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REGULAR ARTICLE

Involvement of glutathione in heat shock– and hydrogen peroxide–induced cadmium tolerance of rice (*Oryza sativa* L.) seedlings

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Abstract The role of reduced glutathione (GSH) in heat shock (HS)- and H_2O_2-induced protection of rice (*Oryza sativa* L., cv. Taichung 1) seedlings from Cd stress was investigated. HS- and H_2O_2-pretreatment resulted in an increase in GSH content in leaves of rice seedlings. Addition of exogenous GSH under non-HS conditions, which resulted in an increase in GSH in leaves, enhanced subsequent Cd tolerance of rice seedlings. Pretreatment with buthionine sulfoximine (BSO), a specific inhibitor of GSH synthesis, which effectively inhibited GSH content induced by HS and H_2O_2, reduced subsequent Cd tolerance. Furthermore, the effect of BSO on HS- and H_2O_2-induced GSH accumulation and toxicity by subsequent Cd stress can be reversed by the addition of GSH. The time-course analyses of HS in rice seedlings demonstrated that the accumulation of H_2O_2 preceded the increase in GSH. Based on the data obtained in this study, it could be concluded that the early accumulation of H_2O_2 during HS signals the increase in GSH content, which in turn protects rice seedlings from oxidative damage caused by Cd.

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

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Abbreviations

APX	Ascorbate peroxidase
BSO	Buthionine sulfoximine
γ-EC	γ-Glutamylcysteine
γ-ECS	γ-EC synthetase
FW	Initial fresh weight
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Glutathione disulfide
HS	Heat shock
ROS	Reactive oxygen species

Introduction

Heavy metal pollution has become a serious environmental and health problem. Cadmium (Cd) is a toxic element found in various amounts in the soil. Plants take up Cd from the soil (Greger and Landberg 2008; Jarvis et al. 1976). Cd damages roots, reduces nutrient and water uptake, impairs photosynthesis, induces chlorosis, inhibits growth and finally results in cell death (Das et al. 1998). Although it does not directly produce hydroxyl radicals through Fenton or Haber-Weiss reactions, Cd can produce reactive oxygen species (ROS) including H_2O_2 and generates an oxidative stress (Cho and Seo 2005; Garnier et al. 2006; Hsu and Kao 2007a; Schützendübel and Polle 2002).

Glutathione (γ-glutamylcystenylglycine, GSH) is widely distributed in eukaryotes and is an essential

component of the cellular antioxidative defence system, which keeps ROS under control (Foyer and Nocotor 2005; Noctor and Foyer 1998; Rouhier et al. 2008). GSH is synthesized from two consecutive ATP-dependent reactions (Mendoza-Cózatl et al. 2005). In the first step γ-glutamylcysteine (γ-EC) is formed from L-glutamate and L-cysteine by γ-EC synthetase (γ-ECS). The second step is catalyzed by glutathione synthetase which adds glycine to the C-terminal of γ-EC forming GSH. When GSH is oxidized as part of its antioxidant activity, it forms glutathione disulfide (GSSG), the oxidized form of GSH. The glutathione reductase (GR) reduces GSSG back to GSH at the expense of NADPH. The ability to synthesize GSH appears to be crucial for protection from Cd, as shown by the increased tolerance of plants with elevated levels of GSH as well as a decreased tolerance in plants with diminished levels of GSH (Howden et al. 1995; Pilon-Smits et al. 2000; Xiang et al. 2001; Zhu et al. 1999).

Prior exposure to heat shock (HS) temperatures has been shown to exhibit an acquired protection against Cd stress (Chen and Kao 1995a; Hsu and Kao 2007b; Neumann et al. 1994; Orzech and Burke 1988). Nieto-Sotelo and Ho (1986) demonstrated that maize roots under HS conditions have higher GSH level than those under non-HS. However, no data are available showing that HS-induced Cd tolerance is due to an enhancement of GSH biosynthesis in plants.

Because H_2O_2 is relatively stable and diffusible through membranes (in contrast with superoxide), it is now considered as a signal molecule 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 tolerance to chilling, heat, salt, and drought stresses (Azevedo Neto et al. 2005; Dat et al. 1998; Gechev et al. 2002; Gong et al. 2001; Lopez-Delgado et al. 1998; Prasad et al. 1994a, b; Uchida et al. 2002; Wahid et al. 2007; Yu et al. 2002; 2003). The H_2O_2-induced chilling tolerance in mung bean was established to be associated with an elevation of GSH (Yu et al. 2002; 20003).

