行政院國家科學委員會專題研究計畫 成果報告

水稻幼苗耐鎘機制之探討(3/3)
研究成果報告(完整版)

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行政院國家科學委員會補助專題研究計畫 ☑成果報告

□期中進度報告

水稻幼苗耐鎘機制之探討（1/3）
水稻幼苗耐鎘機制之探討（2/3）
水稻幼苗耐鎘機制之探討（3/3）

計畫類別：☑ 個別型計畫　□ 整合型計畫

計畫編號：NSC 93－2313－B－002－061

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執行期間：**94 年 8 月 1 日 至 97 年 7 月 31 日**

計畫主持人：**高景輝**

共同主持人：

計畫參與人員：**郭美君 許奕婷**

成果報告類型(依經費核定清單規定繳交)：□精簡報告　☑完整報告

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執行單位：台灣大學農藝系

中　華　民　國　96　年　8　月　1　日

中文摘要

水稻鎘毒害與氧化逆境之關係（93-94）

氯化鎘處理增加不耐鎘品種台中在來 1 號（TN1）過氧化氫與 MDA 含量以及增加抗氧化酵素[superoxide dismutase（SOD），ascorbate peroxidase，glutathione reductase，catalase 與 peroxidase（POX）]之活性，但不影響耐鎘品種台農 67 號（TNG67）葉片過氧化氫與 MDA 含量以及增加抗氧化酵素之活性。本研究之結果似乎說明，氯化鎘處理 TN1 幼苗，其葉片可表現氧化逆境，然而氯化鎘處理 TNG67 幼苗，其葉片不會表現氧化逆境，同時也說明鎘處理後 TN1 幼苗葉片所表現之鎘毒害是經由氧化逆境所引起。

關鍵詞：抗氧化酵素、鎘、水稻、氧化逆境

Paclobutrazol 在水稻幼苗鎘毒害上之保護角色（94-95）

Paclobutrazol（+PBZ）或不經 Paclobutrazol（-PBZ）處理之水稻幼苗，經氯化鎘處理後，顯示 PBZ 能夠克服鎘逆境所引起之傷害。同時也發現，PBZ 導致幼苗鎘耐性增加之機制，可能是由於脫落酸累積所造成。

關鍵詞：脫落酸、鎘、水稻、Paclobutrazol

水稻葉片鎘逆境與多元胺間之關係（95-96）

本年度計畫主要是探討多元胺在水稻葉片鎘逆境下所扮演之功能。Spermidine 與 Spermine 可有效的克服葉片鎘逆境所引起之傷害，但 Putrescine 則不是此功能。Spermidine與Spermine之保護作用，可能是由於其能避免H_2O_2之形成以及降低鎘之吸收所造成。

關鍵詞：鎘、水稻、多元胺

英文摘要

Cd toxicity of rice seedlings in relative to oxidative stress（2004-2005）

Changes in H_2O_2 and malondialdehyde（MDA）contents and antioxidant enzyme activities in Cd-treated rice（*Oryza sativa* L.）seedlings of two cultivars were investigated. On treatment with $CdCl_2$, increases in H_2O_2 and MDA contents and antioxidant enzyme activities [superoxide dismutase（SOD）, ascorbate peroxidase, glutathione reductase, catalase and peroxidase（POX）] were observed in the leaves of Cd-sensitive cultivar （cv. Taichung Native 1, TN1）but not in Cd-tolerant cultivar （cv. Tainung 67, TNG67）. The increased content of MDA and activities of SOD and POX preceded the occurrence of toxicity in $CdCl_2$-treated TN1 leaves. In conclusion, the oxidative stress is differently expressed in TN1 and TNG67 rice seedlings in response to $CdCl_2$,. Results also suggest that $CdCl_2$ causes an oxidative stress and $CdCl_2$-induced toxicity is mediated through oxidative stress in TN1 leaves.

Keywords：cadmium、*Oryza sativa* L.、oxidative stress、antioxidant enzymes

The protective role of paclobutrazol in Cd toxicity of rice seedlings（2005-2006）

Twelve –day-old –PBZ （paclobutrazol）and +PBZ seedlings were treated or not with $CdCl_2$. The results indicated that PBZ applied to seeds was able to protect rice seedlings from Cd toxicity. Accumulated data suggested that the PBZ-induced Cd tolerance of rice seedlings is mediated through an accumulation of ABA.

Keywords：abscilic acid、cadmium、*Oryza sativa* L.、paclobutrazol

The protective role of polyamines in Cd toxicity of rice leaves（2006-2007）

The protective effect of polyamines against Cd toxicity of rice（*Oryza sativa* L.）leaves was investigated. Spermidine（Spd）and Spermine（Spm）, but not putrescine（Put）, were effective in reducing $CdCl_2$-induced toxicity. Results of the present study suggest that Spd and Spm are able to protect Cd-induced oxidative damage and this protection is most likely related to the avoidance of H_2O_2 generation and the reduction of Cd uptake.

Keywords：cadmium、*Oryza sativa* L.、polyamines

報告內容

前言

鎘是一種二價陽離子之重金屬，且為重金屬中毒性很強之一種重金屬。鎘會引起植物生態、生理、生化與構造上發生變化。鎘是一種非氧化還原性金屬（non-redox metal），不會經由 Fenton 或 Haber-Weiss 反應產生活化氧族。然而，許多證據顯示，鎘會間接造成活化氧族之形成。鎘可增加脂質過氧化作用，因此鎘被認為可造成氧化逆境。

Triazoles 類的生長阻礙劑，會抑制 GAs 之合成，且被認為具有保護逆境之作用，其主要機制是抑制氧化逆境。該類生長阻礙劑尚可抑制 ABA 之代謝，而使 ABA 累積。Triazoles 誘導植物對鎘毒害之抗性，研究的不多。因此，triazoles 與鎘毒害間之關係應是值得探討的問題。

多元胺廣泛存在植物體中，被認為與逆境之抗性有關，許多證據也顯示，多元胺可抑制脂質過氧化作用，克服氧化逆境。很少研究探討多元胺與鎘所引起之氧化逆境間之關係，同時也不瞭解植物對鎘毒害之抗性是否與多元胺含量之變化有關。

研究目的

本研究計畫為三年計畫，基本上是探討對鎘逆境之問題。第一年著重於瞭解水稻幼苗對鎘之耐性是否與氧化逆境有關。第二年則進行瞭解 paclobutrazol（triazole 類之一種）是否可增加水稻幼苗對鎘之耐性及其可能機制。第三年則希望多瞭解水稻鎘逆境與多元胺間之關係。

研究方法、結果與討論

本計畫已完成論文發表。第一年計畫發表於 Bot Bull Acad Sin 45:291-299,2004（附件一）；第二年計畫則發表於 Physiol Plant 124:71-80,2005（附件二）；第三年計畫則發表於 Plant Soil 291:27-37,2007（附件三）。

計畫成果自評

三年計畫順利執行，達到預期目標。計畫成果對本研究長期所關注的問題水稻幼苗鎘逆境有深入的瞭解。

附件一（93-94 年成果）

Bot. Bull. Acad. Sin. (2004) **45:** 291-299

Antioxidant enzyme activities are upregulated in response to cadmium in sensitive, but not in tolerant, rice (*Oryza sativa* L.) seedlings

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Abstract. Changes in H_2O_2 and malondialdehyde (MDA) contents and antioxidant enzyme activities in Cd-treated rice (*Oryza sativa* L.) seedlings of two cultivars were investigated. On treatment with $CdCl_2$, increases in H_2O_2 and MDA contents and antioxidant enzyme activities [speroxide dismutase (SOD), ascorbate peroxidase, glutathione reductase, catalase, and peroxidase (POX)] were observed in the leaves of Cd-sensitive cultivar (cv. Taichung Native 1, TN1) but not in Cd-tolerant cultivar (cv. Tainung 67, TNG67). The increased content of MDA and activities of SOD and POX preceded the occurrence of toxicity in $CdCl_2$-treated TN1 leaves. Pretreatment with abscisic acid (ABA) enhanced Cd tolerance and reduced Cd-induced increase in the content of MDA and increase in the activities of SOD and POX in TN1 leaves. Exogenous application of ABA biosynthesis inhibitor, fluridone, decreased Cd tolerance, increased the content of MDA, and increased the activities of SOD and POX in Cd-treated TNG67 leaves. Furthermore, fluridone's effects on toxicity, the content of MDA, and the activities of SOD and POX in Cd-treated TNG67 leaves were reversed by the application of ABA. In conclusion, the oxidative stress is differently expressed in TN1 and TNG67 rice seedlings in response to $CdCl_2$. Results also suggest that $CdCl_2$ causes an oxidative stress and $CdCl_2$-induced toxicity is mediated through oxidative stress in TN1 leaves.

Keywords: Abscisic acid; Cadmium; *Oryza sativa* L.; Oxidative stress.

Abbreviations: ABA, abscisic acid; **APX,** ascorbate peroxidase; **CAT,** catalase; **Flu,** fluridone; **FW,** fresh weight; **GR,** glutathione reductase; **MDA,** malondialdehyde; **POX,** peroxidase; **SOD,** superoxide dismutase; **TN1,** Taichung Native 1; **TNG67,** Tainung 67.

Introduction

Oxygen is essential for the existence of aerobic life, but toxic active oxygen species (AOS), which include the superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2), are generated in all aerobic cells during metabolic processes (Foyer et al., 1994; Asada, 1999). Injury caused by these AOS, known as oxidative stress, is one of the major damaging factors in plants exposed to environmental stress. Plants cope with oxidative stress by using antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), peroxidase (POX), catalase (CAT), and the low molecular weight antioxidants, ascorbic acid and glutathione (Noctor and Foyer, 1998; Asada, 1999).

Cadmium (Cd), a heavy metal toxic to humans, animals, and plants is a widespread pollutant with a long biological half-life (Wagner, 1993). Three lines of evidence indicate that one mechanism of Cd toxicity is related to oxidative stress in plant cells. First, Cd can promote the generation of AOS (Piqueras et al., 1999; Romero-Puertas et al., 1999; Chen et al., 2000; Shah et al., 2001; Sandalio et al., 2001; Schützendübel et al., 2001; Olmos et al., 2003). Second, Cd can inhibit or stimulate the activities of antioxidant enzymes (Shaw, 1995; Gallego et al., 1996; Chaoui et al., 1997; Dixit et al., 2001; Shah et al., 2001; Iannelli et al., 2002; León et al., 2002). Third, treatment with Cd results in cellular oxidative damage or lipid peroxidation (Shaw, 1995; Gallego et al., 1996; Chaoui et al., 1997; Lozano-Rodríguez et al., 1997; Dixit et al., 2001; Shah et al., 2001; Chien et al., 2002).

This study examines H_2O_2 content, lipid peroxidation, and antioxidant enzyme activities in two cultivars of rice (*Oryza sativa* L.) seedlings in response to Cd. One cultivar (cv. Taichung Native 1, TN1) is known to be sensitive to Cd, and the other (cv. Tainung 67, TNG67) is known to show significant tolerance to Cd (Hsu and Kao, 2003a). If a mechanism related to oxidative stress is involved in Cd toxicity, this mechanism should then be differently expressed in plants tolerant and sensitive to Cd. Therefore, the aim of this study was to investigate whether the oxidative stress mechanism is differently expressed in TN1 and TNG67 rice seedlings in response to Cd.

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Materials and Methods

Plant Cultivation and Treatment

Two rice cultivars, an Indica type cultivar, TN1 and a Japonica cultivar, TNG67, obtained from Taiwan Agricultural Research Institute, Taichung, Taiwan, were used in this study. Seeds were sterilized with 2.5% (v/v) 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 without aeration as described previously (Chu and Lee, 1989). The concentrations of N, P, K, S, Ca and Mg in half strength Kimura B solution were 11.5, 2.9, 7.2, 15.0, 7.4, and 8.7 p.p.m., respectively. The hydroponically cultivated seedlings were grown in a Phytotron (Agricultural Experimental Station, National Taiwan university, Taipei, Taiwan) with natural light at 30°C day (12 h)/25°C night (12 h) and 90% relative humidity. Twelve-day-old seedlings with three leaves were used in all experiments.

For Cd, abscisic acid (ABA), and fluridone (Flu) treatment, $CdCl_2$ (0.5 or 1.5 mM), ABA (5 μM), and Flu (0.2 mM) were added directly to the culture solution during experiment.

Cd Determination

For determination of Cd, leaves or roots 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 % (v/v) H_2O_2 at 72°C for 2 h, and dissolved in 0.1 N HCl. Cd was then quantified using an atomic absorption spectrophotometer (Model AA-6800; Shimadzu, Kyoto, Japan). The amount of Cd was expressed on the basis of dry weight (DW).

