peroxide · *Oryza sativa* · Oxidative stress

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

Abbreviations

APX	Ascorbate peroxidase
ASC	Ascorbate
CAT	Catalase
DAB	3, 3-Diaminobenzidine
DW	Dry weight
GR	Glutathione reductase
HS	Heat shock
IMD	Imidazole
MDA	Malondialdehyde
ROS	Reactive oxygen species
SOD	Superoxide dismutase

Introduction

Cadmium (Cd), a heavy metal toxic to humans, animals and plants, is a widespread pollutant. In Cd-sensitive plants, it has been shown that (a) Cd can promote the generation of reactive oxygen species (ROS); (b) Cd can inhibit or stimulate the activities of antioxidant enzymes; and (c) treatment with Cd results in cellular oxidative damage or lipid peroxidation (Chaoui et al. 1997; Chien and Kao 2000; Dixit et al. 2001; Gallego et al. 1996; Kuo and Kao 2004; Olmos et al. 2003; Piqueras et al. 1999; Romero-Puertas et al. 2003; Shah et al. 2001; Shaw 1995). These results indicate that oxidative stress is a major component of Cd stress.

Exposure of plants in the range of 15 min to a few hours at temperature 5 to 15°C above the normal growing temperature is usually considered as heat

shock (HS) treatment. It has been shown that leaf segments exhibited an acquired protection against Cd and other heavy metal toxicity following exposure of the seedlings to HS in the dark (Orzech and Burke 1988). Neumann et al. (1994) demonstrated that a HS treatment preceding Cd stress induced a tolerance effect by preventing the membrane damage. Prior HS exposure of rice seedlings was also observed to inhibit subsequent Cd-induced ethylene production in detached leaves (Chen and Kao 1995a).

In addition to being an endogenous oxidant, H_2O_2 has also been implicated as a diffusible signal for selective induction of defense mechanisms in plant cells (Chen et al. 1993; Prasad et al. 1994a, b). H_2O_2 treatment has been shown to enhance chilling tolerance in maize (Prasad et al. 1994a, b) and in mung bean and *Phalaenopsis* (Yu et al. 2002; 2003). Nodal potato explants subcultured from H_2O_2-treated microplant were resistant to normally lethal exposure of 42°C (Lopez-Delgado et al. 1998). Recent work also demonstrated that H_2O_2 pretreatment resulted in an improvement of salt tolerance (Azevedo Neto et al. 2005; Uchida et al. 2002; Wahid et al. 2007). In mustard seedlings, HS at 45°C caused a significant increase in endogenous H_2O_2 content and improved their tolerance to subsequent heat stress at 55°C (Dat et al. 1998). Gong et al. (2001) reported that a HS pretreatment could simultaneously induce cross adaptation of maize seedlings to chilling, heat, drought and salt stresses. They also showed that HS-induced H_2O_2 accumulation was involved in signaling and triggering of this cross adaptation.

It is not known if H_2O_2 treatment induces Cd stress tolerance in rice seedlings. Neither do we know if HS pretreatment results in an accumulation of H_2O_2 in rice seedlings. In the present study, we showed that a HS pretreatment could induce Cd stress tolerance in rice seedlings and investigated the possible involvement of H_2O_2 in Cd stress tolerance.

Materials and methods

Plant material and treatments

Rice (*Oryza sativa* L., cv. TN1) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. These seeds were then germinated in Petri dishes with wetted filter papers at 37°C in the dark. After 48 h incubation, uniformly germinated seeds were selected and cultivated in a 250 ml beaker containing half-strength Kimura B solution containing the following macro- and micro-elements: 182.3 μM $(NH_4)_2SO_4$, 91.6 μM KNO_3, 273.9 μM $MgSO_4 \cdot 7H_2O$, 91.1 μM KH_2PO_4, 182.5 μM $Ca(NO_3)_2$, 30.6 μM Fe–citrate, 0.25 μM H_3BO_3, 0.2 μM $MnSO_4 \cdot H_2O$, 0.2 μM $ZnSO_4 \cdot 7H_2O$, 0.05 μM $CuSO_4 \cdot 5H_2O$, and 0.07 μM H_2MoO_4. Kimura B solution contains the desired nutrient elements and has been widely used for growing rice plants. Since young rice seedlings were used for the present study, the nutrient solution contains no silicon, although silicon is essential for rice. The nutrient solutions (pH 4.7) were replaced every 3 days. The hydroponically cultivated seedlings were grown in a Phytotron (Agricultural Experimental Station, National Taiwan University, Taipei, Taiwan) with natural sunlight at 30/25°C day/night and 90% relative humidity.