Our recent data (Hsu and Kao 2007b) demonstrated that enhanced tolerance of rice seedlings to Cd stress was obtained by prior treatment of HS (3 h at 45°C in darkness) or H_2O_2. In this study, we investigated the possible involvement of GSH in HS- and H_2O_2-induced Cd tolerance of rice seedlings.

Materials and methods

Plant material and treatments

Rice (*Oryza sativa* L., cv. Taichung Native 1) 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 or H_2O_2 pretreatment and Cd 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. For H_2O_2 pretreatment, 0.5 mM H_2O_2 was added to nutrient solution for 3 h under non-HS conditions. Rice seedlings were then grown in basic nutrient solution with or without 5 μM $CdCl_2$ under natural sunlight at 30/25°C day/night for 7 days. Cd toxicity was evaluated by the chlorosis and lipid peroxidation (increase in the content of malondialdehyde, MDA) of the second leaves.

Determinations of MDA, GSH, and GSSG

MDA was extracted with 5% (*w/v*) trichloroacetic acid and determined by the thiobabituric acid reaction as described by Heath and Packer (1968). GSH and GSSG in 3% sulfosalicylic acid extract were analyzed according to Tsai et al. (2004). GSH and GSSG

contents were expressed on the basis of initial fresh weight (FW).

Extraction and assays of ascorbate peroxidase (APX) and glutathione reductase GR) To measure APX and GR, leaf tissues were homogenized with 0.1 M sodium phosphate buffer (pH 6.8) in a chilled pestle and mortar. For analysis of APX activity, 2 mM ascorbate 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. APX activity was determined according to Nakano and Asada (1981). One unit of activity for APX was defined as the amount of enzyme that degraded 1 μmol of ASC per min. 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 min. 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

HS– and H_2O_2–induced Cd tolerance

To test if pretreatment of rice seedlings with HS would affect subsequent Cd-induced toxicity of the second leaves, rice seedlings were pretreated with HS for 3 h under dark conditions and then treated with 5 μM $CdCl_2$ for 7 days. It was observed that a 3-h HS pretreatment exhibited a complete reduction of Cd-induced leaf chlorosis (Fig. 1a) and lipid peroxidation in the second leaves (Fig. 2a).

To examine the possible involvement of H_2O_2 in the protection of rice seedlings against Cd toxicity, rice seedlings were first pretreated with 0.5 mM H_2O_2 for 3 h and then transferred rice seedlings to nutrient solution with or without 5 μM $CdCl_2$ for 7 days. Results demonstrated that the chlorosis and the increase in the content of MDA caused by $CdCl_2$ were reduced by H_2O_2 pretreatment (Fig. 1b and Fig. 2a). We also observed that HS or H_2O_2 pretreatment exhibited a reduction of Cd-decreased GSH content (Fig. 2b) and Cd-increased APX and GR activities (Fig. 2c,d) in rice leaves.

