

# Toxicity in leaves of rice exposed to cadmium is due to hydrogen peroxide accumulation

Yi Ting Hsu · Ching Huei Kao

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**Abstract** The production of H<sub>2</sub>O<sub>2</sub> in detached rice leaves of Taichung Native 1 (TN1) caused by CdCl<sub>2</sub> was investigated. CdCl<sub>2</sub> treatment resulted in H<sub>2</sub>O<sub>2</sub> production in detached rice leaves. Diphenyleneiodonium chloride (DPI) and imidazole (IMD), inhibitors of NADPH oxidase (NOX), prevented CdCl<sub>2</sub>-induced H<sub>2</sub>O<sub>2</sub> production, suggesting that NOX is a H<sub>2</sub>O<sub>2</sub>-generating enzyme in CdCl<sub>2</sub>-treated detached rice leaves. Phosphatidylinositol 3-kinase inhibitors wortmanin (WM) or LY294002 (LY) inhibited CdCl<sub>2</sub>-induced H<sub>2</sub>O<sub>2</sub> production in detached rice leaves. Exogenous H<sub>2</sub>O<sub>2</sub> reversed the inhibitory effect of WM or LY, suggesting that phosphatidylinositol 3-phosphate is required for Cd-induced H<sub>2</sub>O<sub>2</sub> production in detached rice leaves. Nitric oxide donor sodium nitroprusside (SNP) was also effective in reducing CdCl<sub>2</sub>-induced accumulation of H<sub>2</sub>O<sub>2</sub> in detached rice leaves. Cd toxicity was judged by the decrease in chlorophyll content. The results indicated that DPI, IMD, WM, LY, and SNP were able to reduce Cd-induced toxicity of detached rice leaves. Twelve-day-old TN1 and Tainung 67 (TNG67) rice seedlings were treated with or without CdCl<sub>2</sub>. In

terms of Cd toxicity (leaf chlorosis), it was observed that rice seedlings of cultivar TN1 are Cd-sensitive and those of cultivar TNG67 are Cd-tolerant. On treatment with CdCl<sub>2</sub>, H<sub>2</sub>O<sub>2</sub> accumulated in the leaves of TN1 seedlings but not in the leaves of TNG67. Prior exposure of TN1 seedlings to 45°C for 3 h resulted in a reduction of H<sub>2</sub>O<sub>2</sub> accumulation, as well as Cd tolerance of TN1 seedlings treated with CdCl<sub>2</sub>. The results strongly suggest that Cd toxicity of detached leaves and leaves attached to rice seedlings are due to H<sub>2</sub>O<sub>2</sub> accumulation.

**Keywords** Cadmium · Hydrogen peroxide · NADPH oxidase · *Oryza sativa* · Phosphatidylinositol 3-kinase · Phosphatidylinositol 3-phosphate

## Abbreviations

|      |                                  |
|------|----------------------------------|
| ASC  | Ascorbate                        |
| DAB  | 3,3-Diaminobenzidine             |
| DMSO | Dimethyl sulfoxide               |
| DPI  | Diphenyleneiodonium chloride     |
| HS   | Heat shock                       |
| IMD  | Imidazole                        |
| LY   | 294002                           |
| NO   | Nitric oxide                     |
| NOX  | NADPH oxidase (EC 1.6.99.6)      |
| PI3K | Phosphatidylinositol 3-kinase    |
| PI3P | Phosphatidylinositol 3-phosphate |
| ROS  | Reactive oxygen species          |
| SNP  | Sodium nitroprusside             |

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

|       |                   |
|-------|-------------------|
| TN1   | Taichung Native 1 |
| TNG67 | Tainung 67        |
| WM    | Wortmannin        |

## Introduction

Reactive oxygen species (ROS) have been characterized as the key factor on the responses of plants to both biotic and abiotic stress (Apel and Hirt 2004). Initially, ROS were only regarded as damaging to cells. More recently, ROS emerged as ubiquitous signaling molecules participating in the recognition of and the response to stress factors (Foyer and Noctor 2005).

Inside plant cells, ROS can be produced in chloroplasts, mitochondria, peroxisome, glyoxysomes, byproducts of metabolic processes such as photosynthesis and respiration (Apel and Hirt 2004). Several biochemical mechanisms have been proposed to explain ROS production. Apoplastic H<sub>2</sub>O<sub>2</sub> production can be mediated by cell-wall peroxidase, germin-like oxalate oxidases or amine oxidases (Apel and Hirt 2004; Mittler et al. 2004). However, it has been suggested that a NADPH oxidase (NOX), analogous to that which generated superoxide during the respiratory burst in mammalian phagocytes, can serve the source of the ROS detected in plants upon successful pathogen recognition (Auh and Murphy 1995; Doke 1983; Low and Merida 1996; Murphy and Auh 1996), in abscisic acid-mediated stomatal closure (Pei et al. 2000), in auxin-regulated gravitropic response (Joo et al. 2005; Neill et al. 2002), and under abiotic stresses such as temperature, wounding and ozone (Dat et al. 1998; López-Delgado et al. 1998; Orozco-Cárdenas and Ryan 1999; Sharma et al. 1996).

Cadmium (Cd), a heavy metal toxic to humans, animals, and plants, is a widespread pollutant with a long biological half-life (Wagner 1993). It has been demonstrated that Cd can promote the generation of H<sub>2</sub>O<sub>2</sub> (Hsu and Kao 2004; Kuo and Kao 2004; Olmos et al. 2003; Piqueras et al. 1999; Romero-Puertas et al. 2004; Sandalio et al. 2001; Schützendübel et al. 2001; Shah et al. 2001). The involvement of NOX in Cd-induced H<sub>2</sub>O<sub>2</sub> production has been suggested in tobacco cells (BY-2 line; Olmos et al. 2003), pea leaves (Romero-Puertas et al. 2004), and pea roots (Rodríguez-Serrano et al. 2006).