HS pretreatment and Cd stress treatment

Twelve-day-old seedlings with three leaves were exposed to 30°C (non-HS) and 45°C (HS) for 3 h in the dark. Non-HS and HS seedlings were then grown in basic nutrient solution with or without $CdCl_2$ (0.5 mM or 50 μM) at 30/25°C day/night.

Measurement of chlorophyll, H_2O_2, malondialdehyde, and Cd

Chlorophyll content was determined according to Wintermans and De Mots (1965) after extraction in 96% (*v*/*v*) ethanol. H_2O_2 was visually detected in the leaves by using 3,3-diaminobenzidine (DAB) as substrate (Orozco-Cárdenas and Ryan 1999). Detached rice leaves were supplied through the cut ends with DAB (1 mg ml^{-1}) solution for 24 h under light at 27°C. 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. To verify the specificity of precipitates, before staining with DAB some leaves were immersed for 2 h in solution containing the H_2O_2 scavenger 1 mM ascorbate (ASC) suggested by Romero-Puertas et al. (2004). It was observed that the development of DAB–H_2O_2 reac-

tion product in detached rice leaves could be prevented by ASC (data not shown), which demonstrated the specificity of the reaction of DAB with H_2O_2. The H_2O_2 staining was repeated four times with similar results. The H_2O_2 content was also measured colorimetrically as described by Jana and Choudhuri (1982). H_2O_2 was extracted by homogenizing leaf tissue with phosphate buffer (50 mM, pH 6.5) containing 1 mM hydroxylamine. The homogenate was centrifuged at 6,000×*g* for 25 min. To determine H_2O_2 content, the extracted solution was mixed with 0.1% titanium chloride in 20% (*v*/*v*) H_2SO_4. The mixture was then centrifuged at 6,000×*g* for 25 min. The absorbance was measured at 410 nm. Using this method, we obtained that absorbance increased linearly with the amount of H_2O_2 and addition of H_2O_2 to extracts resulted in the predicted increase of absorbance, i.e. added H_2O_2 was fully recovered (data not shown), was calculated using the extinction coefficient of 0.28 μmol^{-1} cm^{-1}. Malondialdehyde (MDA), routinely used as an indicator of lipid peroxidation, was extracted with 5% (*w*/*v*) trichloroacetic acid and determined by the thioburbituric acid reaction as described by Heath and Packer (1968). 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 ASC 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). 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). One unit of CAT was defined as the amount of enzyme which degraded 1 μmol H_2O_2 per minute. APX activity was determined according to Nakano and Asda (1981). One unit of activity for APX was defined as the amount of enzyme that degraded 1 μmol of ASC per minute. 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 A_{340} per minute. The enzyme extracts were used for determination of protein by the method of Bradford (1976).

Statistical analysis

Statistical differences between measurements (n=4) on different treatments or on different times were analyzed following LSD test.

Results

Effect of prior HS exposure on the tolerance of rice seedling to Cd

In plants, the most general symptom of Cd toxicity is leaf chlorosis (Das et al. 1997). When rice seedlings were treated with 0.5 mM $CdCl_2$, chlorosis was first shown in the second leaves, but not the third leaves of rice seedlings in a short-term experiment (3 days; Hsu and Kao 2003). Thus, in the present study, Cd toxicity was assessed by the chlorosis and the decrease in chlorophyll content of the second leaves.

To test if prior exposure of rice seedlings to HS would affect subsequent Cd-induced toxicity of the second leaves, seedlings were pretreated with HS for 1, 2, and 3 h, respectively, under dark conditions. Rice seedlings pretreated with 1 h HS resulted in no protective effect on subsequent Cd-induced leaf chlorosis (Fig. 1a) and Cd-decreased chlorophyll content (Fig. 1b). However, a 2-h HS pretreatment exhibited a slight but significant reduction of Cd-induced leaf chlorosis (Fig. 1a) and Cd-decreased chlorophyll content (Fig. 1b). Three hours of HS exposure showed a complete inhibition of Cd-induced toxicity in rice seedlings (Fig. 1a,b).