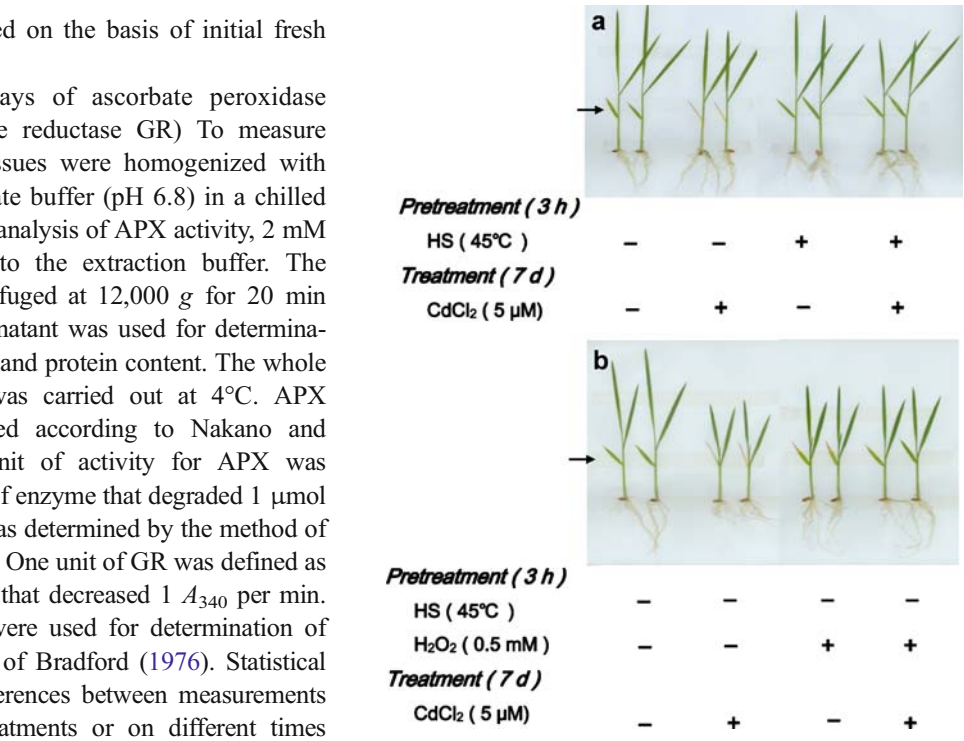

Fig. 1 Effect of $CdCl_2$ on chlorosis in the second leaves of rice seedlings pretreated with HS **a** and H_2O_2 **b**. Rice seedlings were pretreated with HS (45°C) or 0.5 mM H_2O_2 under non-HS (30°C) conditions for 3 h and then treated with or without 5 μM $CdCl_2$ for 7 days. *Arrow* indicates the second leaves

GSH content and the GSH/GSSG ratio in HS– and H_2O_2–treated leaves

Not only GSH content but also the GSH/GSSG ratio was increased in leaves of rice seedlings pretreated with HS or H_2O_2 (Fig. 3). These results suggest that GSH may play an important role in HS– and H_2O_2–induced tolerance of rice to subsequent Cd stress.

Pretreatment with GSH under non-HS conditions

GSH content was increased in leaves of rice seedlings pretreated with 1 mM GSH under non-HS conditions (Fig. 4a). It is also shown that APX and GR activities were increased in leaves of rice seedlings pretreated with GSH under non-HS conditions (Fig. 4b,c). When rice seedlings pretreated with GSH under non-HS conditions were transferred to nutrient solution with or without 5 μM $CdCl_2$ for 7 days, Cd tolerance was greatly improved (Fig. 5a,b).