It has been shown that NOX is activated by binding phosphatidylinositol 3-phosphate (PI3P), a

product of phosphatidylinositol 3-kinase (PI3K), to the PX domain of p40<sup>phox</sup> (Ellson et al. 2001). In plant cells, PI3P is also known to be required for abscisic acid (ABA)-induced H<sub>2</sub>O<sub>2</sub> production in guard cells (Jung et al. 2002; Park et al. 2003) and in leaves (Hung and Kao 2004), methyl jasmonate-induced H<sub>2</sub>O<sub>2</sub> production in leaves (Hung et al. 2006), and auxin-induced ROS production in roots (Joo et al. 2005).

We have previously shown that CdCl<sub>2</sub> increases the content of H<sub>2</sub>O<sub>2</sub> in detached leaves of rice cultivar Taichung Native 1 (TN1; Hsu and Kao 2004). In this article, we investigate the possibilities that NOX and PI3P, as found in animal cells and guard cells, activate H<sub>2</sub>O<sub>2</sub> generation in CdCl<sub>2</sub>-treated detached leaves of TN1 by using NOX inhibitors such as diphenyleneiodonium chloride (DPI) and imidazole (IMD), and PI3K inhibitors wortmannin (WM) and LY294002 (LY). Previously, we demonstrated that rice seedlings of cultivar TNG67 (TNG67) are more tolerant to Cd than those of cultivar TN1 (Hsu and Kao 2003). Evidence has been provided to show that abscisic acid is involved in Cd tolerance of TNG67 rice seedlings (Hsu and Kao 2003). It appears that these two cultivars of rice seedlings with different tolerance to Cd provide a good system to study mechanism of Cd toxicity of rice plants. Thus, using detached rice leaves of TN1 and intact leaves attached to rice seedlings of TN1 and TNG67, the possible link of H<sub>2</sub>O<sub>2</sub> between Cd and subsequent toxicity (chlorophyll loss or leaf chlorosis) was also investigated.

## Materials and methods

### Plant material and treatments

Rice (*Oryza sativa* L., cv. TN1, or TNG67) 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 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. In our laboratory, detached leaves excised from the third leaf of TN1 rice seedlings have long been used as a model system for the studies of stress physiology. Thus, in experiments using detached rice leaves, the apical 3 cm of the third leaves of 12-day-old TN1 rice seedlings was used. A group of 10 segments was floated in a Petri dish containing 10 ml of test solution. Incubation was carried out at 27°C in the light ( $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Test solutions included  $\text{CdCl}_2$  (5 mM),  $\text{H}_2\text{O}_2$  (1 mM), IMD (50  $\mu\text{M}$ ), nitric oxide (NO) donor, sodium nitropruside (SNP, 100  $\mu\text{M}$ ), and PI3K inhibitors, LY (100  $\mu\text{M}$ ) and WM (1  $\mu\text{M}$ ). LY and WM stock solutions were prepared in 100% dimethyl sulfoxide (DMSO). For experiments of using leaves attached to TN1 and TNG67 seedlings,  $\text{CdCl}_2$  was added to half-strength Kimura B solution at the time when the third leaf was fully expanded.  $\text{CdCl}_2$  concentrations at 0.5 mM and 30  $\mu\text{M}$  were applied over a short (2 days) and a longer period (6 days), respectively. For some experiments, seedlings exposed to 30°C and 45°C, respectively, for 3 h in darkness, to serve as non-heat shock (control) and heat shock (HS) treatments before the addition of  $\text{CdCl}_2$  (0.5 mM or 30  $\mu\text{M}$ ).

#### Evaluation of Cd toxicity

For detached rice leaves, Cd toxicity was judged by the decrease in chlorophyll content. Based on our experience from the experiments of Cd effect on rice seedlings, chlorosis is first observed in the second leaf of TN1 seedlings. Thus, Cd toxicity in the second leaves attached to rice seedlings caused by excess Cd was assessed by chlorosis.

#### Determination of chlorophyll and $\text{H}_2\text{O}_2$

Chlorophyll content was determined according to Wintermans and De Mots (1965) after extraction in 96% (v/v) ethanol.  $\text{H}_2\text{O}_2$  was visually detected in the leaves by using 3, 3-diaminobenzidine (DAB) as substrate (Orozco-Cárdenas and Ryan 1999). Cd- and inhibitor-treated detached rice leaves were first rinsed with distilled water and were then supplied through the cut ends with DAB (1 mg  $\text{ml}^{-1}$ ) solution for 24 h under light at 27°C. Leaves were decolorized in boiling ethanol (95%) for 0.5 h. This treatment decolorized the leaves except for the brown polymer-

ization spots produced by DAB with  $\text{H}_2\text{O}_2$ . After cooling, the leaves were extracted at room temperature with fresh ethanol to visualize the brown polymerization product produced by DAB with  $\text{H}_2\text{O}_2$ . To verify the specificity of brown spots, before staining with DAB some leaves were incubated for 2 h in 1 mM ascorbate (ASC), a  $\text{H}_2\text{O}_2$  scavenger. The  $\text{H}_2\text{O}_2$  staining was repeated four times with similar results. The  $\text{H}_2\text{O}_2$  content was also measured colorimetrically as described by Jana and Choudhuri (1982).  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  content, the extracted solution was mixed with 0.1% titanium chloride in 20% (v/v)  $\text{H}_2\text{SO}_4$ . The mixture was then centrifuged at 6,000 *g* for 25 min. The absorbance was measured at 410 nm. Using this method, we found that absorbance increased linearly with the amount of  $\text{H}_2\text{O}_2$  and addition of  $\text{H}_2\text{O}_2$  to extracts resulted in the predicted increase of absorbance, i.e., added  $\text{H}_2\text{O}_2$  was fully recovered (data not shown). The  $\text{H}_2\text{O}_2$  content in leaf extracts was calculated using an extinction coefficient of  $0.28 \mu\text{mol}^{-1} \text{cm}^{-1}$ .