Determination of Chlorophyll, Protein, H_2O_2, and Lipid Peroxidation

Chlorophyll content was determined according to Wintermans and De Mots (1965) after extraction in 96% (v/v) ethanol. For protein determination, leaves were homogenized in a 50 mM sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,600 *g* for 20 min, and the supernatants were used for determination of protein by the method of Bradford (1976) and antioxidant enzyme activities. The H_2O_2 content was measured colorometrically as described by Jana and Choudhuri (1981). 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, 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. The H_2O_2 content was calculated using the extinction coefficient 0.28 $\mu mol^{-1}\ cm^{-1}$. Malondialdehyde (MDA), routinely used as an indicator of lipid peroxidation, was extracted with 5% trichloroacetic acid and determined according Heath and Packer (1968). Chlorophyll, protein, H_2O_2, and MDA contents were expressed on the basis of initial fresh weight (FW).

Enzyme Assays

CAT activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Kato and Shimizu, 1987). The decrease in H_2O_2 was followed as the decline in absorbance at 240 nm, and activity was calculated using the extinction coefficient (40 $mM^{-1}\ cm^{-1}$ for at 240 nm) for H_2O_2 (Kato and Shimizu, 1987). POX activity was measured using a modification of the procedure of MacAdam et al. (1992). Activity was calculated using the extinction coefficient (26.2 $mM^{-1}\ cm^{-1}$ at 470 nm) for tetraguaiacol. SOD was determined according to Paoletti et al. (1986). APX was determined according to Nakano and Asada (1981). The decrease in ascorbate concentration was followed as the decline in absorbance at 290 nm and activity was calculated using the extinction coefficient (2.8 $mM^{-1}\ cm^{-1}$ at 290 nm) for ascorbate. GR was determined by the method Foster and Hess (1980). One unit of activity for CAT, POX, SOD, APX, and GR was defined as the amount of enzyme which degraded 1 μmol tetraguaiacol per min, inhibited by 50% the rate of NADH oxidation observed in control, degraded 1 μmol of ascorbate per min, and decreased 1 A_{340} per min, respectively. Enzyme activities are expressed on the basis of mg protein.

Statistical Analysis

Statistical differences between measurements (n = 4) on different treatments or on different times were analyzed following Duncan's multiple range test or Student's *t*-test.

Results

TNG67 Is More Tolerant to Cd Than TN1

In plants, the most apparent symptom of Cd toxicity is chlorosis of the leaves (Das et al., 1997). When rice seedlings of TN1 and TNG67 were treated with 0.5 mM $CdCl_2$ for 7 d, leaf chlorosis was observed in TN1 seedlings, but not in TNG67 seedlings (Figure 1). In short term (3 d) experiments, chlorosis was first observed in the second leaf of TN1 seedlings. In the present investigation, unless otherwise indicated, Cd toxicity in the second leaf caused by 0.5 mM $CdCl_2$ was assessed by a decrease in chlorophyll and protein contents. Figure 2 shows the time courses of chlorophyll and protein contents in the second leaf of TN1 and TNG67 seedlings treated with or without 0.5 mM $CdCl_2$. It is clear that the decrease in chlorophyll and protein contents in TN1 leaves is more pronounced than in TNG 67 leaves, indicating that TNG67 seedlings are a Cd-tolerant cultivar while TN1 seedlings are Cd-sensitive. Figure 3 shows the changes in Cd content in rice seedling treated with 0.5 mM $CdCl_2$. Cd content in the second leaf and roots of TNG67 seedlings remained unchanged and slightly increased, respectively, after Cd treatment. In contrast, a marked increase in Cd content in Cd-treated TN1 leaves and roots was observed.

It seems that avoiding the build-up of Cd in the second leaf of TNG 67 prevents the toxic effect of Cd.

Oxidative Stress is Induced by Cd in TN1 but Not in TNG67 Leaves

MDA content in the second leaf remained unchanged in TN1 seedlings treated without $CdCl_2$ (Figure 4). It is clear that increase in MDA content in TN1 leaves induced by $CdCl_2$ was evident at 1 d after $CdCl_2$ treatment (Figure 4).

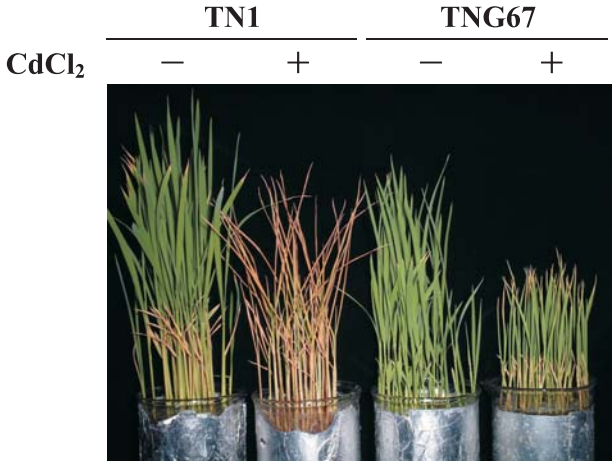

Figure 1. Effect of $CdCl_2$ (0.5 mM) on leaf chlorosis of Taichung Native 1 (TN1) and Tainung 67 (TNG67) rice seedlings. Picture was taken after 7 d of treatment.

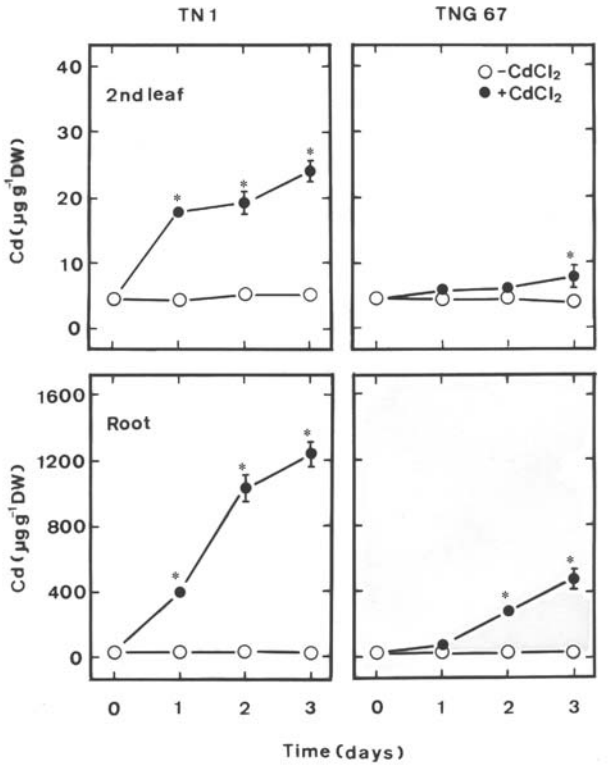

Figure 3. Changes in Cd content in the second leaf and roots of rice seedlings treated with or without $CdCl_2$ (0.5 mM). Bars represent standard errors (n = 4). Asterisks represent values significantly different at $P < 0.05$.

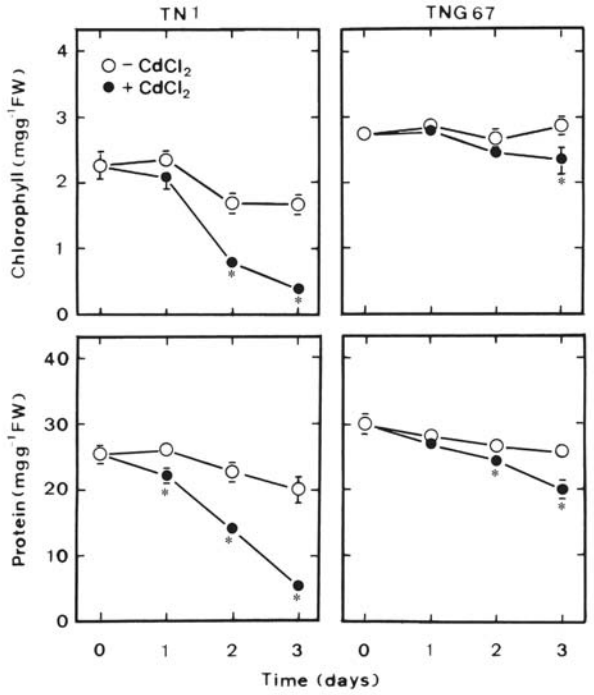

Figure 2. Changes in chlorophyll and protein contents in the second leaf of rice seedlings treated with or without $CdCl_2$ (0.5 mM). Bars represent standard errors (n = 4). Asterisks represent values significantly different at $P < 0.05$.

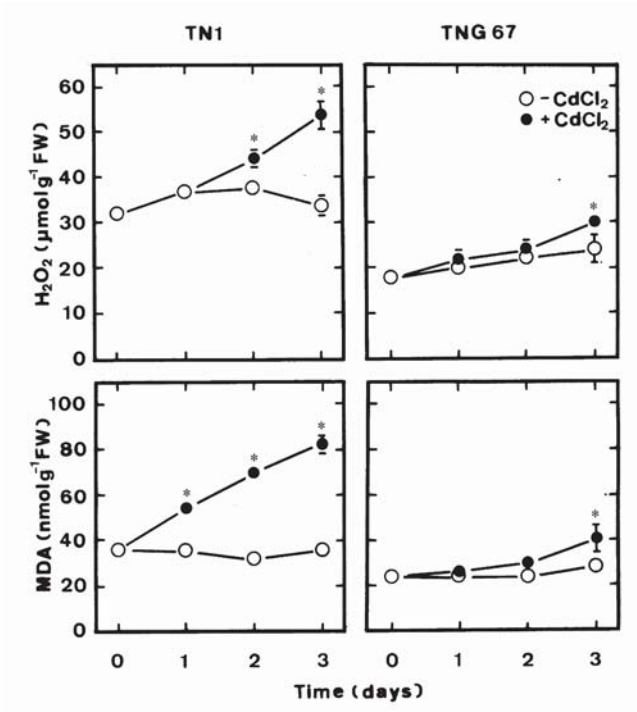

Figure 4. Changes in H_2O_2 and MDA contents in the second leaf of rice seedlings treated with or without $CdCl_2$ (0.5 mM). Bars represent standard errors (n = 4). Asterisks represent values significantly different at $P < 0.05$.

However, no increase in MDA content in TNG 67 leaves was observed throughout the $CdCl_2$ treatment (Figure 4). This showed that the Cd toxicity in TN1 leaves was linked to lipid peroxidation. Lipid peroxidation is caused by AOS (Thompson et al., 1987). $CdCl_2$ treatment also caused an increase in H_2O_2 content in TN1 but not in TNG67 leaves (Figure 4). These results all support the involvement of AOS as the chemical species inducing Cd toxicity in TN1 leaves.

The striking increase in lipid peroxidation seen in TN1 leaves treated with $CdCl_2$ may be a reflection of the changes in antioxidant enzyme activities. As shown in Figures 5 and 6, $CdCl_2$-treated TN1 leaves had higher activities of SOD and POX than the controls at 1 d after treatment. Higher activities of GR, APX, and CAT activities of TN1 leaves was observed at 2 and 3 d, respectively, after treatment. However, no or a slight increase in antioxidant enzyme activities in Cd-treated TNG67 leaves was observed (Figures 5 and 6).

Oxidative Stress in TN1 Leaves Induced by Cd Precedes the Occurrence of Cd Toxicity

In order to know if increase in MDA content and antioxidant enzyme activities caused by $CdCl_2$ in TN1 leaves is a cause or a consequence of Cd toxicity, a short-term experiment (24 h) was conducted. As shown in Figure 7, increase in the content of MDA and the activities of SOD

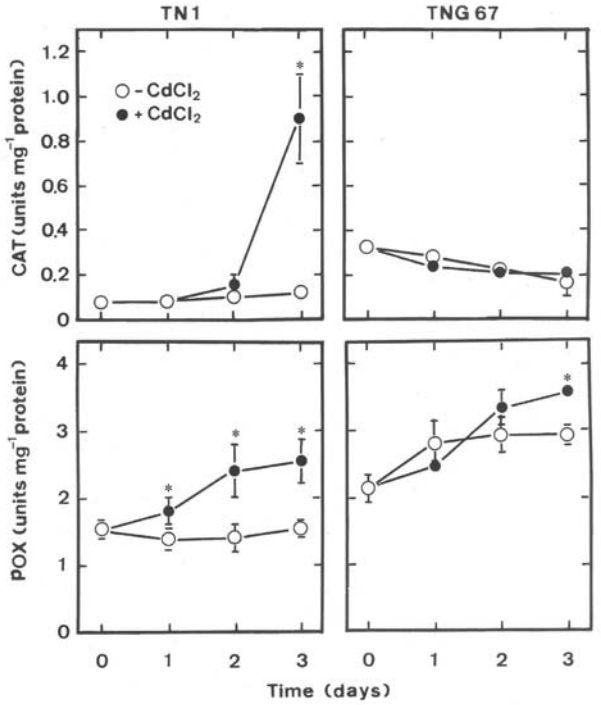

Figure 6. Changes in the activities of CAT and POX in the second leaf of rice seedlings treated with or without $CdCl_2$ (0.5 mM). Bars represent standard errors (n = 4). Asterisks represent values significantly different at $P < 0.05$.