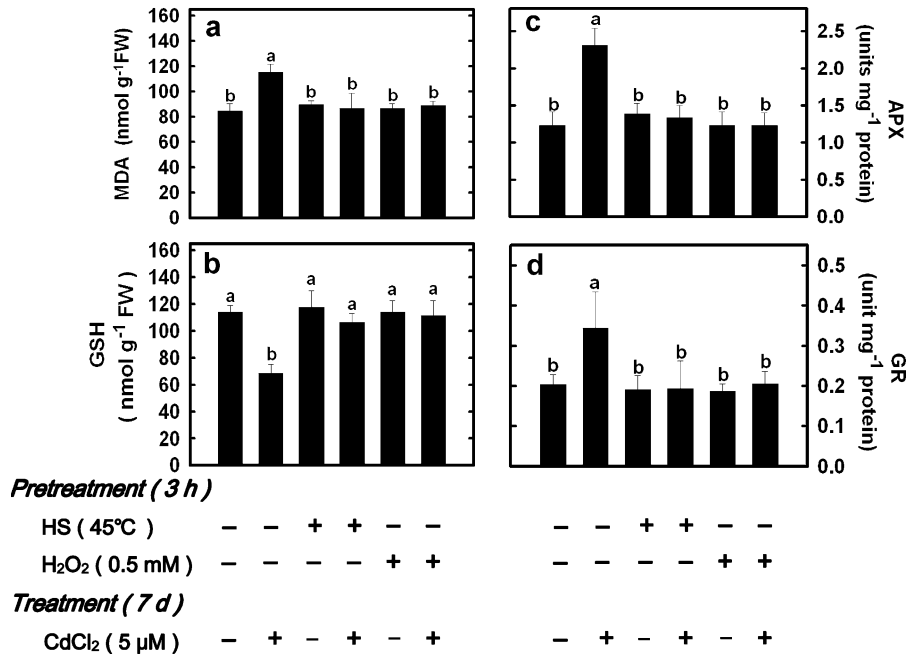

Fig. 2 Effect of $CdCl_2$ on the contents of MDA **a** and GSH **b** and the activities of APX **c** and GR **d** in the second leaves of rice seedlings pretreated with HS and H_2O_2. Rice seedlings were pretreated with HS (45°C) or 0.5 mM H_2O_2 under non-HS conditions for 3 h and then treated with or without 5 μM $CdCl_2$ for 7 days. *Bars* indicate standard error (n=4). *Values with the same letter* are not significantly different at P<0.05

Pretreatment with BSO

BSO is a specific and potent inhibitor of γ-ECS (Griffith and Meister 1979). When rice seedlings were pretreated with 0.5 mM BSO for 3 h under HS conditions, the second leaves had lower GSH content than those pretreated without BSO under HS conditions (Fig. 6a). Results also show that that activities of APX and GR were decreased in leaves of rice seedlings pretreated with BSO under HS conditions (Fig. 6b,c). To confirm the involvement of GSH in HS-induced Cd tolerance, BSO-pretreated rice seedlings under HS conditions were transferred to nutrient solution with 5 μM $CdCl_2$ for 7 days. It was observed that pretreatment of rice seedlings with BSO under HS condition enhanced Cd toxicity (Fig. 7a,b). The effects of BSO under HS conditions on the content of GSH, the activities of APX and GR, and the toxicity of Cd were reversed by the application of GSH (Fig. 6 and Fig. 7).

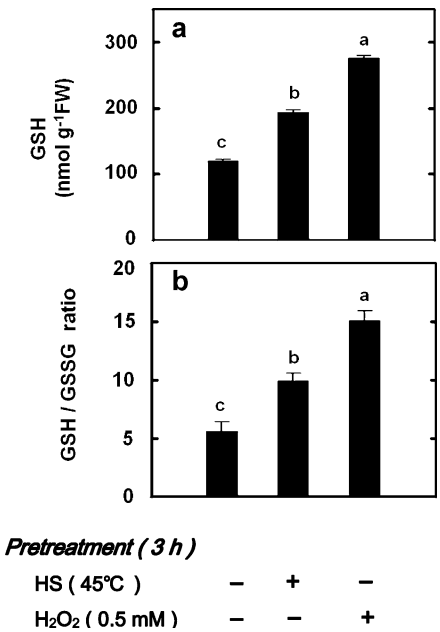

Fig. 3 Effect of HS and H_2O_2 on GSH content **a** and the GSH/GSSG ratio **b** in the second leaves of rice seedlings. Rice seedlings were treated with HS (45°C) or 0.5 mM H_2O_2 under non-HS conditions for 3 h. *Bars* indicate standard error (n=4). *Values with the same letter* are not significantly different at P<0.05

The increase in the content of GSH and the activities of APX and GR in leaves caused by H_2O_2 was reduced by the addition of BSO (Fig. 8a,b,c). Interestingly, rice seedlings pretreated with H_2O_2 + BSO were less tolerant to subsequent Cd stress than those pretreated with H_2O_2 only (Fig. 9a,b). The effects of BSO on H_2O_2-increased GSH content, APX and GR activities, and Cd toxicity can be restored by the addition of GSH (Fig. 8 and Fig. 9).

Change in H_2O_2 and GSH contents during HS

Changes in H_2O_2 and GSH contents after exposure rice seedlings to HS were evaluated. As shown in