#### Statistical analysis

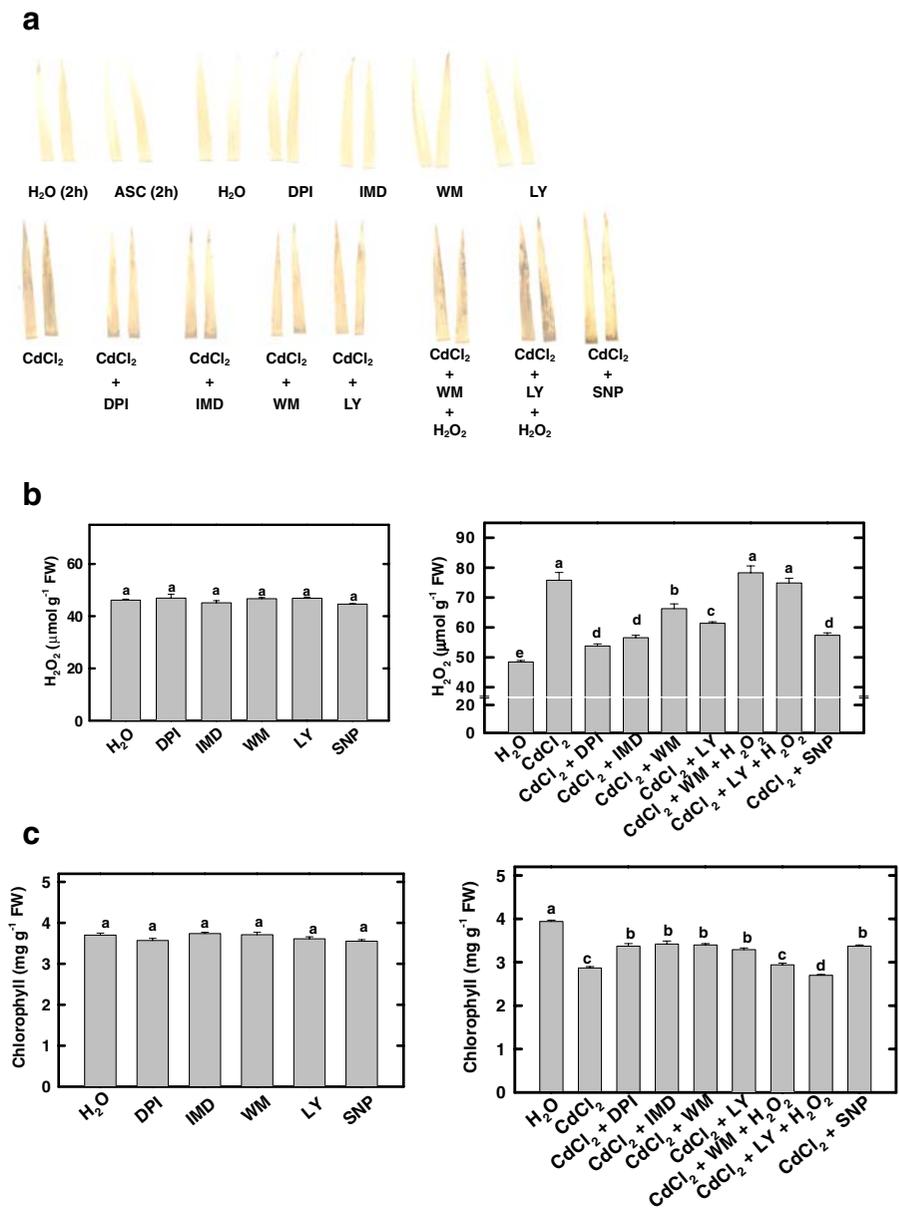
Statistical differences between measurements ( $n=4$ ) on different treatments or on different times were analyzed following LSD test.

## Results

#### $\text{CdCl}_2$ increases $\text{H}_2\text{O}_2$ production and decreases chlorophyll in detached rice leaves

In the present study,  $\text{H}_2\text{O}_2$  production was first visualized by a histochemical method with 3,3-diaminobenzidine (DAB) that is based on the formation by  $\text{H}_2\text{O}_2$  of brown polymerization product. The development of DAB- $\text{H}_2\text{O}_2$  reaction product in detached rice leaves could be prevented by  $\text{H}_2\text{O}_2$  scavengers such as ASC, indicating that the DAB staining method for  $\text{H}_2\text{O}_2$  is specific (Fig. 1a).  $\text{CdCl}_2$  treatment led to an accumulation of DAB- $\text{H}_2\text{O}_2$  reaction product (Fig. 1a). When  $\text{H}_2\text{O}_2$  was measured colorimetrically,  $\text{CdCl}_2$  treatment also increased  $\text{H}_2\text{O}_2$

**Fig. 1** Effect of DPI, IMD, WM, LY, or SNP on the development of DAB-H<sub>2</sub>O<sub>2</sub> reaction product (a), and the contents of H<sub>2</sub>O<sub>2</sub> (b) and chlorophyll (c) in CdCl<sub>2</sub>-treated detached rice leaves (cv. TN1) in the presence or absence of H<sub>2</sub>O<sub>2</sub>. The concentrations of CdCl<sub>2</sub>, DPI, IMD, WM, LY, SNP, and ascorbate (ASC) were 5 mM, 10 μM, 50 μM, 1 μM, 100 μM, 100 μM, and 1 mM, respectively. DAB-H<sub>2</sub>O<sub>2</sub> reaction product, for all treatments except ASC was visualized 24 h after treatment in the light. DAB-H<sub>2</sub>O<sub>2</sub> reaction product for ASC treatment was visualized 2 h after treatment in the light. H<sub>2</sub>O<sub>2</sub> and chlorophyll contents were measured 24 h after treatment in the light



content in detached rice leaves (Fig. 1b). As expected, CdCl<sub>2</sub> treatments resulted in a decrease in chlorophyll content (Fig. 1c).