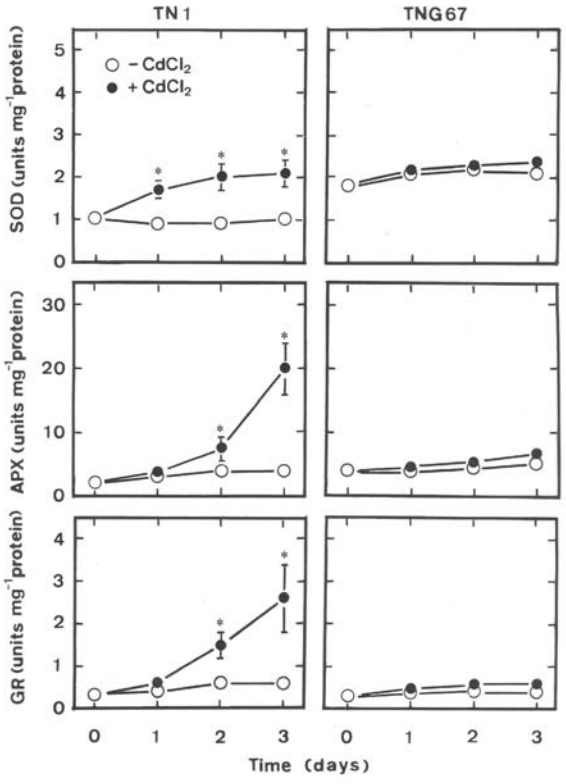

Figure 5. Changes in the activities of SOD, APX, and GR in the second leaf of rice seedlings treated with or without $CdCl_2$ (0.5 mM). Bars represent standard errors (n = 4). Asterisks represent values significantly different at $P < 0.05$.

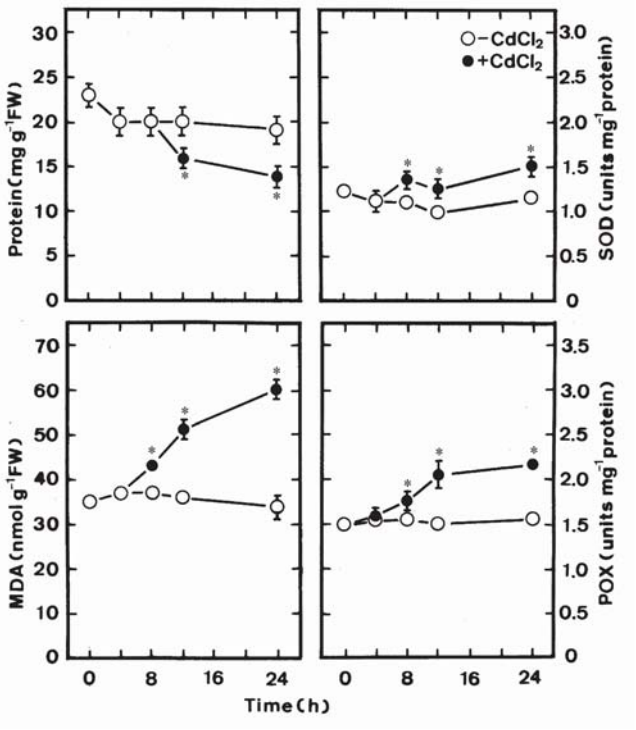

Figure 7. Changes in the contents of protein and MDA and the activities of SOD and POX in the second leaf of TN1 rice seedlings treated with or without $CdCl_2$ (0.5 mM) for 24 h. Bars represent standard errors (n = 4). Asterisks represent values significantly different at $P < 0.05$.

and POX in TN1 seedlings treated with $CdCl_2$ was observed prior to the occurrence of Cd toxicity (decrease in protein content), indicating that the induction of oxidative stress in Cd-treated TN1 leaves is a cause of Cd toxicity.

Pretreatment with Abscisic Acid (ABA) Reduces Cd Toxicity and Cd-induced Oxidative Stress in TN1 Leaves

In a more recent work, we reported that ABA plays an important role in Cd tolerance and pretreatment of TN1 seedlings with ABA counteracting Cd-induced toxicity (Hsu and Kao, 2003a; b). If oxidative stress is an important mechanism in regulating Cd toxicity in TN1 leaves, then pretreatment with ABA is expected to reduce Cd toxicity and Cd-increased content of MDA and activities of SOD and POX in TN1 leaves. Since some chlorosis was observed in the second leaf of TN1 seedlings treated with ABA for 2 d, Cd toxicity was evaluated by using the third leaf and 1.5 mM $CdCl_2$. As expected, ABA pretreatment reduced Cd toxicity and reduced Cd-induced increase in the content of MDA and increase in the activities of SOD and POX in the third leaf of TN1 seedlings (Figure 8 and 9).

Fluridone (Flu) Treatment Enhances Toxicity and Oxidative Stress in Cd-treated TNG67 Leaves

Previously, we have shown that treatment with Flu, an inhibitor of ABA biosynthesis, inhibited the increase in ABA content and enhanced toxicity in the second leaf of TNG67 seedlings treated with $CdCl_2$ (Hsu and Kao, 2003a; b). Here, we also observed that Flu enhanced toxicity, increased the content of MDA, and increased the activities of SOD and POX in Cd-treated TNG 67 leaves (Figures 10 and 11). It is interesting to note that the effect of Flu on Cd toxicity and oxidative stress in TNG67 can be reversed by the application of ABA (Figures 10 and 11).

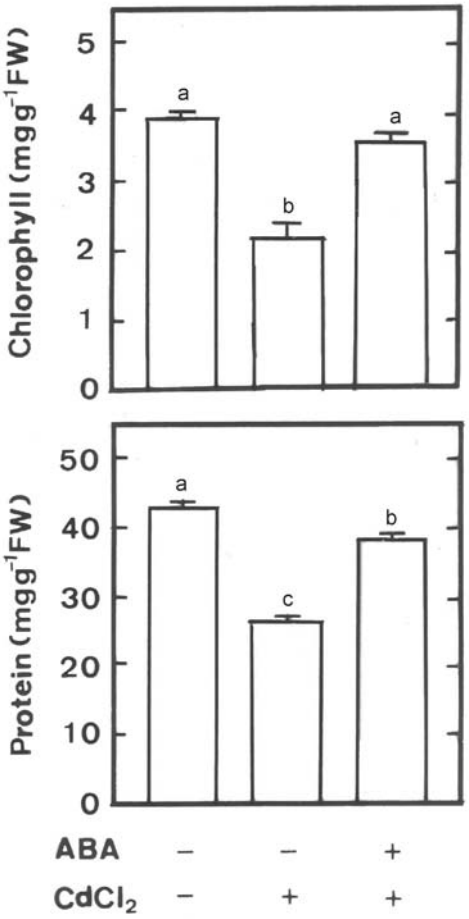

Figure 8. Effect of ABA-pretreatment on chlorophyll and protein contents in the third leaf of TN1 rice seedlings treated with or without $CdCl_2$ (1.5 mM). Taichung Native 1 (TN1) rice seedlings were pretreated with ABA (5 μM) for 2 d and then treated with or without $CdCl_2$ (1.5 mM) for 2 d. Bars represent standard errors (n = 4). Values with the same letter are not significantly different at $P < 0.05$.

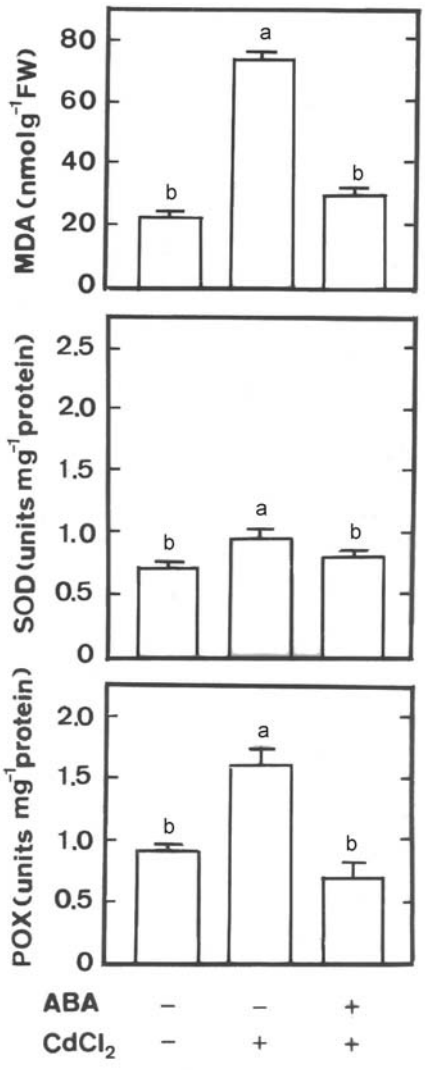

Figure 9. Effect of ABA-pretreatment on the content of MDA and the activities of SOD and POX in the third leaf of Taichung Native 1 (TN1) rice seedlings treated with or without $CdCl_2$ (1.5 mM). TN1 rice seedlings were pretreated with ABA (5 μM) for 2 d and then treated with or without $CdCl_2$ (1.5 mM) for 2 d. Bars represent standard errors (n = 4). Values with the same letter are not significantly different at $P < 0.05$.

Discussion

Plants have a range of potential mechanisms at the cellular level that might be involved in detoxification and thus tolerance to heavy metals. These all appear to be involved primarily in avoiding the build-up of toxic concentrations at sensitive sites within the cell and thus preventing damage (Hall, 2002). Obviously Cd-tolerant plant must be able to prevent the absorption of excess Cd. Of the two rice cultivars used in the present study, more absorption of Cd was detected in Cd-sensitive TN1 seedlings than in Cd-tolerant TNG67 (Figure 3). Jarvis et al. (1976) examined the Cd distribution in 23 species and found that the higher up in the plant one checked, the lower the Cd concentration, which decreased in the following order; fibrous roots > storage roots > stems > leaves. We also observed that Cd content was higher in roots than leaves in both TN1 and TNG67 seedlings (Figure 3).

It has been documented that Cd can cause an increased production of AOS, including H_2O_2 (Piqueras et al., 1999; Romero-Puertas et al., 1999; Shah et al., 2001; Sandalio et al., 2001; Schützendübel et al., 2001; Olmos et al., 2003) and induce lipid peroxidation (Shaw, 1995; Gallego et al., 1996; Benavides and Tomaro, 1996; Chaoui et al., 1997; Lozano-Rodríquez et al., 1997; Dixit et al., 2001; Shah et al., 2001; Chien et al., 2002). This suggests that Cd treatment causes oxidative stress in plants. In the present study, five lines of evidence indicated that oxidative stress seems to be involved in toxicity of rice seedlings caused by $CdCl_2$. First, on treatment with $CdCl_2$, lipid peroxidation, H_2O_2 content, and antioxidant enzyme activities increased in the leaves of the Cd-sensitive TN1 but not in the Cd-tolerant TNG67 (Figures 4, 5, and 6). Second, Cd-increased lipid peroxidation, and the activities of SOD and POX preceded the appearance of Cd toxicity in TN1 leaves (Figure 7). Third, pretreatment with ABA enhanced Cd tolerance and reduced Cd-increased lipid peroxidation and the activities of SOD and POX in TN1 leaves (Figures 8 and 9). Fourth, exogenous application of ABA biosynthesis

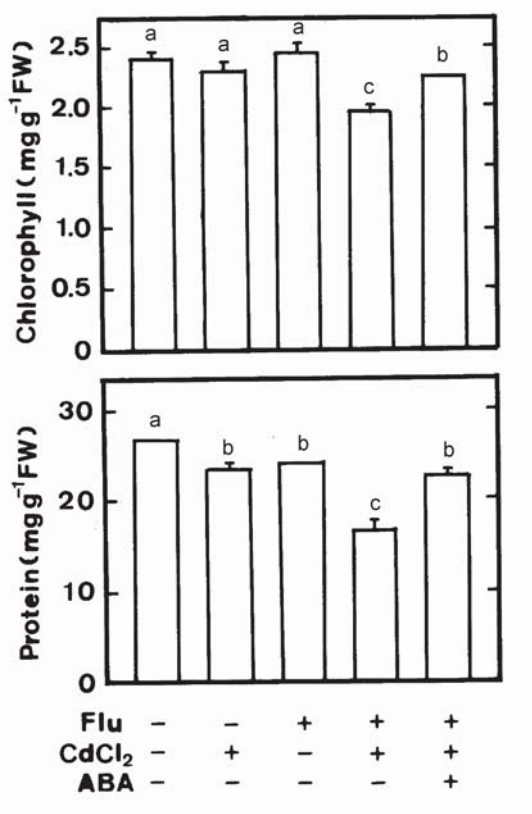

Figure 10. Effect of fluridone (Flu, 0.2 mM) on the contents of chlorophyll and protein in the second leaf of Tainung 67 (TNG67) rice seedlings treated with $CdCl_2$ (0.5 mM) and ABA (5 μM). Chlorophyll and protein contents were measured after 2 d of treatments. Bars represent standard errors (n = 4). Values with the same letter are not significantly different at $P<0.05$.