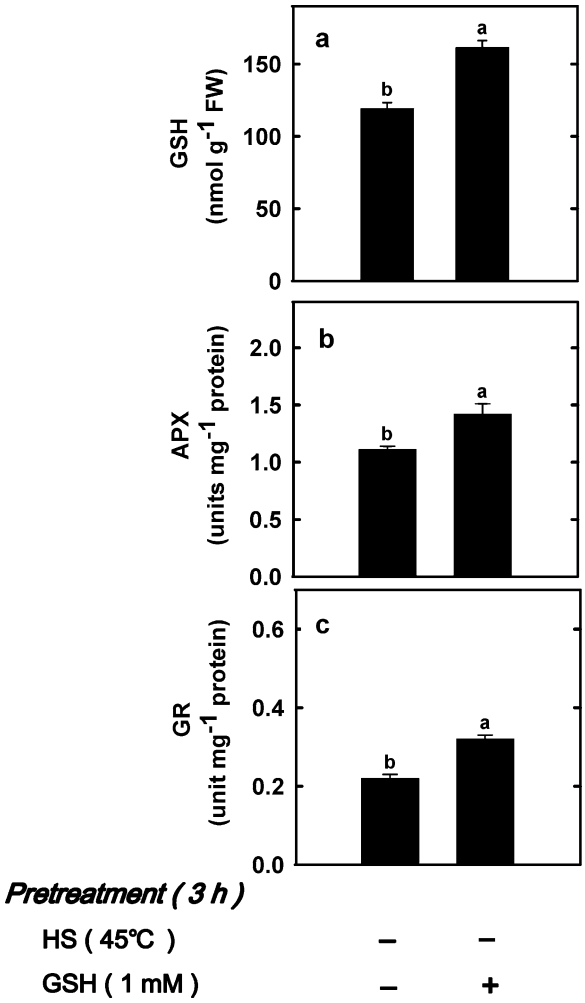

Fig. 4 Effect of GSH pretreatment on the content of GSH **a** and the activities of APX **b** and GR **c** in the second leaves of rice seedlings. Rice seedlings were pretreated with 1 mM GSH for 3 h under non-HS conditions. *Bars* indicate standard error (n=4). *Values with the same letter* are not significantly different at P<0.05

(Fig. 10a), H_2O_2 content increased in leaves 1 h after HS exposure and subsequently decreased slightly. GSH content in leaves of HS seedlings was higher than their respective non-HS, but it occurred 3 h after treatment (Fig. 10b).

Discussion

The most general symptom of Cd toxicity of plants is leaf chlorosis (Das et al. 1997). When rice seedlings were treated with $CdCl_2$, chlorosis was first observed in the second leaves (Hsu and Kao 2003). The formation of MDA (thiobarbituric acid reactive substances) is an indicator of ROS generation in the tissues, and it may be used as a reliable index of lipid peroxidation in biological systems (Heath and Packer 1968). In our previous work, it has been shown that Cd can induce oxidative stress in rice leaves, characterized by an increase in the content of MDA (Hsu and Kao 2005; Kuo and Kao 2004). Thus, in the present study, Cd toxicity was assessed by the chlorosis and lipid peroxidation of the second leaves. On the basis of these criteria, we demonstrated that HS pretreatment protected rice seedlings from subsequent stress caused by 5 μM $CdCl_2$ (Fig. 1a and Fig. 2a). It has previously been shown that Cd treatment causes a decrease in APX and GR activities in leaves of rice seedlings (Hsu and Kao 2005; Kuo and Kao 2004). Here we observed that these Cd-

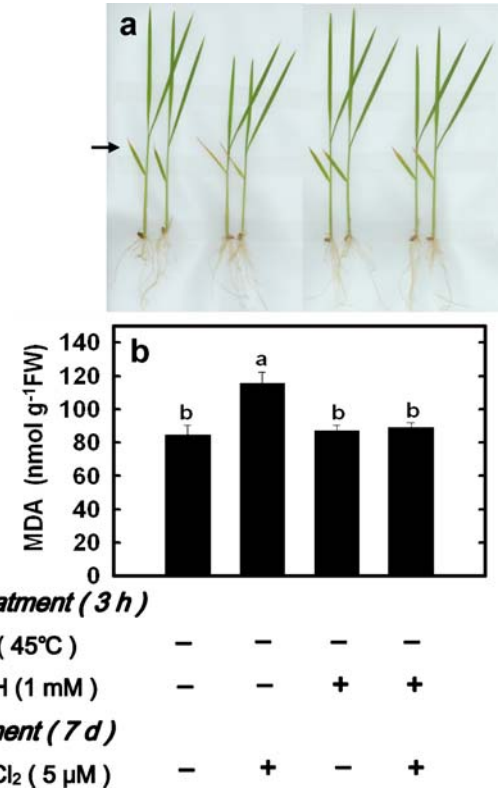

Fig. 5 Effect of $CdCl_2$ on chlorosis and lipid peroxidation in the second leaves of rice seedlings pretreated with or without GSH. Rice seedlings were pretreated with 1 mM GSH under non-HS (30°C) conditions for 3 h and then treated with or without 5 μM $CdCl_2$ for 7 days. *Arrow* indicates the second leaves