#### The effect of NOX inhibitors

The role of NOX in the Cd-stimulated H<sub>2</sub>O<sub>2</sub> production was investigated using NOX inhibitors such as diphenyleneiodonium chloride (DPI) and imidazole (IMD). When 1 μM DPI or 100 μM IMD

was added to detached rice leaves simultaneously with CdCl<sub>2</sub>, a reduction of Cd-induced H<sub>2</sub>O<sub>2</sub> accumulation was observed (Fig. 1a,b). The decrease in chlorophyll content in detached rice leaves by CdCl<sub>2</sub> was reduced by DPI and IMD (Fig. 1c).

#### The effect of phosphatidylinositol 3-kinase inhibitors

It has been shown that PI3P is important in NOX-mediated H<sub>2</sub>O<sub>2</sub> production during ABA-induced

stomatal closure (Jung et al. 2002), during ABA-promoted leaf senescence (Hung and Kao 2005), during methyl jasmonate-induced leaf senescence (Hung et al. 2006) and during auxin-induced root gravitropic responses (Joo et al. 2005). Thus, it is of great interest to understand whether PI3P is also important in CdCl<sub>2</sub>-induced H<sub>2</sub>O<sub>2</sub> production in rice leaves. Wortmannin (WM) and LY294002 (LY) are inhibitors of PI3K, a product of which is PI3P. When detached rice leaves were treated with a solution WM (1 μM) or LY (100 μM), CdCl<sub>2</sub>-induced accumulation of H<sub>2</sub>O<sub>2</sub> in detached rice leaves was reduced (Fig. 1a,b). WM or LY also reduced Cd-induced decrease in chlorophyll content (Fig. 1c). Exogenous H<sub>2</sub>O<sub>2</sub> (1 mM) was observed to be able to reverse the inhibitory effect of WM or LY on H<sub>2</sub>O<sub>2</sub> production and chlorophyll content (Fig. 1a-c).

The effect of nitric oxide donor sodium nitroprusside

Nitric oxide (NO) is a bioactive free radical implicated in a number of physiological functions, including intra-cellular mediation of some animal responses (Anbar 1995). In plants, NO is involved in many physiological responses, such as pathogen response, programmed cell death, growth, germination, root organogenesis, phytoalexin production, internal iron availability, and ABA-dependent stomatal closure (Lamattina et al. 2003; Neill et al. 2003). We have previously demonstrated that Cd toxicity in detached rice leaves is reduced by NO (Hsu and Kao 2004). If H<sub>2</sub>O<sub>2</sub> is responsible for Cd toxicity in detached rice leaves, then H<sub>2</sub>O<sub>2</sub> accumulation is expected to be reduced by NO donor sodium nitroprusside SNP. As indicated in Fig. 1a, b, it is indeed the case. In the present study, we also show that the decrease in chlorophyll content in detached rice leaves caused by Cd was reduced by SNP (Fig. 1c).

CdCl<sub>2</sub> induces H<sub>2</sub>O<sub>2</sub> accumulation in the leaves of cultivar TN1 seedlings but not in cultivar TNG67

Figure 2 shows the effect of 0.5 mM CdCl<sub>2</sub> on the chlorosis of the second leaves of rice seedlings. It is clear that CdCl<sub>2</sub> treatment resulted in Cd toxicity of the second leaves of TN1, but not in TNG67 seedlings (Fig. 2) at 2 days after Cd treatment. If H<sub>2</sub>O<sub>2</sub> is important in regulating Cd toxicity, then



**CdCl<sub>2</sub>**            -        +            -        +  
**(0.5 mM, 2 d)**

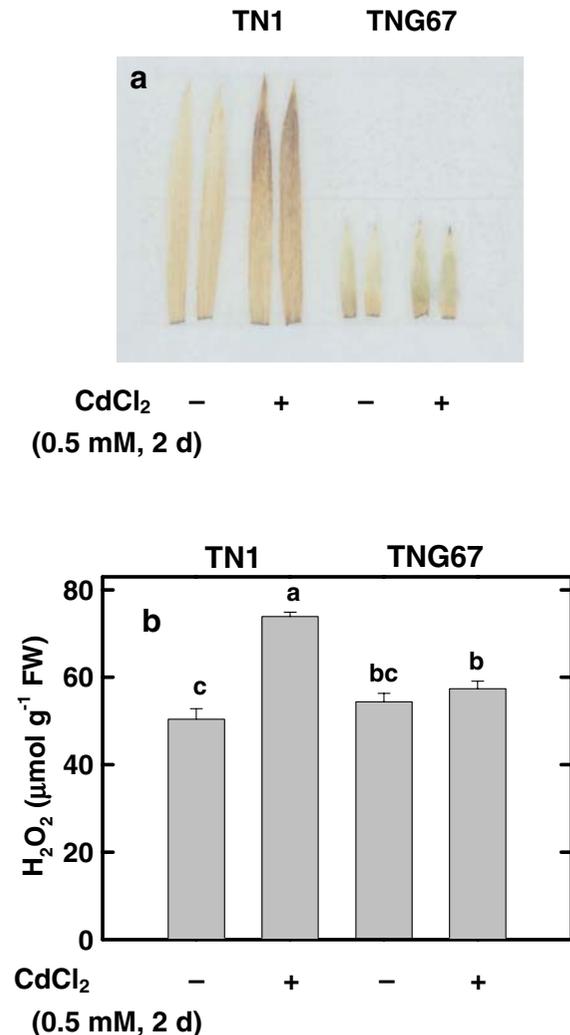
**Fig. 2** Effect of CdCl<sub>2</sub> (0.5 mM) on the toxicity (chlorosis) in the second leaves of TN1 and TNG67 rice seedlings. Rice seedlings were cultivated in half-strength Kimura B solution in a Phytotron with natural sunlight at 30°C (day)/25°C (night) and 90% relative humidity. CdCl<sub>2</sub> (0.5 mM) was added to half-strength Kimura B solution at the time when the third leaves of both TN1 and TNG67 seedlings were fully expanded. Pictures were taken 2 days after the addition of CdCl<sub>2</sub> (0.5 mM). Arrow indicates the second leaves