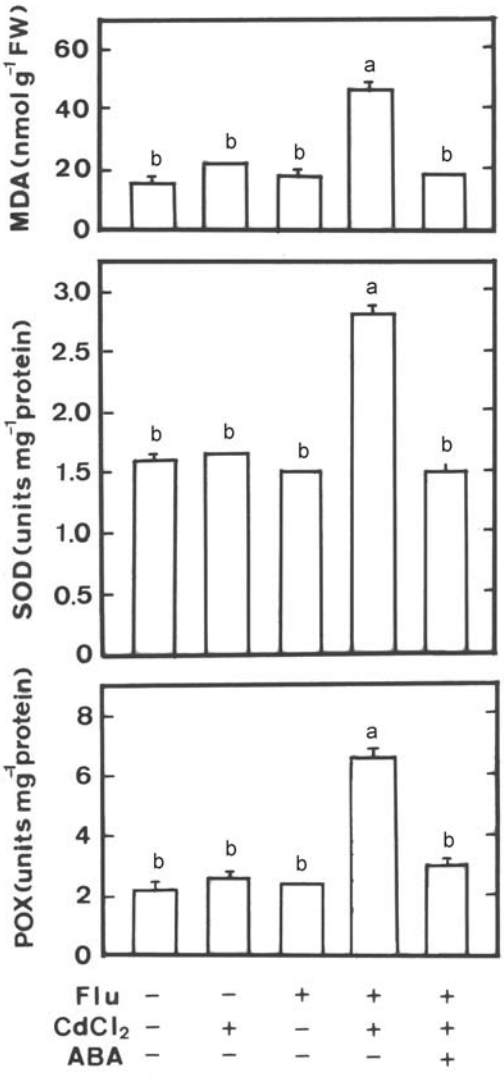

Figure 11. Effect of fluridone (Flu, 0.2 mM) on the content of MDA and the activities of SOD and POX in the second leaf of Tainung 67 (TNG67) rice seedlings treated with $CdCl_2$ (0.5 mM) and ABA (5 μM). Chlorophyll and protein contents were measured after 2 d of treatments. Bars represent standard errors (n = 4). Values with the same letter are not significantly different at $P < 0.05$.

inhibitor, Flu, decreased Cd tolerance, increased lipid peroxidation, and increased SOD and POX activities in Cd-treated TNG67 leaves (Figures 10 and 11). Fifth, Flu effect on Cd toxicity, lipid peroxidation, and the activities of SOD POX in Cd-treated TNG67 leaves was reversed by the application of ABA (Figures 10 and 11).

Cadmium produced an enhancement of MDA content in TN1 leaves (Figure 4), which is an indicator of lipid peroxidation and, therefore, of oxidative stress. Similar increases in MDA content by Cd treatment have been observed in *Phaseolus vulgaris* (Shaw, 1995; Chaoui et al., 1997), *Helianthus annuus* (Gallego et al., 1996), *Pisum sativum* (Lozano-Rodríguez et al., 1997; Dixit et al., 2001), and *Oryza sativa* plants (Shah et al., 2001). The peroxidation of cell membranes severely affects its functionality and integrity and can produce irreversible damage to cell function (Thompson et al., 1987). Lipid peroxidation can be initiated by AOS (Thompson et al., 1987). Unlike Cu and Fe, Cd is not a redox metal and therefore cannot catalyze Fenton-type reactions yielding AOS. However, Cd can induce oxidative stress indirectly by producing a disturbance in chloroplasts. Thus, Cd produced degradation of chlorophyll and carotenoids as well as an inhibition of their biosynthesis (Bazzaz et al., 1992), which can produce disturbances in electron transport rates of PSI and PSII, leading to generation of AOS. Ascorbate is a major antioxidant in photosynthetic and non-photosynthetic tissues which reacts directly with AOS and is utilized as a substrate for APX catalysed H_2O_2 detoxification (Noctor and Foyer, 1998). Reduced glutathione (GSH) is involved in ascorbate regeneration and functions also as a direct antioxidant of AOS (Noctor and Foyer, 1998). We have shown that $CdCl_2$ treatment significantly reduced the contents of GSH and ascorbate in rice leaves in TN1 leaves (Hsu and Kao, 2004). In a recent review, Schützendübel and Polle (2002) also suggest that the depletion of GSH is apparently a critical step in Cd-induced AOS.

Our results indicated an increase in antioxidant enzyme activities in Cd-treated TN1 leaves (Figures 4, 5 and 6). In line with our observations, a Cd-induced increase in antioxidant enzyme activities was also reported by Dixit et al. (2001), Schützendübel et al. (2001), Shah et al. (2001), and Iannelli et al. (2002). Nitric oxide, a bioactive free radical, is known to act as an AOS scavenger (Lamattina et al., 2003). In a recent report, we were able to show that nitric oxide effectively reduced Cd-increased MDA content and antioxidant enzyme activities in TN1 leaves (Hsu and Kao, 2004). It appears that Cd-induced increase in antioxidant enzyme activities is a consequence of AOS overproduction (Thompson et al., 1987).

Information is limited about the mechanism with which Cd leads to the generation of H_2O_2. In cultured tobacco cells (BY-2 line), diphenyleneiodonium and imidazole, both inhibitors of neutrophil NADPH oxidase, prevented the generation of H_2O_2 induced by Cd (Olmos et al., 2003). These data suggest the involvement of an NADPH oxidase-like enzyme leading to H_2O_2 production through O_2^- dismutation by SOD. We have not investigated whether H_2O_2 production in Cd-treated TN1 leaves was augmented by the stimulation of NADPH oxidase-like enzyme. However, this is likely to occur because Cd treatment resulted in an increase in H_2O_2 content and in SOD activity in TN1 leaves (Figures 4 and 5).

The most novel aspects of this report are the findings that ABA reduces oxidative stress indicators in Cd sensitive cultivar (TN1) and Flu increases indicators in Cd tolerant cultivar (TNG67) (Figures 9 and 11). The simplest explanation of this finding is that ABA closed stomata and reduced Cd uptake while Flu opened stomata and increased Cd uptake. Indeed, in our previous work, we have shown that exogenous ABA application could result in the decrease in transpiration rate and Cd content of TN1 seedlings and Flu treatment caused not only the block of ABA biosynthesis but also the increase in transpiration rate and Cd content in TNG67 seedlings (Hsu and Kao, 2003a).

In conclusion, we provide evidence that oxidative stress is differently expressed in TN1 and TNG67 rice seedlings in response to $CdCl_2$. Results also suggest that $CdCl_2$ causes oxidative stress and $CdCl_2$-induced toxicity is mediated through oxidative stress in TN1 leaves.

Acknowledgements. This work was financially supported by the National Science Council of the Republic of China.

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鎘所誘導之抗氧化酵素活性變化：耐鎘與不耐鎘水稻幼苗之比較

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本研究探討鎘處理對水稻兩個品種過氧化氫與 malondialdehyde（MDA）含量以及抗氧化酵素活性變化之影響。氯化鎘處理增加不耐鎘品種台中在來一號（TN1）葉片之過氧化氫與 MDA 含量以及增加抗氧化酵素［superoxide dismutase (SOD), ascorbate peroxidase, glutathione reductase, catalase 與 peroxidase (POX)］之活性，但不影響耐鎘品種台農 67 號 (TNG67) 葉片過氧化氫與 MDA 含量以及抗氧化酵素之活性。氯化鎘處理之 TN1 葉片，其 MDA 含量與 SOD 及 POX 活性之增加早於毒害之發生。以離層酸前處理 TN1 幼苗，增加 TN1 對鎘的耐性，同時降低鎘所誘導之 MDA 含量與 SOD 及 POX 活性之增加。以離層酸合成抑制劑（fluridone）與鎘同時處理 TNG67 幼苗，可降低 TNG67 對鎘之耐性，增加 MDA 含量與 SOD 及 POX 之活性。同時，此種 fluridone 處理 TNG67 幼苗之效應又可被離層酸處理所恢復。本研究之結果似乎說明，氯化鎘處理 TN1 幼苗，其葉片可表現氧化逆境，然而鎘處理 TNG67 幼苗，其葉片不會表現氧化逆境，同時也說明鎘處理後 TN1 幼苗葉片所表現之鎘毒害是經由氧化逆境所造成。

關鍵詞：離層酸；鎘；水稻；氧化逆境。

附件二（94-95 年成果）

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Abscisic acid accumulation and cadmium tolerance in rice seedlings

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Rice (*Oryza sativa* L.) seeds were soaked for 18 h in distilled water in the absence (–PBZ) or presence (+PBZ, a triazole) of 100 mg l^{-1} paclobutrazol and then air dried. These air-dried seeds were germinated in the dark and then cultivated in a Phytotron. Twelve-day-old –PBZ and +PBZ seedlings were treated or not with $CdCl_2$. Cd toxicity was judged by the decrease in biomass production, decrease in chlorophyll and protein content, increase in NH_4^+ content and induction of oxidative stress. The results indicated that PBZ applied to seeds was able to protect rice seedlings from Cd toxicity. On treatment with $CdCl_2$, the abscisic acid (ABA) content increased in +PBZ leaves, but not in –PBZ leaves. The decrease in the transpiration rate of –PBZ seedlings by $CdCl_2$ was less than that of +PBZ seedlings. Exogenous application of the ABA biosynthesis inhibitor, fluridone (Flu), reduced ABA accumulation, increased the transpiration rate and Cd content, and decreased the Cd tolerance of +PBZ seedlings. The effects of Flu on the Cd toxicity, transpiration rate and Cd content were reversed by the application of ABA. It seems that the PBZ-induced Cd tolerance of rice seedlings is mediated through an accumulation of ABA.

Introduction

Paclobutrazol [PBZ, (2*RS*,3*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazolyl)-pentan-3-ol], a member of the triazole plant growth regulator group, is an inhibitor of gibberellin (GA) biosynthesis (Graebe 1987, Rademacher et al. 1987, Davis and Curry 1991) and a retardant of shoot growth (Cox and Keever 1988, Davis et al. 1988, Davis and Curry 1991). The primary mode of action of PBZ is inhibition of *ent*-kaurene oxidase to *ent*-kaurenoic acid in the early pathway of GA biosynthesis (Graebe 1987, Rademacher et al. 1987). PBZ has been used to provide plant protection against environmental stresses, such as chilling (Whitaker and Wang 1987, Lurie et al. 1994, Pinhero and Fletcher 1994, Pinhero et al. 1997), heat (Kraus and Fletcher 1994, Pinhero and Fletcher 1994, Kraus et al. 1995, Gilley and Fletcher 1998, Vettakkorumakankav et al. 1999), flooding (Webb and Fletcher 1996), salt (Abou El-Khashab et al. 1997) and gaseous sulphur dioxide (Lee et al. 1985).

Cadmium (Cd) is a heavy metal that is toxic to humans, animals and plants, and is a widespread pollutant with a long biological half-life (Wagner 1993). This metal enters the environment mainly from industrial processes and phosphate fertilizers and is transferred to animals and humans through the food chain (Wagner 1993). Taken up in excess by plants, Cd directly or indirectly inhibits physiological processes, such as

Abbreviations – ABA, abscisic acid; AOS, active oxygen species; APX, ascorbate peroxidase; ASC, ascorbate; CAT, catalase; d. wt., dry weight; ELISA, enzyme-linked immunosorbent assay; Flu, fluridone; f. wt., initial fresh weight; GA, gibberellin; GR, glutathione reductase; GS, glutamine synthetase; GSH, reduced glutathione; MDA, malondialdehyde; PAL, phenylalanine ammonia-lyase; PBZ, paclobutrazol; POX, peroxidase; SOD, superoxide dismutase; TN1, Taichung Native 1.

respiration, photosynthesis, cell elongation, plant–water relationships, nitrogen metabolism and mineral nutrition, resulting in poor growth and low biomass (Sanitá di Toppi and Gabbrielli 1999).

In Taiwan, inappropriate disposal of industrial waste has given rise to widespread Cd contamination of irrigated water (higher than 10 mg l^{-1}). Thus, there is an urgent need to study the mechanism of Cd tolerance of rice plants.

It has been demonstrated that uniconazole, another potent member of the triazole family, induces Cd tolerance in wheat (Singh 1993). However, this is the only report describing the protective effect of triazole against Cd toxicity. Here, we show that PBZ protects rice seedlings from Cd toxicity, confirming the ability of PBZ to induce stress tolerance in plants.

In higher plants, abscisic acid (ABA) is a well-known stress hormone leading to the induction of various protective reactions, which are adaptations for coping with abiotic environmental stresses, such as ozone (Lin et al. 2001), freezing (Guy 1990), chilling (Lee et al. 1993), drought (Zeevaart and Creelman 1988) and salt (La Rosa et al. 1987). Recently, a role of ABA in the Cd tolerance of rice seedlings has been demonstrated (Hsu and Kao 2003a, 2003b, Kuo and Kao 2004). We found that rice seedlings of cultivar Tainung 67 were more tolerant to Cd than were those of cultivar Taichung Native 1 (TN1), and the increase in endogenous ABA content was closely related to the Cd tolerance of rice seedlings (Hsu and Kao 2003a). It is not known whether PBZ-induced Cd tolerance of rice seedlings is mediated through ABA accumulation. Thus, we also investigated the role of ABA in the PBZ-induced Cd tolerance of TN1 rice seedlings.