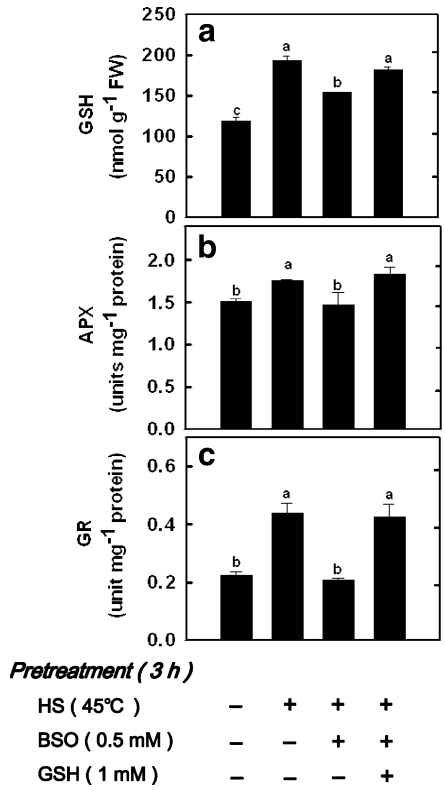

Fig. 6 Effect of BSO and GSH pretreatments on the content of GSH **a** and the activities of APX **b** and GR (**c**) in the second leaves of rice seedlings under HS (45°C) conditions. All measurements were made 3 h after pretreatment. The concentrations of GSH and BSO are 1 mM and 0.5 mM, respectively. *Bars* indicate standard error (n=4). *Values with the same letter* are not significantly different at $P<0.05$

induced changes can be reduced by a HS pretreatment (Fig. 2b,c,d). We have recently reported that a 3-h HS exposure prevents the toxicity of high $CdCl_2$ concentrations (50 μM and 500 μM) (Hsu and Kao 2007b). Basically, our results are in agreement with the idea that HS protects against Cd-dependent oxidative stress (Gong et al. 2001; Orzech and Burke 1988).

GSH plays an important role in physiological functions such as redox regulation, conjugation of metabolites, detoxification of xenobiotics and homeostasis and cellular signaling that trigger adaptive responses (Foyer and Noctor 2005; Noctor et al. 2002; Rouhier et al. 2008). Years ago it has been shown that GSH in mammalian cells increases during HS (Mitchel et al. 1983). Nieto-Sotelo and Ho (1986) were the first to show that elevated synthesis of GSH occurs during HS in plant cells. In the present study, we demonstrated that GSH content and the GSH/GSSG ratio increased in leaves of rice seedlings pretreated with HS (Fig. 3). We also observed that BSO reduced HS-induced GSH accumulation (Fig. 6a), suggesting that HS-increased GSH content may be due to the increase in GSH synthesis. Yu et al. (2002, 2003) demonstrated that H_2O_2 treatment induced GSH accumulation in mung bean and *Phalaenopsis*. Our results also show that H_2O_2 treatment was able to increase the synthesis of GSH under non-HS conditions (Fig. 3, Fig. 8a).

Several authors have shown that an elevated GSH content is correlated with the ability of plants to withstand Cd-induced oxidative stress (Chen and Goldsbrough 1994; Chen and Kao 1995b; Howden et al. 1995; Pilon-Smits et al. 2000; Rüegsegger et al.1990; Xiang et al. 2001; Zhu et al. 1999). The present study indicates that HS–and H_2O_2–induced protection against subsequent Cd stress of rice seedlings are mediated through GSH. This conclusion was based on the observations that (i) HS–and H_2O_2–pretreatment resulted in an increase in GSH content in leaves of rice seedlings (Fig. 3) and enhancement of subsequent Cd tolerance (Fig. 1 and Fig. 2), (ii) exogenous application of GSH under non-HS conditions, which resulted in an increase in GSH in leaves (Fig. 4 a), enhanced subsequent Cd tolerance