H<sub>2</sub>O<sub>2</sub>-DAB reaction product and H<sub>2</sub>O<sub>2</sub> content are expected to be more and higher, respectively, in CdCl<sub>2</sub>-treated TN1 seedlings than in TNG67. As indicated in Fig. 3, it is indeed the case.

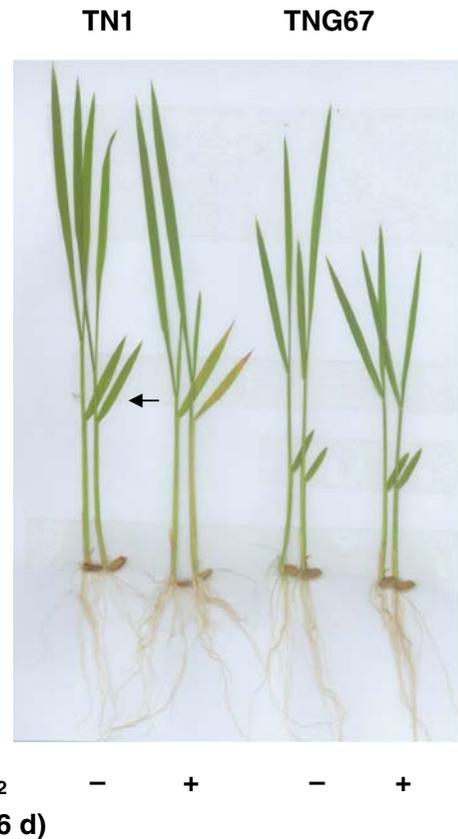
The concentration of CdCl<sub>2</sub> used in the aforementioned study was 0.5 mM. We also conducted experiments with lower CdCl<sub>2</sub> concentration, 30 μM, applied over a longer period (6 days). Cd toxicity and H<sub>2</sub>O<sub>2</sub> generation was also observed to be more pronounced in TN1 seedlings than TNG67 treated with lower concentration (30 μM) CdCl<sub>2</sub> for 6 days (Figs. 4 and 5). Thus, the responses to lower CdCl<sub>2</sub> concentration are basically in accordance with those to higher CdCl<sub>2</sub> concentration.

Prior high temperature exposure of TN1 seedlings reduces Cd-induced H<sub>2</sub>O<sub>2</sub> accumulation

Exposure in the range of 2 min to a few hours at temperature 5°C to 15°C above the normal growing temperature is usually called HS treatment. Prior exposure to HS has been shown to increase the tolerance of plants to subsequent Cd stress (Chen and



**Fig. 3** Effect of CdCl<sub>2</sub> (0.5 mM) on DAB-H<sub>2</sub>O<sub>2</sub> reaction product (a) and H<sub>2</sub>O<sub>2</sub> content (b) in the second leaves of TN1 and TNG67 rice seedlings. Rice seedlings were cultivated in half-strength Kimura B solution in a Phytron with natural sunlight at 30°C (day)/25°C (night) and 90% relative humidity. CdCl<sub>2</sub> (0.5 mM) was added to half-strength Kimura B solution at the time when the third leaves of both TN1 and TNG67 seedlings were fully expanded. The second leaves were excised to visualize H<sub>2</sub>O<sub>2</sub>-DAB reaction product and measure H<sub>2</sub>O<sub>2</sub> content 2 days after the addition of CdCl<sub>2</sub> (0.5 mM)



**Fig. 4** Effect of CdCl<sub>2</sub> (30 μM) on the toxicity (chlorosis) in the second leaves of TN1 and TNG67 rice seedlings. Rice seedlings were cultivated in half-strength Kimura B solution in a Phytotron with natural sunlight at 30°C (day)/25°C (night) and 90% relative humidity. CdCl<sub>2</sub> (30 μM) was added to half-strength Kimura B solution at the time when the third leaves of both TN1 and TNG67 seedlings were fully expanded. Pictures were taken 2 days after the addition of CdCl<sub>2</sub> (30 μM). Arrow indicates the second leaves