In previous work, it has been demonstrated that Cd can induce oxidative stress in rice leaves, characterized by an increase in the contents of H_2O_2 and MDA (an indicator of lipid peroxidation; Hsu and Kao 2004;

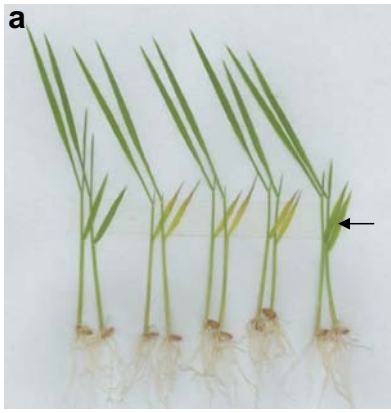


Fig. 1 Effects of $CdCl_2$ (0.5 mM) on chlorosis (**a**) and chlorophyll content (**b**) in the second leaves of rice seedlings pretreated with HS (45°C) for 1, 2, and 3 h, respectively, under dark conditions. *Arrow* indicates the second leaves. *Bars* indicate standard error (n=4). *Values with the same letter* are not significantly different at P<0.05

Kuo and Kao 2004). In the present study, we observed that the increase in the contents of H_2O_2 (Fig. 2a) and MDA (Fig. 2b) caused by $CdCl_2$ was more pronounced in non-HS leaves than in HS leaves.

The concentration of $CdCl_2$ used in the aforementioned study was 0.5 mM. We also conducted experiments with lower $CdCl_2$ (50 μM) applied over a longer period (6 days). Rice seedlings pretreated with 3 and 6 h HS was observed to exhibit protective effect on subsequent Cd-induced chlorosis (Fig. 3). Thus, the effects of HS on the response to lower $CdCl_2$ concentration are basically in accordance with those to higher $CdCl_2$ concentration.

Effect of HS pretreatment on Cd concentration in leaves of rice seedlings

Figure 4 shows that rice seedlings pretreated with 3 h HS had similar Cd concentration in the second leaves caused by $CdCl_2$ as those non-HS. These results suggest that the protective effect of HS on subsequent Cd-induced toxicity of the leaves is unlikely due the inhibition of Cd uptake or transport.

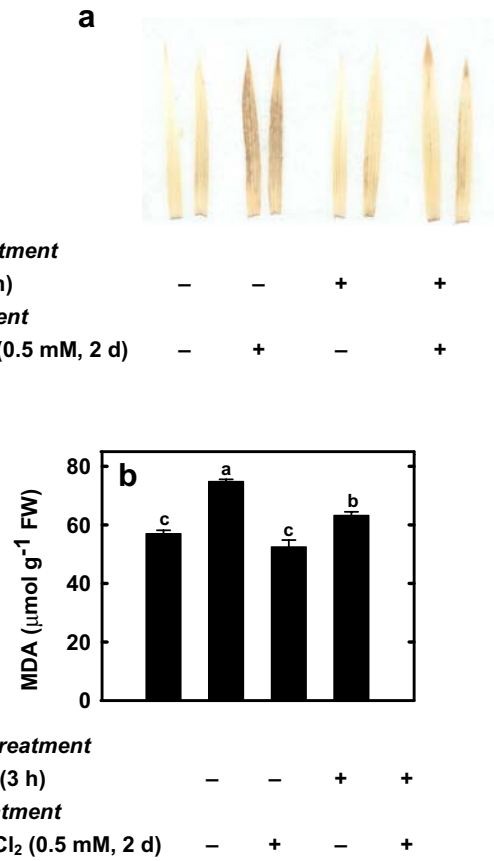


Fig. 2 Effects of $CdCl_2$ (0.5 mM) on H_2O_2 (**a**) and MDA (**b**) contents in the second leaves of rice seedlings pretreated with or without HS (45°C) for 3 h under dark conditions. The H_2O_2 content was visually detected in the second leaves by using DAB as substrate after 2 days of $CdCl_2$ treatment. To visualize DAB–H_2O_2 reaction product, leaves were decolorized in boiling ethanol. *Bars* indicate standard error (n=4). *Values with the same letter* are not significantly different at P<0.05

Pretreatment						
HS (h)	**0**	**0**	**3**	**3**	**6**	**6**
Treatment						
$CdCl_2$ (50 μM, 6 d)	**–**	**+**	**–**	**+**	**–**	**+**

Fig. 3 Effect of lower $CdCl_2$ concentration (50 μM) on chlorosis in the second leaves of rice seedlings pretreated with HS (45°C) for 3 and 6 h, respectively, under dark conditions