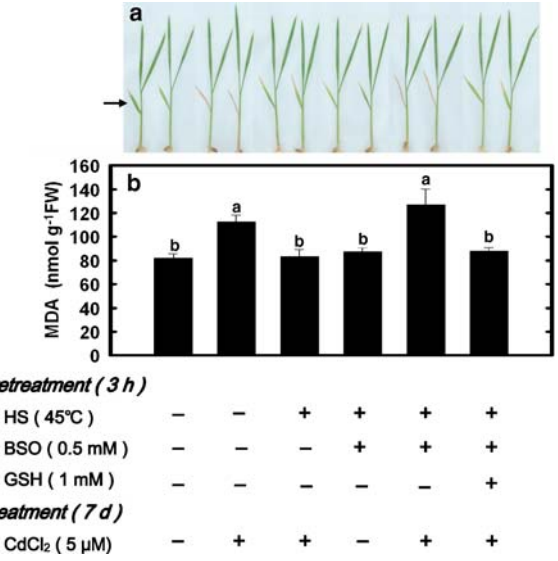

Fig. 7 Effect of $CdCl_2$ on chlorosis **a** and lipid peroxidation **b** in the second leaves of rice seedlings pretreated with BSO and BSO + GSH under HS (45°C) conditions. Rice seedlings were pretreated with BSO and BSO + GSH for 3 h and then treated with $CdCl_2$ for 7 days. The concentrations of GSH, BSO, and $CdCl_2$ are 1 mM, 0.5 mM, and 5 μM, respectively. *Arrow* indicates the second leaves

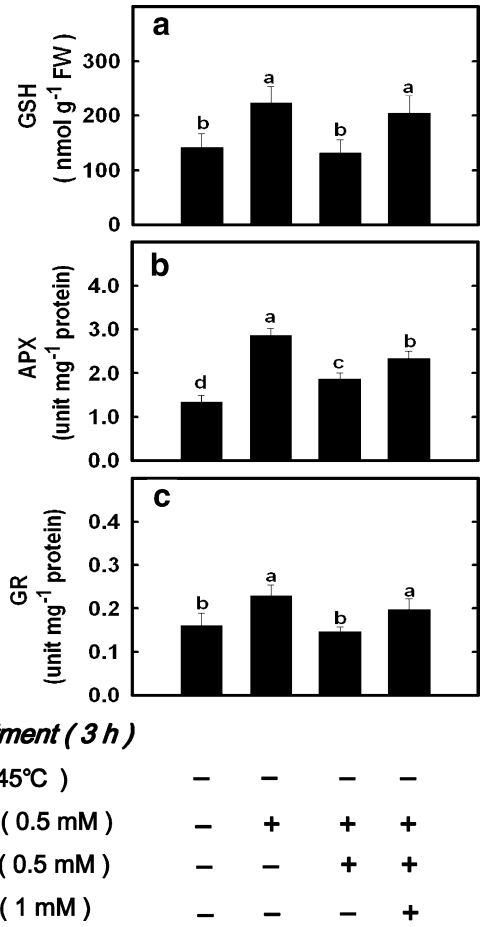

Fig. 8 Effect of H_2O_2, BSO, and BSO + GSH pretreatments on the content of GSH **a** and the activities of APX **b** and GR **c** in the second leaves of rice seedlings under non-HS (30°C) conditions. All measurements were made 3 h after pretreatment. The concentrations of H_2O_2, GSH and BSO are 0.5 mM, 1 mM, and 0.5 mM, respectively. *Bars* indicate standard error (n=4). *Values with the same letter* are not significantly different at P<0.05