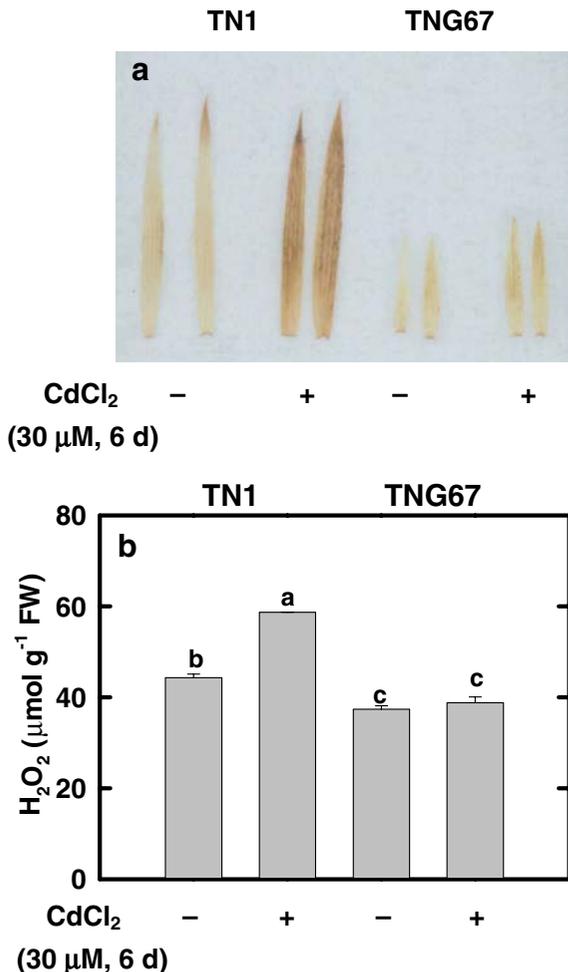
Kao 1995; Orzech and Burke 1988). To test if prior high temperature exposure of TN1 seedlings affects subsequent Cd stress, TN1 seedlings were pretreated at 45°C for 3 h. It was observed that HS pretreatment induces tolerance of Cd stress (0.5 mM or 30 μM) in TN1 seedlings (Figs. 6 and 8). Figures 7 and 9 also show that HS pretreatment resulted in the production of less H<sub>2</sub>O<sub>2</sub> in the second leaves of TN1 seedlings treated with CdCl<sub>2</sub> than that non-HS (30°C) pretreatment.

## Discussion

In the present study, 0.5 mM or 30 μM CdCl<sub>2</sub> was used to evaluate Cd toxicity of leaves attached to rice

seedlings. In a recent study on 64 soils (urban, forest, and agricultural soils) containing various levels of Cd contamination, free dissolved Cd concentrations ranged from 0.1 to 2,000 nM (Sauvé et al. 2000). Thus, the 30  $\mu\text{M}$   $\text{CdCl}_2$  used in some of our experiments can be considered to be very high. Basically, rice seedlings in our study were considered to be suffering from acute Cd toxicity.

It has been shown that Cd is able to produce  $\text{H}_2\text{O}_2$  (Hsu and Kao 2004; Kuo and Kao 2004; Olmos et al.



**Fig. 5** Effect of  $\text{CdCl}_2$  (30  $\mu\text{M}$ ) on DAB- $\text{H}_2\text{O}_2$  reaction product (a) and  $\text{H}_2\text{O}_2$  content (b) in the second leaves of TN1 and TNG67 rice seedlings. Rice seedlings were cultivated in half-strength Kimura B solution in a Phytotron with natural sunlight at 30°C (day)/25°C (night) and 90% relative humidity.  $\text{CdCl}_2$  (30  $\mu\text{M}$ ) was added to half-strength Kimura B solution at the time when the third leaves of both TN1 and TNG67 seedlings were fully expanded. The second leaves were excised to visualize  $\text{H}_2\text{O}_2$ -DAB reaction product and measure  $\text{H}_2\text{O}_2$  content 6 days after the addition of  $\text{CdCl}_2$  (30  $\mu\text{M}$ )



**Pretreatment**

HS - - + +

**Treatment**

$\text{CdCl}_2$  (0.5 mM, 2 d) - + - +

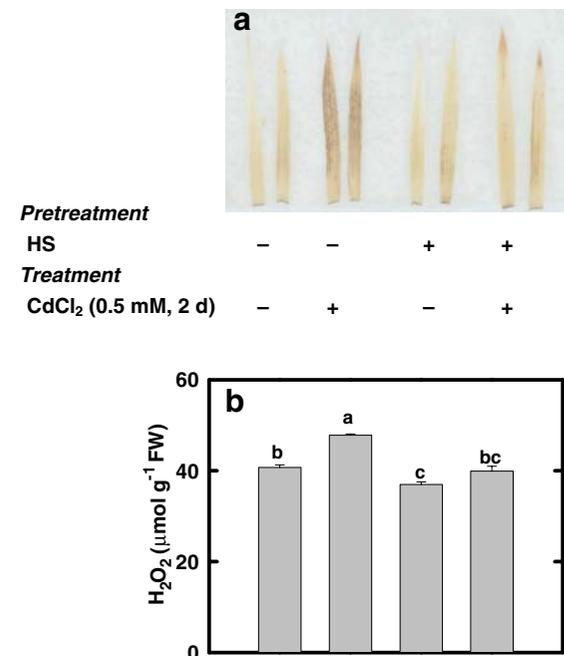
**Fig. 6** Effect of HS pretreatment on the toxicity (chlorosis) in the second leaves of rice seedlings (cv. TN1) in the presence or absence of  $\text{CdCl}_2$  (0.5 mM). Rice seedlings at the time when the third leaves fully expanded were transferred to 30°C (non-HS) or 45°C (HS) in the dark for 3 h. Rice seedlings were then cultivated in a Phytotron with natural sunlight at 30°C (day)/25°C (night) and 80% relative humidity. Pictures were taken 2 days after the addition of  $\text{CdCl}_2$  (0.5 mM) to half-strength Kimura B solution. Arrow indicated the second leaves

2003; Piqueras et al. 1999; Romero-Puertas et al. 2004; Sandalio et al. 2001; Schützendübel et al. 2001; Shah et al. 2001). Here, we also show that  $\text{CdCl}_2$  induced  $\text{H}_2\text{O}_2$  production in detached rice leaves by histochemistry with DAB (Fig. 1a) and colorimetric methods (Fig. 1b). Wounding is known to induce  $\text{H}_2\text{O}_2$  production (Orozco-Cárdenas and Ryan 1999). When detached rice leaves are used, wounding is always a problem. However in the present study, each long and narrow rice leaf was cut transversely; thus the area of wounding was very small. Therefore,  $\text{H}_2\text{O}_2$  generation of detached rice leaves induced by  $\text{CdCl}_2$  is unlikely to be complicated the wounding effect.