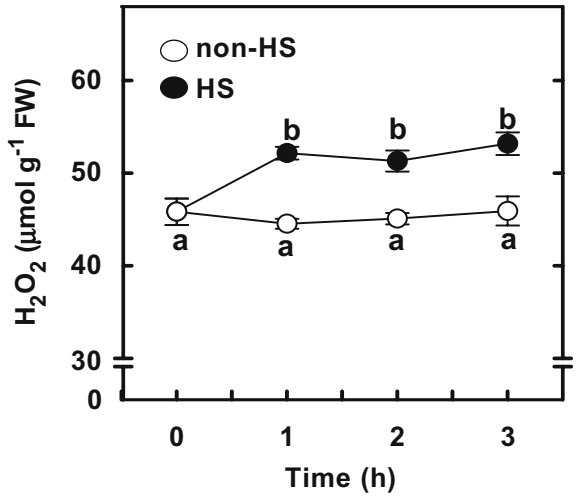
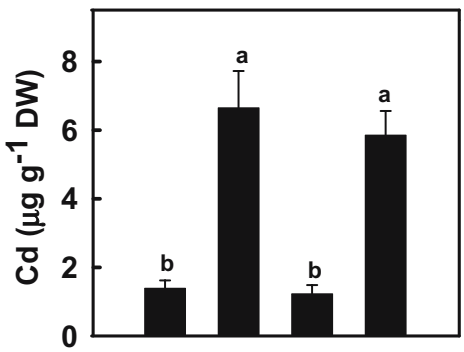


Fig. 5 Changes in H_2O_2 content in the second leaves of rice seedlings pretreated with or without HS (45°C) under dark conditions. Seedlings were exposed to HS for 1, 2, and 3 h and the second leaves were taken for determination of H_2O_2 contents. *Bars* indicate standard error (n=4). *Values with the same letter* are not significantly different at P<0.05

H_2O_2 content and antioxidant enzyme activities in response to HS

Changes in H_2O_2 content and antioxidant enzymes (SOD, APX, GR, and CAT) after exposure rice seedlings to HS were evaluated. As shown in Fig. 5, H_2O_2 content increased in leaves 1 h after HS exposure and subsequently remained unchanged. There was no difference in SOD and CAT activities between HS- and non-HS leaves (Fig. 6a,d). However, APX and GR activities in leaves of HS seedlings were higher than their respective non-HS, and it occurred after 2 h of HS treatment (Fig. 6b,c).



Pretreatment				
HS (3 h)	**-**	**-**	**+**	**+**
Treatment				
$CdCl_2$ (0.5 mM, 2 d)	**-**	**+**	**-**	**+**

Fig. 4 Effect of $CdCl_2$ (0.5 mM) on Cd concentration in the second leaves of rice seedlings pretreated with or without HS (45°C) for 3 h under dark conditions. Cd was determined 2 days after $CdCl_2$ treatment. *Bars* indicate standard error (n=4). *Values with the same letter* are not significantly different at P<005

The effect of NADPH oxidase inhibitor

To test if NADPH oxidase is responsible for H_2O_2 production during HS, imidazole (IMD), an inhibitor of NADPH oxidase inhibitor, was added to half-strength Kimura B solution at the time when the third leaves was fully expanded. Results in Fig. 7 show that HS-induced H_2O_2 production and protection against subsequent Cd stress can be counteracted by IMD (0.1 mM).

Effect of H_2O_2 pretreatment under non-HS condition on the tolerance of rice seedlings to Cd

To test if HS-increased H_2O_2 content is involved in the protection of rice seedlings against Cd toxicity, rice seedlings were first pretreated with H_2O_2 (in the range of 0.1–1 mM) for 3 h under non-HS conditions and then transferred to nutrient solution with or without $CdCl_2$. It was observed that pretreatment of rice