Materials and methods

Plant material and treatment

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 soaked for 18 h at room temperature in 100 mg l^{-1} PBZ or in distilled water (–PBZ), as described by Fletcher and Hosfsta (1990), and then were air dried for 5 days. These air-dried seeds were then germinated in Petri dishes with wet filter papers at 37°C in the dark. After 48 h of incubation, uniformly germinated seeds were selected and cultivated in a 250 ml beaker containing half-strength Kimura B solution including the following macro- and microelements: 182.3 μ*M* $(NH_4)_2SO_4$, 91.6 μ*M* KNO_3, 273.9 μ*M* $MgSO_4{\cdot}7H_2O$, 91.1 μ*M* KH_2PO_4, 182.5 μ*M* $Ca(NO_3)_2$, 30.6 μ*M* Fe-citrate, 0.25 μ*M* H_3BO_3, 0.2 μ*M* $MnSO_4{\cdot}H_2O$, 0.2 μ*M* $ZnSO_4.7H_2O$, 0.05 μ*M* $CuSO_4{\cdot}5H_2O$ and 0.07 μ*M* H_2MoO_4 (Chu and Lee 1989). The hydroponically cultivated seedlings were grown 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. Twelve-day-old seedlings with three leaves were used in all experiments.

For Cd, ABA or fluridone (Flu) treatment, 12-day-old seedlings were grown in basic nutrient solution plus 1.5 m*M* $CdCl_2$, 5 μ*M* ABA or 0.2 m*M* Flu.

Growth analysis

At the end of treatment, the seedlings were divided into their separate parts (shoot, adventitious roots and primary roots). The length of the shoot and primary roots and the fresh weight (f. wt.) of the shoot and roots (adventitious roots plus primary roots) were then measured. For dry weight (d. wt.) estimation, the shoot and roots were dried at 65°C for 48 h.

Cd determination

For the 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 was then quantified using an atomic absorption spectrophotomer (Model AA-6800, Shimadzu, Kyoto, Japan). The amount of Cd was expressed on a d. wt. basis.

Determination of chlorophyll, protein, NH_4^+, malondialdehyde (MDA), ascorbate (ASC) and reduced glutathione (GSH)

The chlorophyll content was determined according to Wintermans and De Mots (1965) after extraction in 96% (v/v) ethanol. For protein determination, leaves were homogenized in a 50 m*M* sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17 600 *g* for 20 min, and the supernatants were used for determination by the method of Bradford (1976). NH_4^+ was measured in the crude extract by the Berthelot reaction, modified according to Weatherburn (1967). The detailed procedure has been described previously (Lin and Kao 1996). MDA, routinely used as an indicator of lipid peroxidation, was extracted with 5% (w/v) trichloroacetic acid and determined according to Heath and Packer (1968). ASC in 5% (w/v) trichloroacetic acid

and GSH in 3% sulphosalicylic acid extract were determined as described by Laws et al. (1983) and Smith (1985), respectively.

Enzyme extraction and assays

For the extraction of enzymes, leaf tissues were homogenized with 0.1 *M* sodium phosphate buffer (pH 6.8) in a chilled pestle and mortar. The homogenate was centrifuged at 12 000 *g* for 20 min and the resulting supernatant was used for the determination of the enzyme activity. 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 inhibited by 50% the rate of NADH oxidation observed in a blank sample. Peroxidase (POX) activity was measured using a modification of the procedure of MacAdam et al. (1992). The activity was calculated using the extinction coefficient (26.2 mM^{-1} cm^{-1} at 470 nm) for tetraguaiacol. One unit of POX was definedas the amount of enzyme that caused the formation of 1 μmol tetraguaiacol per minute. Glutamine synthetase (GS) was assayed by the method of Oaks et al. (1980). One unit of GS was defined as the amount of enzyme that caused the formation of 1 μmol L-glutamate γ-monohydroxamate per minute. Phenylalanine ammonia-lyase (PAL) was extracted and determined according to Hyodo and Fujinami (1989). The activity was calculated using the extinction coefficient (9500 M^{-1} cm^{-1} at 290 nm) for *trans*-cinnamate. One unit of PAL was defined as the amount of enzyme that caused the formation of 1 μmol *trans*-cinnamate per hour.

ABA determination

For the extraction of ABA, leaves were homogenized with a pestle and mortar in extraction solution (80% methanol containing 2% glacial acetic acid). To remove plant pigments and other non-polar compounds which could interfere in the immunoassay, extracts were first passed through a polyvinylpyrrolidone column and C18 cartridges. The eluates were concentrated to dryness by vacuum evaporation and resuspended in Tris-buffered saline before enzyme-linked immunosorbent assay (ELISA). ABA was quantified by ELISA (Walker-Simmons 1987). The ABA immunoassay detection kit (PGR-1) was purchased from Sigma Chemical Co. (St. Louis, MO) and was specific for (+)-ABA. By evaluating ^{3}H-ABA recovery, ABA loss was less than 3% by the method described here.

Transpiration rate

The transpiration rate was measured according to Greger and Johansson (1992). The transpiration rate was calculated from the water loss during each interval and converted to a per day per seedling basis.

Expression of data and statistical analysis

In the present study, the third leaves of rice seedlings were used to determine chlorophyll, protein, NH_4^+, MDA, ASC, GSH and ABA. As the f. wt. of –PBZ leaves was no different from that of +PBZ leaves, data were expressed on the basis of initial fresh weight (f. wt.). Statistical differences between measurements ($n = 4$) for different treatments or for different times were analysed following the LSD test.

Results

Growth analysis

PBZ-treated rice seedlings exhibited typical characteristics of triazole treatment, such as reduced shoot length and f. wt. and enhanced primary root length (Table 1; Pinhero and Fletcher 1994). Shoot d. wt., root d. wt. and root f. wt. of PBZ-treated rice seedlings were not significantly different from those of the controls (–PBZ) (Table 1). Less adventitious roots were visually observed in +PBZ than in –PBZ seedlings. Both adventitious roots and primary roots were used to determine the f. wt. and d. wt. of roots. This may explain why the d. wt. and f. wt. of +PBZ roots were not significantly different from those of –PBZ roots.

Evaluation of Cd toxicity

Biomass production

Cd is readily taken up by rice seedlings, leading to growth reduction (Chen and Kao 1995). Thus, in the

Table 1. Growth analysis of rice seedlings 12 days after planting. Seeds were soaked for 18 h in water (–PBZ) or PBZ (+PBZ) and dried. The seedlings were cultivated for 12 days in a Phytotron with natural sunlight at 30°C (day)/25°C (night) and 90% relative humidity. Significant difference at $^{a}P < 0.01$ and $^{b}P < 0.05$, respectively. PBZ, paclobutrazol.

Parameter	–PBZ	+PBZ
Shoot length (cm)	13.7 ± 0.3	9.4 ± 0.1[a]
Root length (cm)	7.9 ± 0.2	13.2 ± 0.3[a]
Shoot fresh weight (mg seedling^{-1})	53.6 ± 3.1	44.0 ± 1.7[b]
Shoot dry weight (mg seedling^{-1})	12.7 ± 0.2	12.9 ± 0.1
Root fresh weight (mg seedling^{-1})	47.1 ± 3.9	40.5 ± 3.2
Root dry weight (mg seedling^{-1})	6.1 ± 0.4	6.7 ± 0.2

present study, Cd toxicity was first evaluated by biomass production (shoot and root d. wt.). The effect of $CdCl_2$ concentration on shoot and root d. wt. of rice seedlings is presented in Fig. 1. In –PBZ seedlings, the shoot d. wt. was decreased by 0.5 m*M* $CdCl_2$ and no further decrease was observed at 1 and 1.5 m*M* (Fig. 1A). However, $CdCl_2$ (0.5 – 1.5 m*M*) had no effect on the shoot d. wt. of +PBZ seedlings (Fig. 1B). Increasing concentrations of $CdCl_2$ from 0.5 to 1.5 m*M* progressively decreased the root d. wt. of –PBZ seedlings, but had no effect on the root d. wt. of +PBZ rice seedlings. Fig. 2 shows the time courses of biomass production of –PBZ and +PBZ seedlings in the presence or absence of 1.5 m*M* $CdCl_2$. The d. wt. of the shoot and roots of –PBZ seedlings treated with $CdCl_2$ was significantly lower than that of the –PBZ seedlings not treated with $CdCl_2$. However, the d. wt. of the shoot and roots of +PBZ seedlings was only slightly affected by $CdCl_2$. All of these results suggest that +PBZ seedlings are Cd tolerant.

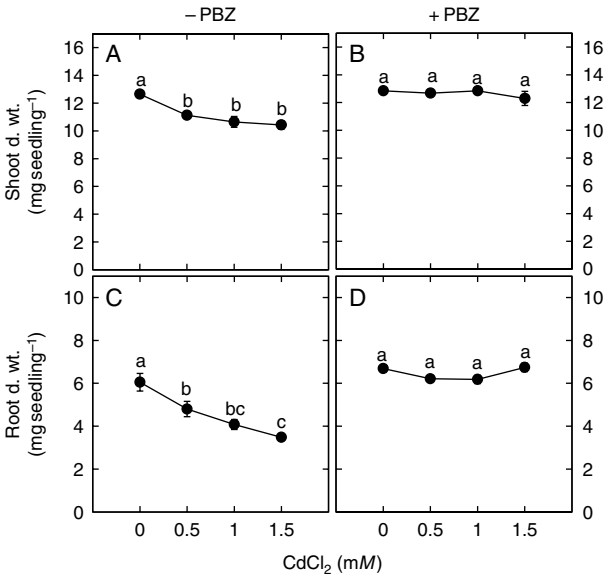

Fig. 1. Effect of $CdCl_2$ concentration on the dry weight (d. wt.) of shoot (A, B) and roots (C, D) of –PBZ and +PBZ rice seedlings (PBZ, paclobutrazol). The d. wt. of shoot and roots were measured 2 days after treatment. Bars indicate the standard error ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$.

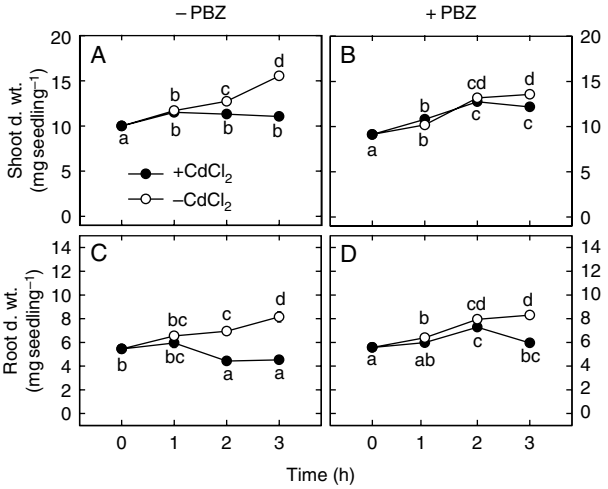

Fig. 2. Changes in the dry weight (d. wt.) of shoot (A, B) and roots (C, D) of –PBZ and +PBZ rice seedlings (PBZ, paclobutrazol) in the presence or absence of $CdCl_2$ (1.5 m*M*). Bars indicate the standard error ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$.

Chlorophyll and protein loss

In plants, the most general symptom of Cd toxicity is chlorosis (Das et al. 1997). In previous work, we have shown that rice seedlings treated with $CdCl_2$ at high (0.5 – 1.5 m*M*) and low (10–50 μ*M*) concentrations show chlorosis and protein loss (Hsu and Kao 2003a). However, a longer period (more than 6 days) was required to show chlorosis when rice seedlings were treated with low concentrations of $CdCl_2$ (Hsu and Kao 2003a). When seedlings were treated with 0.5 m*M* $CdCl_2$, 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 2004). In order to show chlorosis in the third leaves in a short-term experiment, 1.5 m*M* $CdCl_2$ was required. Thus, in the present study, Cd toxicity in the third leaves exposed to 1.5 m*M* $CdCl_2$ was assessed by the decrease in chlorophyll and protein content. A marked decrease in chlorophyll and protein was observed in –PBZ leaves after Cd treatment (Fig. 3A,C). In contrast, only a slight decrease in chlorophyll and protein content caused by $CdCl_2$ was observed in +PBZ leaves (Fig. 3B,D).