A role for plasma membrane NOX in the production of the  $\text{H}_2\text{O}_2$  has been a recent focus in ROS signaling (Sagi and Flurhr 2006). Here, we show that DPI and IMD, inhibitors of NOX, reduced  $\text{CdCl}_2$ -induced  $\text{H}_2\text{O}_2$  accumulation (Fig. 1a,b). It has been shown that a high concentration of DPI can affect

other enzymes potentially involved in the production of ROS, including cell wall peroxidase and NO synthase (Bolwell et al. 1998). The fact that CdCl<sub>2</sub>-induced H<sub>2</sub>O<sub>2</sub> accumulation in detached rice leaves can be inhibited by low concentration of DPI (1 μM) and can be inhibited by both DPI and IMD (Fig. 1a,b) strongly suggest that CdCl<sub>2</sub>-dependent H<sub>2</sub>O<sub>2</sub> production originated, at least in part, from plasma membrane NOX. The involvement of NOX in Cd-induced H<sub>2</sub>O<sub>2</sub> production has also been suggested in tobacco cells (Olmos et al. 2003), pea leaves (Romero-Puertas et al. 2004), and pea roots (Rodriguez-Serrano et al. 2006).

It has been shown that zinc deficiency enhanced NAD(P)H-dependent superoxide radical production in



**Fig. 7** Effect of HS pretreatment on the H<sub>2</sub>O<sub>2</sub>-DAB reaction product (**a**) and H<sub>2</sub>O<sub>2</sub> content (**b**) in the second leaves of rice seedlings (cv. TN1) in the presence or absence of CdCl<sub>2</sub> (0.5 mM). Rice seedlings at the time when the third leaves fully expanded were transferred to 30°C (non-HS) or 45°C (HS) in the dark for 3 h. Rice seedlings were then cultivated in a Phytotron with natural sunlight at 30°C (day)/25°C (night) and 80% relative humidity. The second leaves were excised to visualize H<sub>2</sub>O<sub>2</sub>-DAB reaction product and measure H<sub>2</sub>O<sub>2</sub> content, 2 days after the addition of CdCl<sub>2</sub> (0.5 mM) to half-strength Kimura B solution



**Pretreatment**

**HS** - - + +

**Treatment**

**CdCl<sub>2</sub> (30 μM, 6 d)** - + - +

**Fig. 8** Effect of HS pretreatment on the toxicity (chlorosis) in the second leaves of rice seedlings (cv. TN1) in the presence or absence of CdCl<sub>2</sub> (30 μM). Rice seedlings at the time when the third leaves fully expanded were transferred to 30°C (non-HS) or 45°C (HS) in the dark for 3 h. Rice seedlings were then cultivated in a Phytotron with natural sunlight at 30°C (day)/25°C (night) and 90% relative humidity. Pictures were taken 6 days after the addition of CdCl<sub>2</sub> (30 μM) to half-strength Kimura B solution. *Arrow* indicated the second leaves

plasma membrane vesicles isolated from roots of bean plants (Pinton et al. 1994). Since cadmium toxicity has been shown to be associated zinc deficiency (Sandalo et al. 2001), thus the possibility that a Cd-induced reduction in Zn availability could stimulate superoxide radical production in leaves of rice seedlings cannot be excluded.

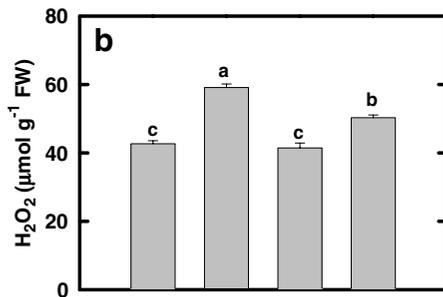
The mechanism of ROS production and the molecules involved have been well investigated in animal cells, particularly in neutrophils. The NOX, which consists of many components, is responsible for ROS production in neutrophil cells and is activated by the binding of PI3P to one of the components (Ellson et al. 2001). In plant cells, PI3P is also known to be required for ABA-induced H<sub>2</sub>O<sub>2</sub> production in guard cells (Jung et al. 2002; Park et al. 2003) and in detached rice leaves (Hung and Kao 2005), methyl jasmonate-induced H<sub>2</sub>O<sub>2</sub> production in detached rice leaves (Hung et al. 2006), and auxin-induced ROS production in roots (Joo et al. 2005). It

**Pretreatment**

|           |   |   |   |   |
|-----------|---|---|---|---|
| <b>HS</b> | - | - | + | + |
|-----------|---|---|---|---|

**Treatment**

|                                      |   |   |   |   |
|--------------------------------------|---|---|---|---|
| <b>CdCl<sub>2</sub> (30 μM, 6 d)</b> | - | + | - | + |
|--------------------------------------|---|---|---|---|

**Pretreatment**

|           |   |   |   |   |
|-----------|---|---|---|---|
| <b>HS</b> | - | - | + | + |
|-----------|---|---|---|---|

**Treatment**

|                                      |   |   |   |   |
|--------------------------------------|---|---|---|---|
| <b>CdCl<sub>2</sub> (30 μM, 6 d)</b> | - | + | - | + |
|--------------------------------------|---|---|---|---|