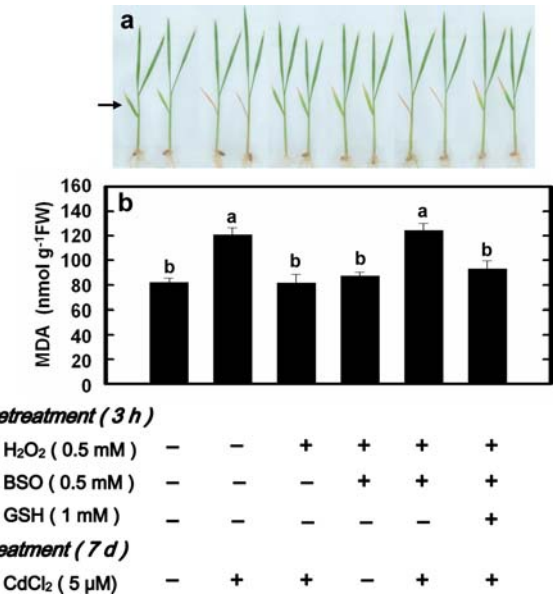

Fig. 9 Effect of $CdCl_2$ on chlorosis **a** and lipid peroxidation **b** in the second leaves of rice seedlings pretreated with H_2O_2, BSO or BSO + GSH under non-HS (30°C) conditions. Rice seedlings were pretreated with BSO and BSO + GSH for 3 h and then treated with $CdCl_2$ for 7 days. The concentrations of GSH, BSO, and $CdCl_2$ are 1 mM, 0.5 mM, and 5 μM, respectively. *Arrow* indicates the second leaves

of rice seedlings (Fig. 5), (iii) BSO, a specific inhibitor of GSH synthesis (Griffith and Meister 1979), pretreatment, which effectively inhibited the increase GSH content caused by HS (Fig. 6a) and H_2O_2 (Fig. 8a), reduced subsequent Cd tolerance (Fig. 7 and Fig. 9), and (iv) the effect of BSO on HS– and H_2O_2–induced GSH accumulation and toxicity to subsequent Cd stress can be reversed by the addition of GSH (Fig 6a, Fig. 7, Fig. 8, and Fig. 9).

The involvement of GSH as an inducer of the stress response has been described before (Hérouart et al. 1993; Noctor et al. 2002; Wingsle and Karpinski 1996). In their work with maize seedlings, Kocsy et al.

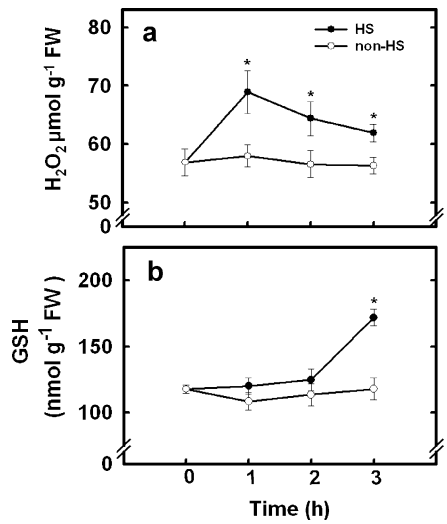

Fig. 10 Changes in the contents of H_2O_2 **a** and GSH **b** in the second leaves of rice seedlings treated with or without HS (45°C) under dark conditions. Seedlings were exposed to HS for 1, 2, and 3 h and the second leaves were taken for determination of H_2O_2 and GSH contents. *Bars* indicate standard error (n=4). *Values with the same letter* are not significantly different at P< 0.05

(2000, 2001) established a linear relationship between GSH content and GR activity. Here, we observed that treatment with GSH under non-HS conditions resulted in an increase in the content of GSH and the activities of APX and GR in leaves of rice seedlings (Fig. 4). BSO treatment, which reduced the content of GSH in leaves of rice seedlings increased by HS and H_2O_2 treatments (Fig. 6a and Fig. 8a), caused the decrease in the activities of APX and GR (Fig. 6b,c and Fig. 8b,c). The effect of BSO on the content of GSH and the activities of APX and GR was reversed by the application of exogenous GSH (Fig. 6 and 8). It appears that GSH is involved in regulating the activities of APX and GR in rice leaves.

The time courses of responses after HS (Fig. 10) suggest that the following sequence of events may take place: HS → H_2O_2 → GSH. Exogenously supplied H_2O_2 to rice seedlings under non-HS conditions also increased GSH content (Fig. 3) and protected against subsequent Cd stress (Fig. 1b and Fig. 2). All these results have led us to conclude that early accumulation of H_2O_2 during HS signals the increase in GSH content, which in turn prevents rice seedlings from oxidative damage by Cd.

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