NH_4^+ accumulation

NH_4^+ is a central intermediate of nitrogen metabolism in plants (Miflin and Lea 1976), but a high content of NH_4^+ is known to have toxic effects on plant cells (Givan 1979). Recent studies have demonstrated that NH_4^+ accumulation in the leaves of rice seedlings is linked to Cd toxicity (Hsu and Kao 2003b). In this study, we showed that, on treatment with $CdCl_2$, the NH_4^+ content increased markedly in –PBZ leaves (Fig. 4A), but only slightly in +PBZ leaves (Fig. 4B). These results suggest that PBZ protects rice seedlings from NH_4^+ accumulation caused by Cd toxicity.

GS is the key enzyme in NH_4^+ assimilation and catalyses the ATP-dependent condensation of NH_4^+

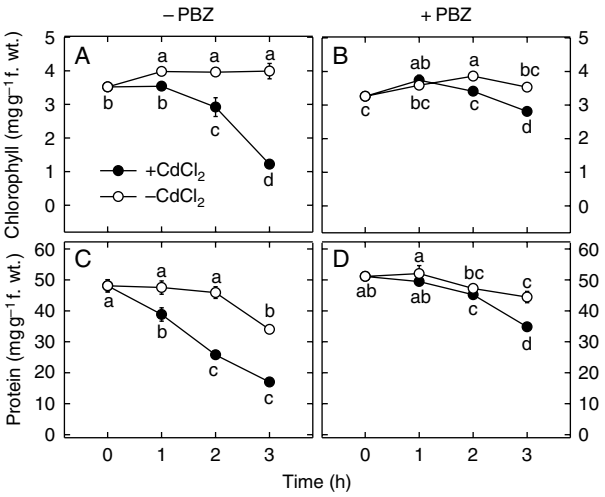

Fig. 3. Changes in the contents of chlorophyll (A, B) and protein (C, D) in the third leaves of –PBZ and +PBZ rice seedlings (PBZ, paclobutrazol) in the presence or absence of $CdCl_2$ (1.5 m*M*). Bars indicate the standard error ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$. f. wt., fresh weight.

with glutamate to produce glutamine (Miflin and Lea 1976). PAL catalyses the elimination of NH_4^+ from phenylalanine and produces *trans*-cinnamate (Hahlbrock and Grisebach 1979). NH_4^+ released from PAL reaction has been shown to be trapped in glutamine molecules by the action of GS (Razel et al. 1996, Van Heerden et al. 1996). Sakurai et al. (2001) have provided evidence to show that GS is partly coupled to the reaction of PAL in developing rice leaves. Previous

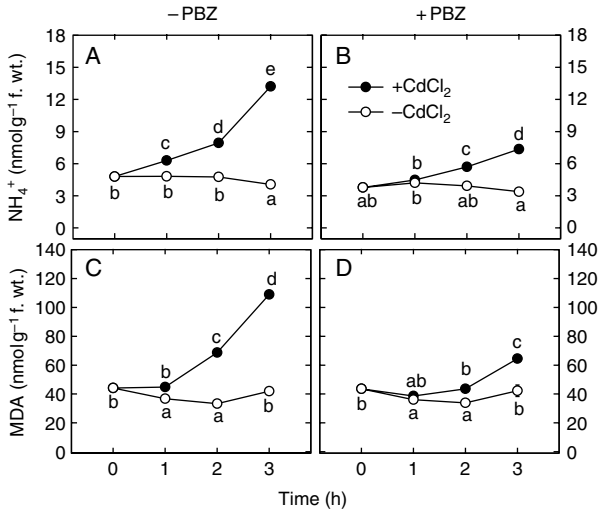

Fig. 4. Changes in the contents of NH_4^+ (A, B) and malondialdehyde (MDA) (C, D) of –PBZ and +PBZ rice seedlings (PBZ, paclobutrazol) in the presence or absence of $CdCl_2$ (1.5 m*M*). Bars indicate the standard error ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$. f. wt., fresh weight.

work has indicated that PAL and GS are the enzymes responsible for NH_4^+ accumulation in rice leaves caused by Cd toxicity (Hsu and Kao 2003b). In this study, we demonstrated that the increase in PAL specific activity and the decrease in GS activity caused by $CdCl_2$ were more pronounced in –PBZ than in +PBZ leaves (Fig. 5A,B). These results further support the conclusion that PBZ-treated seedlings are Cd tolerant.

NH_4^+ can also be produced during nitrate reduction and photorespiration (Miflin and Lea 1976). It is not known whether NH_4^+ accumulation in rice leaves caused by Cd toxicity is mediated via the promotion of nitrate reduction and photorespiration. Further research in this area is likely to be highly rewarding.

Oxidative stress

Unlike Cu and Fe, Cd is not a redox metal, and therefore cannot catalyse Fenton-type reactions yielding active oxygen species (AOS). However, Cd can induce oxidative stress indirectly by producing a disturbance in chloroplasts. Thus, Cd produces the degradation of chlorophyll and carotenoids, as well as an inhibition of their biosynthesis (Bazzaz et al. 1974), which can produce disturbances in the electron transport rates of PSI and PSII, leading to the generation of AOS. In a

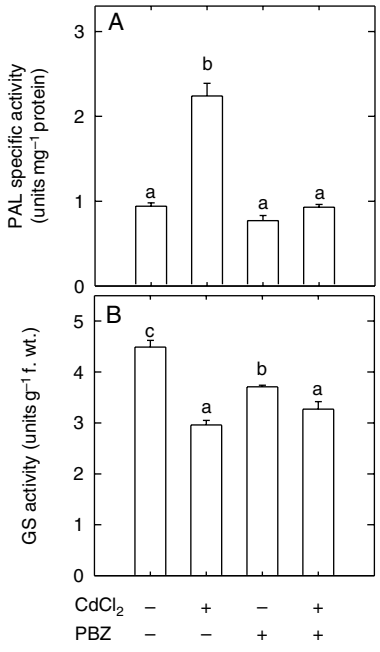

Fig. 5. Effect of $CdCl_2$ on phenylalanine ammonia-lyase (PAL) specific activity (A) and glutamine synthetase (GS) activity (B) in the third leaves of –PBZ and +PBZ rice seedlings (PBZ, paclobutrazol). Rice seedlings were either untreated or treated with $CdCl_2$ (1.5 m*M*) for 2 days. Bars indicate the standard error ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$. f. wt., fresh weight.

recent review, Schützendübel and Polle (2002) suggested that the depletion of GSH was a critical step in Cd-induced AOS generation.

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) (Hsu and Kao 2004, Kuo and Kao 2004). In the present study, we observed that the increase in the content of MDA (Fig. 4) caused by $CdCl_2$ was more pronounced in –PBZ leaves (Fig. 4C) than in +PBZ leaves (Fig. 4D).

The striking increase in lipid peroxidation seen in –PBZ leaves treated with $CdCl_2$ (Fig. 4C) may reflect changes in the activities of antioxidant enzymes and contents of antioxidants. Previously, it has been observed that increases in SOD and POX specific activities in rice seedlings take place prior to the occurrence of Cd toxicity (decrease in protein content) (Kuo and Kao 2004). In this study, we showed that the increase in the specific activities of SOD and PO, caused by $CdCl_2$, was more pronounced in –PBZ leaves than in +PBZ leaves (Fig. 6A,B). ASC is a major antioxidant in photosynthetic and non-photosynthetic tissues, which reacts directly with AOS and is utilized as a substrate for APX-catalysed H_2O_2 detoxification (Noctor and Foyer 1998). GSH is involved in ASC regeneration and also functions as a direct scavenger of AOS (Noctor and Foyer 1998). It is clear from Figs 6C and 6D that the decrease in ASC and GSH contents caused by $CdCl_2$ was greater in –PBZ leaves than in +PBZ leaves. However, the depletion of ASC and GSH caused by $CdCl_2$ could not be prevented completely in +PBZ leaves, suggesting that there was still some oxidative stress in +PBZ seedlings when exposed to $CdCl_2$. All of the results presented here indicate that PBZ can be used to protect rice seedlings from oxidative stress caused by Cd toxicity.

ABA accumulation in +PBZ leaves

Previously, we have shown that an increase in endogenous ABA content is closely related to the Cd tolerance of rice seedlings (Hsu and Kao 2003a). In this study, we showed that $CdCl_2$ treatment resulted in an increase in endogenous ABA in +PBZ leaves, but not in –PBZ leaves (Table 2), suggesting that ABA may play a role in Cd tolerance.

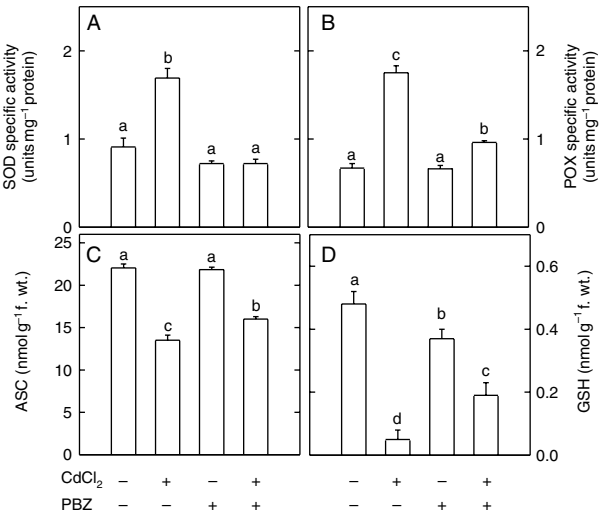

Fig. 6. Effect of $CdCl_2$ on the specific activities of superoxide dismutase (SOD) (A) and peroxidase (POX) (B) and the contents of ascorbate (ASC) (C) and reduced glutathione (GSH) (D) in the third leaves of –PBZ and +PBZ rice seedlings (PBZ, paclobutrazol). Rice seedlings were either untreated or treated with $CdCl_2$ (1.5 m*M*) for 2 days. Bars indicate the standard error ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$. f. wt., fresh weight.

Flu treatment

The role of ABA in PBZ-induced Cd tolerance was tested by using an inhibitor of ABA biosynthesis, Flu, which blocks the conversion of phytoene to phytofluene in the carotenoid biosynthesis pathway (Kowalczyk-Schröder and Sandmann 1992). Flu was observed to inhibit the increase in ABA content (Fig. 7E) and to enhance Cd toxicity (as judged by biomass production and the contents of chlorophyll and protein) in +PBZ seedlings (Fig. 7A – D). The effect of Flu on Cd toxicity in +PBZ seedlings was reversed by the application of

Table 2. Effect of $CdCl_2$ on abscisic acid (ABA) content, transpiration rate and Cd content in the third leaves of –PBZ and +PBZ rice seedlings. Rice seedlings were either untreated or treated with $CdCl_2$ (1.5 m*M*) for 2 days. Values with the same letter are not significantly different at $P < 0.05$. d. wt., dry weight; f. wt., fresh weight; PBZ, paclobutrazol.

Treatment		ABA content (pmol g^{-1} f. wt.)	Transpiration rate (mg H_2O seedling^{-1} day^{-1})	Cd content (μg g^{-1} d. wt.)
–PBZ	– $CdCl_2$	334.8 ± 21.0 (b)	566 ± 39.0 (d)	4.15 ± 0.34 (a)
	+ $CdCl_2$	337.6 ± 20.7 (b)	145 ± 9.1 (b)	33.68 ± 1.24 (c)
+PBZ	– $CdCl_2$	255.1 ± 27.6 (a)	456 ± 20.5 (c)	5.33 ± 0.84 (a)
	+ $CdCl_2$	328.1 ± 19.5 (b)	55 ± 6.1 (a)	20.13 ± 0.71 (b)

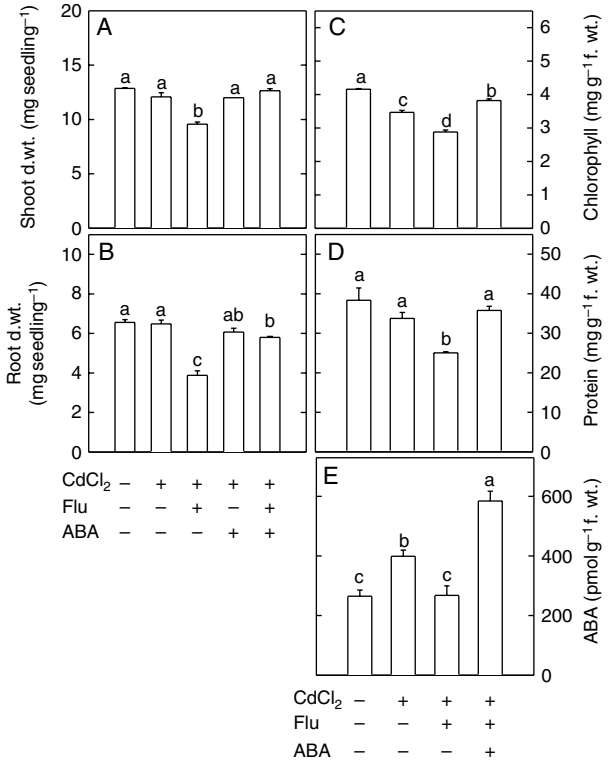

Fig. 7. Effect of fluridone (Flu, 0.2 m*M*) and abscisic acid (ABA) (5 μ*M*) on the dry weight (d. wt.) of shoot (A) and root (B) and the contents of chlorophyll (C), protein (D) and ABA (E) in the third leaves of +PBZ rice seedlings (PBZ, paclobutrazol) treated or not with $CdCl_2$ (1.5 m*M*). All measurements were made 2 days after treatment. Bars indicate the standard error ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$. f. wt., fresh weight.