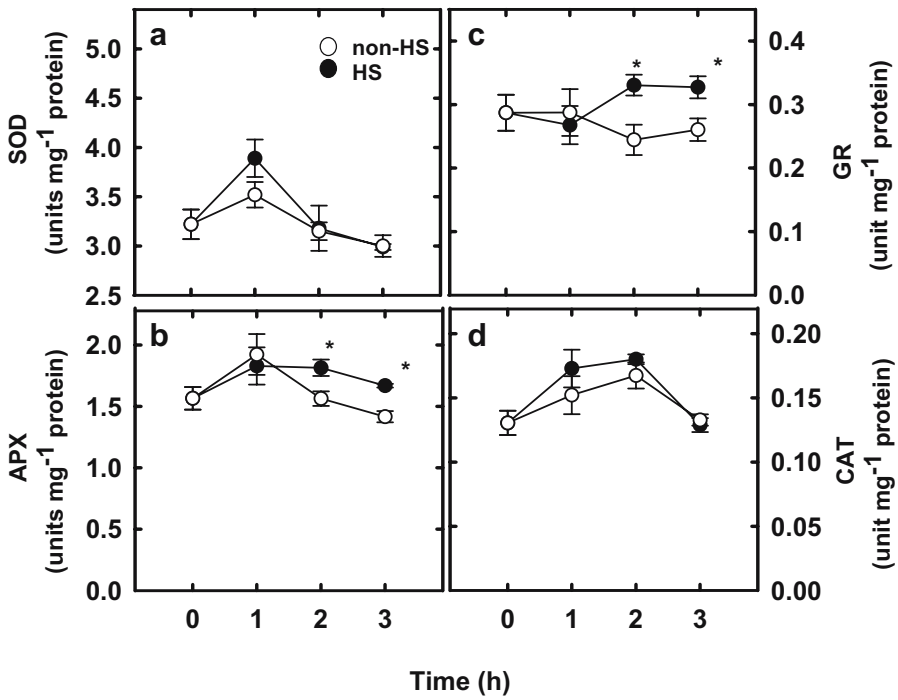


Fig. 6 Changes in the activities of SOD (**a**), APX (**b**), GR (**c**), and CAT (**d**) in the second leaves of rice seedlings pretreated with or without HS (45°C) under dark conditions. Rice seedlings were exposed to HS for 1, 2, and 3 h and the second leaves were taken for determination of each enzyme activity. *Bars* indicate standard error (n=4). *Values with the same letter* are not significantly different at $P<0.05$

seedlings with H_2O_2 greatly improved tolerance of rice seedlings to Cd (Fig. 8a,b). Figure 9a shows that addition of H_2O_2 (0.5 mM) to the basic nutrient solution of rice seedlings for 3 h under non-HS conditions resulted in an increase in H_2O_2 in the second leaves. Furthermore, rice seedlings pretreated with H_2O_2 under non-HS conditions had no effect on SOD activity (Fig. 9e) but showed an enhancement in APX, GR, and CAT activities in the second leaves (Fig. 9b–d).

Discussion

Cd causes chlorosis or chlorophyll loss (Hsu and Kao 2003) and oxidative stress (Kuo and Kao 2004) in rice seedlings. In the present study, we evaluated Cd toxicity by the chlorosis, decrease in chlorophyll content, and increase in H_2O_2 and MDA contents. On the basis of these criteria, we demonstrated that HS pretreatment of rice seedlings was able to protect rice seedlings from subsequent Cd stress [high $CdCl_2$ concentration (0.5 mM for 2 days) or low concentration (50 μM) for 6 days]. The protective effect of HS against subsequent Cd toxicity has also been described previously (Chen and Kao 1995a, b; Neumann et al. 1994; Orzech and Burke 1988).

It has been shown that a reduction of Cd-induced ethylene production was less effective in detached leaves from rice seedlings pretreated with HS for 24 h as compared with those for 6 and 12 h (Chen and Kao 1995a). In the present study, we also observed that the protective effect on Cd-induced chlorosis was more pronounced in a 3-h HS pretreatment than a 6-h HS (Fig. 3). All these results are basically in accordance with the idea that only a short duration of HS is required for seedlings to exhibit an acquired protection against Cd and other heavy metal toxicity (Gong et al. 2001; Orzech and Burke 1988).

The protective effect of HS against subsequent Cd toxicity of rice seedlings is unlikely due to inhibition of Cd uptake or transport. Because rice seedlings pretreated with 3 h HS had similar Cd concentration in leaves cause by $CdCl_2$ as those non-HS (Fig. 4).