**Fig. 9** Effect of HS pretreatment on the H<sub>2</sub>O<sub>2</sub>-DAB reaction product (a) and H<sub>2</sub>O<sub>2</sub> content (b) in the second leaves of rice seedlings (cv. TN1) in the presence or absence of CdCl<sub>2</sub> (30 μM). Rice seedlings at the time when the third leaves fully expanded were transferred to 30°C (non-HS) or 45°C (HS) in the dark for 3 h. Rice seedlings were then cultivated in a Phytotron with natural sunlight at 30°C (day)/25°C (night) and 90% relative humidity. The second leaves were excised to visualize H<sub>2</sub>O<sub>2</sub>-DAB reaction product and measure H<sub>2</sub>O<sub>2</sub> content 6 days after the addition of CdCl<sub>2</sub> (30 μM) to half-strength Kimura B solution

appears that PI3P is important in CdCl<sub>2</sub>-induced H<sub>2</sub>O<sub>2</sub> production based on two lines of evidence. First, LY or WM, inhibitor of PI3K, was able to reduce CdCl<sub>2</sub>-induced H<sub>2</sub>O<sub>2</sub> production (Fig. 1a,b). Second, exogenous H<sub>2</sub>O<sub>2</sub> reversed the inhibitory effect of the PI3K inhibitors on CdCl<sub>2</sub>-induced H<sub>2</sub>O<sub>2</sub> accumulation (Fig. 1a,b). These results supported further that NOX is involved in CdCl<sub>2</sub>-induced H<sub>2</sub>O<sub>2</sub> production. In neutrophils, PI3P regulated H<sub>2</sub>O<sub>2</sub> production by binding the non-catalytic component p40<sup>phox</sup> of the NOX (Ellson et al. 2001). However, a rice homolog

of p40<sup>phox</sup> has not been reported. Therefore, the detailed mechanism of the action of PI3P during H<sub>2</sub>O<sub>2</sub> production in the rice leaves needs further investigation.

The fact that NOX and PI3K inhibitors, which reduced the Cd-induced H<sub>2</sub>O<sub>2</sub> production (Fig. 1a,b), were able to prevent Cd-decreased chlorophyll content in detached rice leaves (Fig. 1c) suggests that Cd toxicity of detached rice leaves is due to H<sub>2</sub>O<sub>2</sub> accumulation.

Several reports convincingly demonstrate that NO is able to counteract the toxicity of paraquat and diquat, which are known to generate ROS in potato and rice leaves (Beligni and Lamattina 1999; Hung et al. 2002), and block H<sub>2</sub>O<sub>2</sub> production induced by jasmonic acid in tomato leaves (Orozco-Cárdenas and Ryan 2002). Here we also showed that SNP, a NO donor, blocks H<sub>2</sub>O<sub>2</sub> production and reduces Cd toxicity (Fig. 1a,b). It has been shown that polyamines are able to protect against oxidative damage caused by paraquat (Chang and Kao 1997), acid rain (Velikova et al. 2000) and heavy metals such as Cd and Cu (Groppa et al. 2001). Recently, we reported that polyamines (spermidine and spermine) are able to protect Cd-induced toxicity of detached rice leaves and this protection is most likely related to the avoidance of H<sub>2</sub>O<sub>2</sub> generation and Cd uptake (Hsu and Kao 2007). All these results support further that Cd toxicity is due to H<sub>2</sub>O<sub>2</sub> production in detached rice leaves. In plants, the most general symptom of Cd toxicity is chlorosis (Das et al. 1997). Based on chlorosis and chlorophyll loss of the second leaves of rice seedlings, it was demonstrated that rice seedlings of cultivar TNG67 are more tolerant to Cd than those of cultivar TN1 (Hsu and Kao 2003; Kuo and Kao 2004). Evidence has also been provided to show that Cd content in the second leaf and roots of TNG67 seedlings remained unchanged and slightly increased, respectively, after Cd treatment (Hsu and Kao 2003; Kuo and Kao 2004). In contrast, a marked increase in Cd content in Cd-treated TN1 leaves and roots was observed (Hsu and Kao 2003; Kuo and Kao 2004). We also observed that higher content of Cd in TN1 than TNG67 leaves results in more production of H<sub>2</sub>O<sub>2</sub> in TN1 than TNG67 leaves (Kuo and Kao 2004). The fact that on treatment with CdCl<sub>2</sub> (0.5 mM for 2 days or 30 μM for 6 days), the H<sub>2</sub>O<sub>2</sub> content increased in Cd-sensitive TN1 seedlings but not in Cd-tolerant TNG67 (Figs. 3 and 5) supports the idea

that Cd toxicity is due to H<sub>2</sub>O<sub>2</sub> accumulation caused by Cd. Prior exposure to HS has been shown to increase tolerance of plants to subsequent Cd stress (Chen and Kao 1995; Orzech and Burke 1988). Here we also demonstrated that HS pretreatment of TN1 seedlings resulted in the reduction of H<sub>2</sub>O<sub>2</sub> production as well as Cd toxicity of TN1 seedlings treated with CdCl<sub>2</sub> (Figs. 6, 7, 8, and 9). H<sub>2</sub>O<sub>2</sub> production is believed to be the consequence of lower capacity of antioxidant system to avoid H<sub>2</sub>O<sub>2</sub> accumulation. In this connection, HS pretreatment may increase the capacity of antioxidant system to reduce H<sub>2</sub>O<sub>2</sub> production under the subsequent Cd treatment. Our unpublished data indeed show that HS pretreatment of rice seedlings resulted in higher contents of reduced glutathione and ascorbate and higher activities of glutathione reductase and ascorbate peroxidase than non-HS.

Based on the results obtained from detached rice leaves and intact leaves attached to rice seedlings, we conclude that Cd toxicity in leaves of rice seedlings is due to H<sub>2</sub>O<sub>2</sub> accumulation. This conclusion is basically consistent with the results of Cho and Seo (2005), who reported that a lower H<sub>2</sub>O<sub>2</sub> accumulation confers Cd-tolerance in *Arabidopsis* seedlings.

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