ABA (Fig. 7A – D). It should be noted that $CdCl_2$ +ABA treatment resulted in a similar biomass production as $CdCl_2$ treatment (Fig. 7A,B). These results suggest that the ABA content in the leaves of +PBZ seedlings treated with $CdCl_2$ is sufficient to exert an effect on biomass production.

Cd content

Table 2 shows the effect of $CdCl_2$ on the Cd content in –PBZ and +PBZ leaves. The Cd content in +PBZ leaves increased about four-fold after Cd treatment (Table 2). However, an eight-fold increase in Cd content in Cd-treated –PBZ leaves was observed (Table 2). Flu treatment caused an increase in Cd content in the leaves of +PBZ seedlings exposed to $CdCl_2$ (Fig. 8A). The effect of Flu on the Cd content in Cd-treated leaves of +PBZ seedlings was reversed by the application of ABA (Fig. 8A).

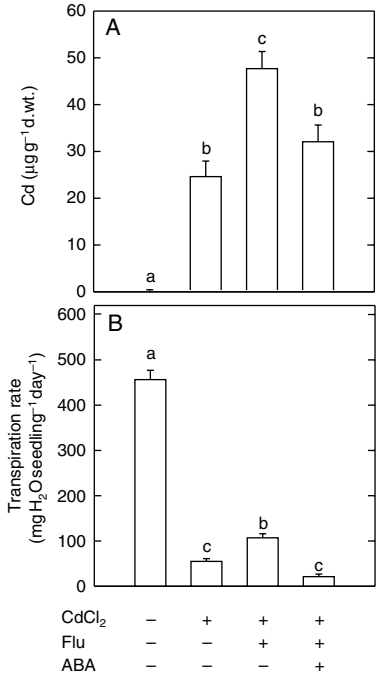

Fig. 8. Effect of fluridone (Flu, 0.2 m*M*) and abscisic acid (ABA) (5 μ*M*) on the Cd content (A) in the third leaves and the transpiration rate (B) of +PBZ rice seedlings (PBZ, paclobutrazol) treated or not with $CdCl_2$ (1.5 m*M*). The Cd content was measured 2 days after treatment, and the transpiration rate was measured 1 day after treatment. Bars indicate the standard error ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$. d. wt., dry weight.

Transpiration rate

In the absence of $CdCl_2$, the transpiration rate of –PBZ seedlings was observed to be higher than that of +PBZ seedlings (Table 2). Cd treatment decreased the transpiration rate in both –PBZ and +PBZ seedlings (Table 2). However, the decrease in the transpiration rate in response to $CdCl_2$ was less pronounced in –PBZ seedlings than in +PBZ seedlings (Table 2). Flu treatment resulted in an increase in the transpiration rate in +PBZ seedlings treated with $CdCl_2$ (Fig. 8B). The effect of Flu on the transpiration rate in +PBZ seedlings treated with $CdCl_2$ was reversed by ABA application (Fig. 8B).

Discussion

Cd causes biomass reduction (Chen and Kao 1995), chlorophyll and protein loss (Hsu and Kao 2003a), NH_4^+ accumulation (Hsu and Kao 2003b) and oxidative stress (Kuo and Kao 2004) in rice seedlings. In the present study, we evaluated Cd toxicity by the decrease in biomass production, decrease in chlorophyll and protein content, increase in NH_4^+ content and

induction of oxidative stress. On the basis of these criteria, we demonstrated that PBZ applied to seeds was able to protect rice seedlings from Cd toxicity. The protective effect of uniconazole, a member of the triazole family, against Cd stress has also been described previously (Singh 1993).

The present study indicated that ABA was involved in the Cd tolerance of +PBZ seedlings. This conclusion was based on the following observations: (1) the increase in the endogenous ABA content in response to Cd in +PBZ leaves was more pronounced than that in –PBZ leaves (Table 2); (2) Flu treatment led to a decrease in the ABA content, as well as Cd tolerance, of +PBZ seedlings (Fig. 7); and (3) the effect of Flu on the Cd toxicity of +PBZ seedlings was reversed by the application of ABA (Fig. 7). These results suggest that the regulation of endogenous ABA biosynthesis under Cd stress is correlated with the tolerance of +PBZ seedlings. As Flu is an inhibitor of ABA biosynthesis through the carotenoid pathway (Kowalczyk-Schröder and Sandmann 1992), the effects of this inhibitor on +PBZ leaves may imply that the ABA biosynthesis pathway in response to Cd appears to be the same as that established in other stress conditions (Zeevaart and Creelman 1988, Seo and Koshiba 2002). In addition, the defect in ABA accumulation in –PBZ leaves may account for the Cd intolerance of –PBZ seedlings.

Plants have a range of potential mechanisms at the cellular level that may be involved in the detoxification of, and thus tolerance to, heavy metals. These all appear to be involved primarily in avoiding the build-up of toxic concentrations at sensitive sites within the cell, and thus preventing damaging effects (Hall 2002). In this context, reduced translocation of Cd to the shoot appears to be a possible mechanism of Cd tolerance in the shoot. Cd translocation to the shoot has been suggested to be driven by transpiration (Salt et al. 1995). Cd has been shown to decrease the transpiration rate in several plants (Bazzaz et al. 1974, Kirkham 1978, Lamoreaux and Chaney 1978, Hagemeyer et al. 1986, Schlegel et al. 1987, Hsu and Kao 2003a). Here, we also observed that Cd treatment decreased the transpiration rate of –PBZ and +PBZ seedlings (Table 2). Cd treatment decreased the transpiration rate in –PBZ and +PBZ seedlings to about 74% and 88% of the control value, respectively (Table 2). Thus, the decrease in the transpiration rate of –PBZ seedlings (which were unable to accumulate ABA) caused by Cd was less than that in +PBZ seedlings (which accumulated ABA), and consequently resulted in a higher Cd content in –PBZ than in +PBZ seedlings (Table 2). The effect of Flu on +PBZ seedlings indicated that not only was ABA biosynthesis blocked, but the transpiration rate and Cd content were increased (Figs 7E and 8). Furthermore, the effect of Flu on the transpiration rate and Cd content of +PBZ seedlings was reversed by the application of ABA (Fig. 8). It appears that the increase in endogenous ABA content is closely related to the Cd tolerance of +PBZ seedlings. ABA may exert its regulatory effect on the transpiration rate, decreasing the translocation of Cd to the shoot.

Stress-tolerant plants often grown more slowly than stress-intolerant plants. It has been hypothesized that +PBZ plants show a better quality of growth than –PBZ plants under stress conditions as a result of the slower growth rate or metabolism of the former (Abou El-Khashab et al. 1997). Thus, the possibility that ABA may also exert its effect on the metabolism of +PBZ seedlings cannot be excluded.

PBZ inhibits GA biosynthesis (Graebe 1987, Rademacher et al. 1987). It has been shown that the application of GA counters both the growth inhibitory and stress-protective effects of triazoles (Guoping 1997, Vettakkorumakankav et al. 1999, Sarkar et al. 2004). An interesting question then arises: is a decrease in both endogenous GA content and shoot length essential to enhance Cd tolerance in rice seedlings? Future work will focus on this question by examining the Cd tolerance in GA-responsive and GA non-responsive dwarf mutants of rice.

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ORIGINAL PAPER

Cadmium-induced oxidative damage in rice leaves is reduced by polyamines

Yi Ting Hsu · Ching Huei Kao

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Abstract The protective effect of polyamines against Cd toxicity of rice (*Oryza sativa*) leaves was investigated. Cd toxicity to rice leaves was determined by the decrease in protein content. $CdCl_2$ treatment results in (1) increased Cd content, (2) induction of Cd toxicity, (3) increase in H_2O_2 and malondialdehyde (MDA) contents, (4) decrease in ascorbic acid (ASC) and reduced glutathione (GSH) contents, and (5) increase in the activities of antioxidative enzymes (superoxide dismutase, glutathione reductase, ascorbate peroxidase, catalase, and peroxidase). Spermidine (Spd) and spermine (Spm), but not putrescine (Put), were effective in reducing $CdCl_2$-induced toxicity. Spd and Spm prevented $CdCl_2$-induced increase in the contents of H_2O_2 and MDA, decrease in the contents of ASC and GSH, and increase in the activities of antioxidative enzymes. Spd and Spm pretreatments resulted in a decrease in Cd content when compared with H_2O pretreatment, indicating that Spd and Spm may reduce the uptake of Cd. Results of the present study suggest that Spd and Spm are able to protect Cd-induced oxidative damage and this protection is most likely related to the avoidance of H_2O_2 generation and the reduction of Cd uptake.

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Keywords Cadmium · Oxidative stress · Putrescine · Rice · Spermidine · Spermine

Abbreviations

APX	Ascorbate peroxidase
ASC	Ascorbic aicd
CAT	Catalase
DAB	3,3′-Diaminobenzidine
DHA	Dehydroascorbate
DW	Dry weight
FW	Fresh weight
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
POX	Peroxidase
Put	Putrescine
ROS	Reactive oxygen species
SOD	Superoxide dismutase
Spd	Spermidine
Spm	Spermine

Introduction

Cadmium (Cd), a heavy metal toxic to humans, animals, and plants, is a widespread pollutant with a long biological half-life (Wagner 1993). Cd is readily taken up by plants, leading to toxic symptoms such as growth reduction (Chen and

Kao 1995). Cd damages the photosynthetic apparatus (Krupa 1988; Siedlecka and Baszynski 1993), lowers chlorophyll (Stobart et al. 1985; Larsson et al. 1998), and alters proline and polyamine contents (Sharma and Dietz 2006).

Oxygen is essential for the existence of aerobic life, but toxic reactive oxygen species (ROS), which include the superoxide anion O_2^-, hydroxyl radical (OH•) and hydrogen peroxide (H_2O_2), are generated in all aerobic cells during metabolic processes (Asada 1999; Foyer et al. 1994, 1997). Initially, ROS were only regarded as damaging to cells (Apel and Hirt 2004). More recently, ROS emerged as ubiquitous signaling molecules participating in the recognition of and the response to stress factors (Foyer and Noctor 2005).

Injury caused by these ROS, known as oxidative stress, is one of the major damaging factors in plants exposed to environmental stress. Plants cope with oxidative stress by using antioxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), peroxidase (POX), catalase (CAT), and the low molecular weight antioxidants, ascorbic acid (ASC) and glutathione (GSH) (Asada 1999; Noctor and Foyer 1998). Three lines of evidence indicate that one mechanism of Cd toxicity is related to oxidative stress in plant cells. First, Cd can promote the generation of ROS (Kuo and Kao 2004; Olmos et al. 2003; Piqueras et al. 1999; Romero-Puertas et al. 2003, 2004; Sandalio et al. 2001; Schützendübel et al. 2001; Shah et al. 2001). Second, Cd can inhibit or stimulate the activities of antioxidant enzymes (Chaoui et al. 1997; Dixit et al. 2001; Gallego et al. 1996; Innelli et al. 2002; Kuo and Kao 2004; León et al. 2002; Shah et al. 2001; Shaw 1995). Third, treatment with Cd results in cellular oxidative damage or lipid peroxidation (Chaoui et al. 1997; Chien et al. 2002; Dixit et al. 2001; Gallego et al. 1996; Kuo and Kao 2004; Lozano-Rodríguez et al. 1997; Shah et al. 2001; Shaw 1995).

The polyamines putrescine (Put), spermidine (Spd), and spermine (Spm) are polycationic cellular molecules and are present in all living organisms. Experimental evidence now indicates that polyamines are involved in a number of cellular and molecular processes in plants (Bouchereau et al. 1999; Wallace et al. 2003). The levels of polyamines in plants are altered in response to heavy metals (Sharma and Dietz 2006). Weinstein et al. (1986) showed an up to 10-fold increase in Put content with a marginal rise in Spd and Spm contents in Cd-treated oat seedlings and detached oat leaves. Similar results were obtained in Cd-treated detached rice leaves (Hou and Kao 1993). It has been shown that polyamines are able to protect against oxidative damage caused by paraquat (Benavides et al. 2000; Chang and Kao 1997; Kurepa et al. 1998; Minton et al. 1990), acid rain (Velikova et al. 2000) and heavy metals such as Cd and Cu (Groppa et al. 2001). Borrell et al. (1997) demonstrated that polyamines inhibited lipid peroxidation in senescing oat leaves. Evidence has been provided to show that polyamines are effective radical scavengers in a number of chemical and in vitro enzyme systems (Drolet et al. 1986) and that the reduction in polyamine content in leaves of *Glycyrrhiza inflata* under osmotic stress promoted the increase in the production of ROS (Li and Wang 2004). A close interrelationship between polyamines and oxidative stress was documented by the finding that leaf necrosis caused by ozone in tomato plants could be suppressed by an exogenous supply of polyamines (Ormrod and Beckerson 1986). However, Bors et al. (1989) claimed that the scavenging of radicals by polyamines cannot explain the protection against ozone damage observed after exogenous application. Recently, Tang et al. (2004) demonstrated that exogenously added polyamines recover browning tissues into normal callus cultures of Virginia pine by decreasing oxidative damage. In the present study, we investigated the effect of polyamines on Cd toxicity of rice leaves, and we observed that oxidative damage caused by $CdCl_2$ is reduced by Spd and Spm.