Doke (1997) showed that early peak in H_2O_2 was observed within 15 min after HS of cell suspensions of potato leaf tissues. Exposure of whole tobacco seedlings to 40°C for 1 h in the light induced a significant increase in H_2O_2 (Foyer et al. 1997). A peak in H_2O_2 content in mustard seedlings was observed within 5 min transfer to the 45°C acclimation temperature (Dat et al. 1998). More recently, Gong et al. (2001) reported that the HS (42°C) pretreatment for 4 h produced an endogenous H_2O_2 peak in maize

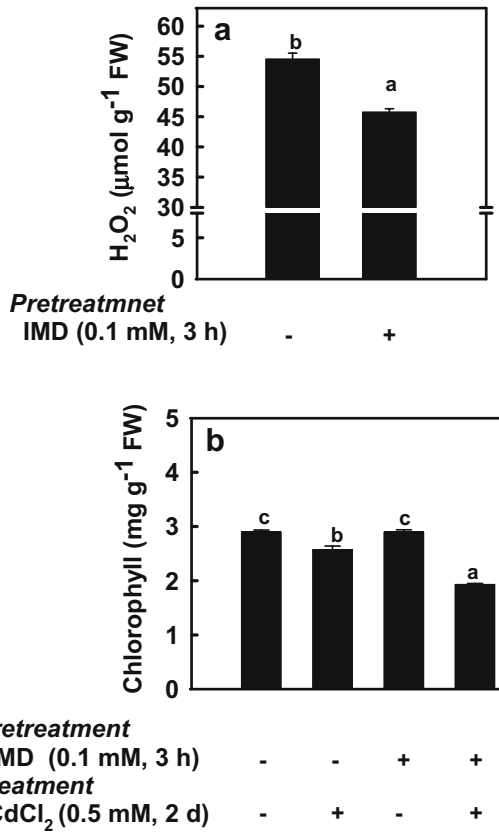


Fig. 7 Effect of IMD (0.1 mM) on HS-induced H_2O_2 production (**a**) and $CdCl_2$-incuced chlorophyll loss (**b**) in the second leaves of rice seedlings. Rice seedlings were pretreated with 3 h HS under dark conditions and then treated with or without 0.5 mM $CdCl_2$ for 2 days. IMD was added to half-strength Kimura B solution during 3 h HS. *Bars* indicate standard error (n=4). *Values with the same letter* are not significantly different at P<0.05

seedlings. Here, we show that HS pretreatment of rice seedlings resulted in an increase in H_2O_2 in 1 h (Fig. 5).

There is limited information about the mechanism of HS-induced H_2O_2 production. HS-induced accumulation of H_2O_2 in mustard seedlings has been suggested to be due to HS-deactivated CAT activity (Dat et al. 1998). This does not seem to be the case in rice seedlings, because HS had no effect on CAT activity (Fig. 6d). In several mode systems investigated in plants, the accumulation of H_2O_2 appears to be mediated by the activation of a plasma-membrane-bound NADPH oxidase complex (Orozco-Cárdenas 1999; Pei et al. 2000; Tsai et al. 2004; Zhang et al. 2001). It is not known whether HS-induced H_2O_2 accumulation in rice seedlings is mediated through NADPH oxidase complex. The fact that HS-induced H_2O_2 production in leaves of rice seedlings can be inhibited by IMD (Fig. 7a) suggests that HS-dependent H_2O_2 generation in rice leaves originated, at least in part, from plasma membrane NADPH oxidase. The involvement of NADPH oxidase in HS-induced H_2O_2 production in *Arabidopsis* has also been described recently (Volkov et al. 2006).

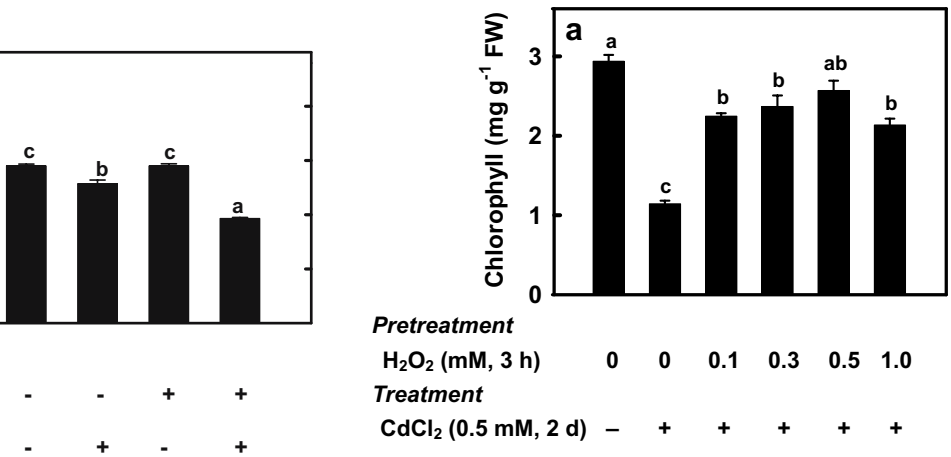


Fig. 8 Effects of $CdCl_2$ (0.5 mM) on chlorophyll content (**a**) and chlorosis (**b**) in the second leaves of rice seedlings pretreated with H_2O_2 for 3 h under non-HS (30°C) and dark conditions. Seedlings were pretreated with various H_2O_2 concentrations (0.1, 0.3, 0.5, and 1 mM). *Arrow* indicates the second leaves. *Bars* indicate standard error (n=4). *Values with the same letter* are not significantly different at P<0.05

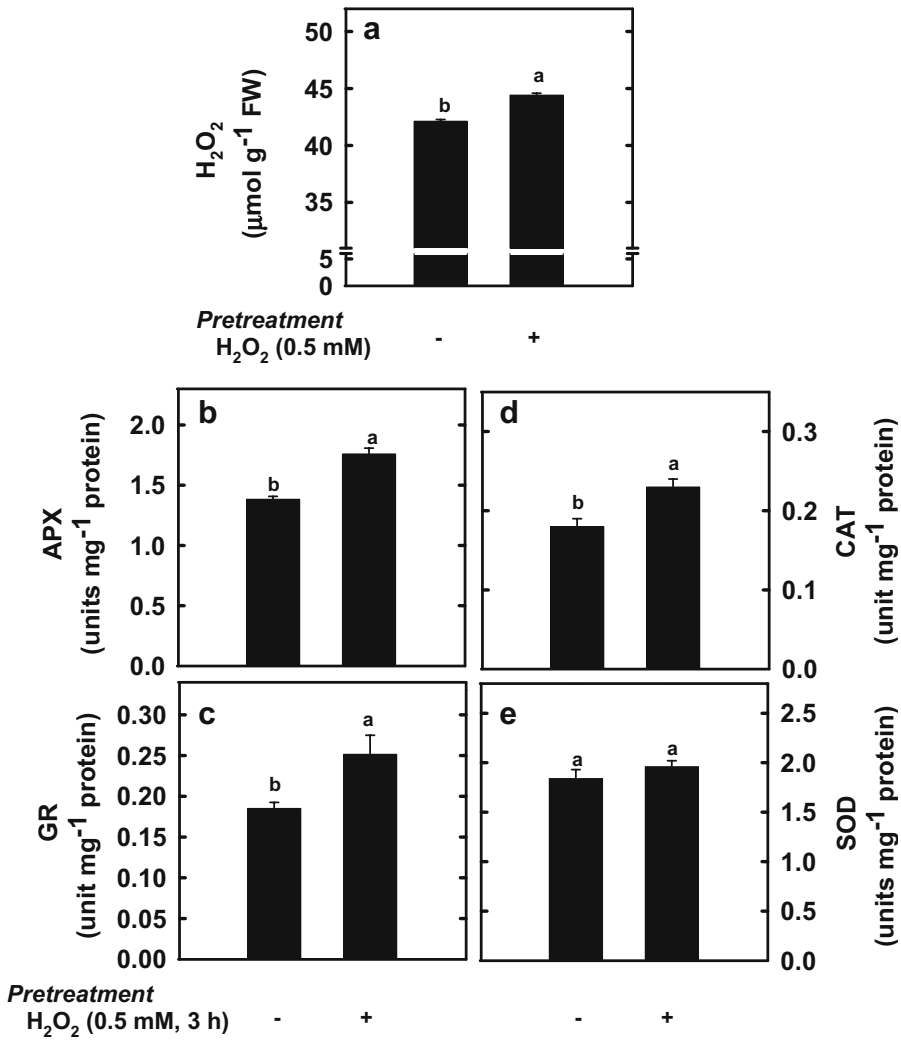


Fig. 9 Effects of H_2O_2 on the content of endogenous H_2O_2 (**a**) and the activities of APX (**b**), GR (**c**), CAT (**d**), and SOD (**e**) in the second leaves of rice seedlings. Seedlings pretreated with 0.5 mM H_2O_2 for 3 h under non-HS (30°C) and dark conditions. *Bars* indicate standard error (n=4). *Values with the same letter* are not significantly different at $P<0.05$

Gong et al. (2001) proposed that H_2O_2 is involved in HS-induced cross adaptation to heat, chilling, drought, and salt stress in maize seedlings. The present study also indicated that H_2O_2 was involved in HS-induced protection against subsequent Cd stress of rice seedlings. This conclusion was based on the observations that (a) endogenous H_2O_2 was higher in leaves of rice seedlings during HS than non-HS (Fig. 5), (b) HS-induced H_2O_2 production and protection against subsequent Cd stress can be counteracted by IMD, an inhibitor of NAPDH oxidase (Fig. 7a,b), and (c) pretreatment of rice seedlings with exogenous H_2O_2 under non-HS conditions, which increased endogenous H_2O_2 content, greatly improved tolerance of rice seedlings to Cd (Figs. 8 and 9a).

It has been shown that various abiotic stresses induce oxidative stress and improvement of stress tolerance is often related to increase in activities of antioxidant enzymes (Alscher et al. 1997; Noctror and Foyer 1998). Heat protection of wheat is improved by keeping APX and GR activities high (Kraus and Flecher 1994), and GR activity is significantly enhanced during heat-induced thermoprotection in mustard seedlings (Dat et al. 1998). Exposure of alfalfa to supra-optimal temperature leads to increases in APX and CAT (Badiani et al. 1997). Evidence was also provided to show that HS induction of *APXa* gene could be the possible cause of reduced chilling injury in rice seedlings (Sato et al. 2001). Here, we report that HS pretreatment in rice seedlings resulted in higher APX and GR activities than non-HS (Fig. 6b,c).

Because H_2O_2 is relatively stable and diffusable through membranes (in contrast with superoxide), it may be a perfect candidate to act as a signal molecule during stress responses (Van Breusegem et al. 2001). In fact, H_2O_2 is now considered as a signal molecule that induces gene expression of antioxidant enzymes related to stress tolerance (Foyer et al. 1997; Morita et al. 1999; Prasad et al. 1994a, b).

Elevated levels of SOD, CAT, and APX have been shown to be correlated with the development of HS-induced chilling tolerance (Kang and Saltveit 2001). In previous work, we demonstrated that Cd can induce oxidative stress in rice leaves (Hsu and Kao 2004; Kuo and Kao 2004). Cho and Seo (2005) reported that seedlings of Cd-resistant *Arabidopsis* had higher activities of SOD, APX, and GR and experienced lower oxidative stress from Cd exposure.

The time-course analyses of HS in rice seedlings clearly indicated that H_2O_2 accumulation occurs first and then APX and GR activities increase (Figs. 5 and 6). Exogenously supplied H_2O_2 to rice seedlings under non-HS conditions also increased APX and GR activities (Fig. 9b,c) and protected against subsequent Cd stress (Fig. 8). All these results have led us to conclude that early accumulation of H_2O_2 during HS signals the increase in APX and GR activities, which in turn prevents rice seedlings from the oxidative damage caused by Cd.

In the present study, we show that HS pretreatment had no effect on CAT activity in rice leaves (Fig. 6d). However, exogenous application of H_2O_2 under non-HS conditions enhanced CAT activity (Fig. 9d). The discrepancy in the regulation of CAT activity of rice seedlings in response to HS and exogenous H_2O_2 under non-HS conditions is more probable due to the difference in the amount of H_2O_2 in leaves (Figs. 5 and 9a).

Glutathione (GSH) is an important compound of the antioxidant system, which scavenge ROS under oxidative conditions. One mechanism of increasing tolerance to Cd or other heavy metals may be the formation of GSH. The protective role of GSH on chilling stress and in reducing Cd or Ni toxicity has been reported (Chen and Kao 1995b; Freeman et al. 2004; Kocsy et al. 2000; Xiang et al. 2001). Nieto-Sotelo and Ho (1986) demonstrated that maize roots under HS conditions had high GSH content than those under non-HS. Furthermore, H_2O_2 treatment induced GSH accumulation has also been described previously (Yu et al. 2002; 2003). An alternative mechanism to explain the HS-acquired Cd tolerance may be related to HS-produced heat shock proteins (Kochhar and Kochhar 2005; Neumann et al. 1994; Susuki et al. 2001; Volkov et al. 2006). It is important to note that the participation of H_2O_2 in HS-induced expression of heat shock proteins has been demonstrated (Volkov et al. 2006). Whether HS-induced Cd tolerance of rice seedlings is due to H_2O_2-mediated GSH biosynthesis and/or expression of heat shock proteins remains to be established.

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