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 2005). 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. The apical 3 cm of the third leaf was used in all experiments. Detached rice leaves were pretreated with distilled water or polyamines for 6 h at 27°C in darkness and then transferred to distilled water or 5 mM $CdCl_2$ for 4, 8, 12, and 18 h at 27°C in the light (40 μmol m^{-2} s^{-1}).

Determination of protein, H_2O_2, lipid peroxidation, GSH, oxidized glutathione (GSSG), ASC, dehydroascorbate (DHA), and Cd

For protein determination, leaf segments were homogenized in a 50 mM sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,600 × *g* for 20 min, and the supernatants were used for determination of protein by the method of Bradford (1976) and antioxidative enzyme activities. The H_2O_2 content was 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 15 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 leaf extracts resulted in the predicted increase of absorbance, i.e. added H_2O_2 was fully recovered (data not shown). The H_2O_2 content in leaf extracts was calculated using the extinction coefficient of 0.28 μmol^{-1} cm^{-1}. In some experiments, H_2O_2 was also 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 them 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. The H_2O_2 staining was repeated four times with similar results.

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). GSH and GSSG in 3% sulfosalicylic acid extract and ASC and DHA in 5% (w/v) trichloracetic acid extract were determined as described previously (Hsu and Kao 2005). For determination of Cd, leaves were dried at 65°C for 48 h and the dried material ashed at 550°C for 4 days. 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 was then quantified using an atomic absorption spectrophotometer (Model AA-6800, Shimadzu, Kyoto, Japan).

Polyamine determination

Leaf tissues were homogenized with 5 ml of 5% (w/v) perchloric acid. Polyamine contents were determined using high performance liquid chromatography (Waters 484, Milford, USA) after benzoylation as described previously (Chen and Kao 1991).

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 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. The whole extraction procedure was carried out at 4°C. 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. POX activity was measured using a modification of the procedure of MacAdam et al. (1992). The activity was calculated using the extinction coefficient (26.2 mM^{-1} cm^{-1} at 470 nm) for tetraguaiacol. One unit of POX was defined as the amount of enzyme that caused the formation of 1 μmol tetraguaiacol per min. 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 min. 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 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.

Statistical analysis

Statistical differences between measurements ($n = 4$) on different treatments or on different times were analyzed following the Duncan's multiple range test or Student's t-test.

Results

Cd promotes protein loss

In plants, the most general symptom of Cd toxicity is chlorosis (Das et al. 1997). In rice, we have shown that detached leaves and seedlings treated with $CdCl_2$ show chlorosis and protein loss (Chien and Kao 2000; Hsu and Kao 2003, 2005). In the present study, Cd toxicity in detached rice leaves caused by excess Cd was assessed by a decrease in protein content. Increasing concentration of $CdCl_2$ from 0.1 to 5 mM progressively decreased protein content in detached rice leaves in the light and no further decrease was observed at 10 mM $CdCl_2$ (data not shown). Thus, 5 mM $CdCl_2$ was used in the present investigation. The promotion of the loss of protein by $CdCl_2$ was evident 8 h after treatment (Fig. 1A). Cd concentration in the control leaves remained unchanged during 18 h of incubation (Fig. 1C). However, Cd concentration in $CdCl_2$-treated leaves increased with increasing duration of incubation (Fig. 1C). The increase in Cd concentration in $CdCl_2$-treated leaves was evident 4 h after treatment (Fig. 1C).

Cd induces oxidative stress

MDA content in $CdCl_2$-treated detached rice leaves was observed to be greater than that in water-treated controls at 8 h after treatment (Fig. 1B). This showed that Cd toxicity in detached rice leaves was linked to lipid peroxidation. Lipid peroxidation is caused by ROS (Thompson et al. 1987). $CdCl_2$ treatment also

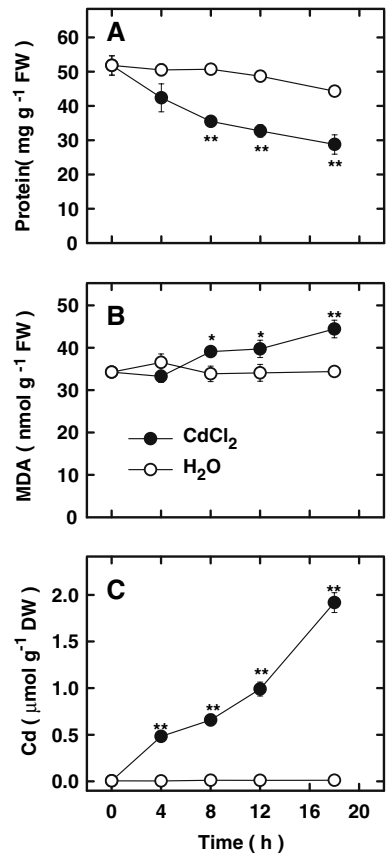

Fig. 1 Changes in the contents of protein (A), MDA (B), and Cd (C) in rice leaves treated with $CdCl_2$. Detached rice leaves were pretreated with H_2O for 6 h in the dark and then treated with H_2O or 5 mM $CdCl_2$ for 4, 8, 12, and 18 h in the light. * and ** represent values that are significantly different between H_2O and $CdCl_2$ treatment at $P < 0.05$ and $P < 0.01$, respectively

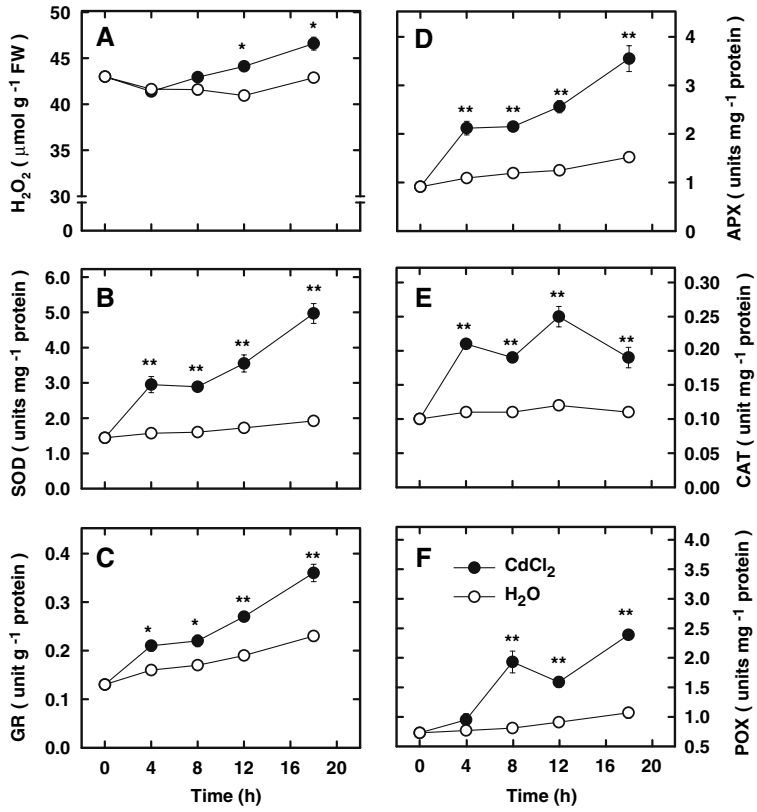

Fig. 2 Changes in the contents of H_2O_2 (A) and the activities of SOD (B), GR (C), APX (D), CAT (E), and POX (F) in rice leaves treated with $CdCl_2$. Detached rice leaves were pretreated with H_2O for 6 h in the dark and then treated with H_2O or 5 mM $CdCl_2$ for 4, 8, 12, and 18 h in the light. * and ** represent values that are significantly different between H_2O and $CdCl_2$ treatment at $P < 0.05$ and $P < 0.01$, respectively

caused an increase in H_2O_2 content (Fig. 2A). To verify in situ the increase in H_2O_2 in leaves treated with $CdCl_2$, a histochemical method with DAB that is based on the formation by H_2O_2 of brown polymerization product was used. The development of DAB-H_2O_2 reaction product in H_2O- and $CdCl_2$-treated leaves is shown in Fig. 3. It is clear that the DAB-H_2O_2 reaction product was observed after H_2O_2 and $CdCl_2$ treatments. All these results support the involvement of ROS as the chemical species inducing Cd toxicity in rice leaves.

$CdCl_2$-treated rice leaves had higher activities of SOD, GR, APX, and CAT than the controls at 4 h after treatment (Figs. 2B–E). Higher activities of POX were observed at 8 h after treatment (Fig. 2F). GSH, GSSG, and ASC contents were observed to be lower than the controls at 4 h after treatment (Figs. 4A–C). However, DHA content in Cd-treated leaves was observed to be higher than the contents at 18 h after treatment (Fig. 4D). The increased activities of antioxidative enzymes

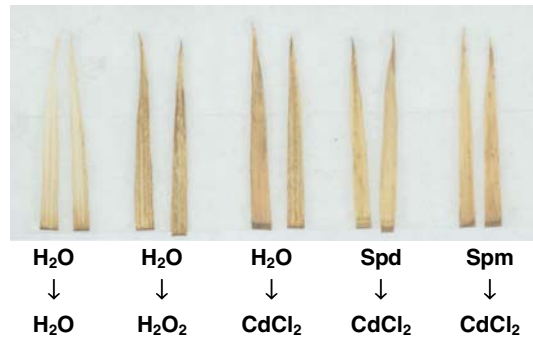

Fig. 3 Histochemical detection of H_2O_2 with DAB staining in rice leaves. Detached leaves were pretreated with H_2O, Spd, and Spm, respectively, for 6 h in the dark, and then treated with either H_2O, H_2O_2, or $CdCl_2$ for 18 h in the light. The concentrations of Spd, Spm, H_2O_2, and $CdCl_2$ were 5, 5, 1, and 5 mM, respectively

and the decreased contents of ASC and GSH in response to $CdCl_2$ are further suggestive of strong induction of oxidative stress.

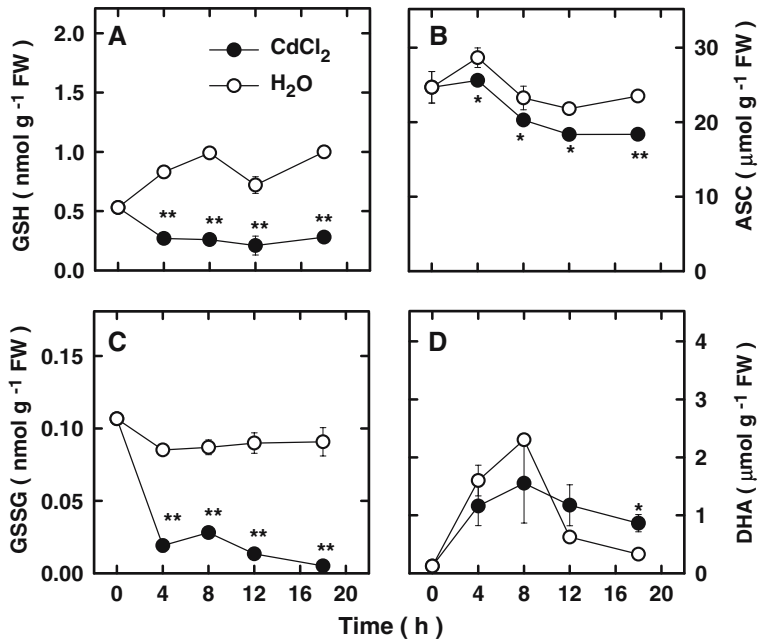

Fig. 4 Changes in contents of GSH (A) and ASC (B) in rice leaves treated with $CdCl_2$. Detached rice leaves were pretreated with H_2O for 6 h in the dark and then treated with H_2O or 5 mM $CdCl_2$ for 4, 8, 12, and 18 h in the light. * and ** represent values that are significantly different between H_2O and $CdCl_2$ treatment at $P < 0.05$ and $P < 0.01$, respectively

Spd and Spm reduce Cd-induced oxidative damage

To test if polyamines could reduce the toxicity caused by $CdCl_2$, as judged by the changes in protein levels, detached rice leaves were pretreated with either water or polyamines for 6 h in the dark and then transferred to either water or $CdCl_2$ for 18 h in the light. Spd and Spm, but not Put, pretreatments reduced Cd toxicity (Fig. 5). We also observed that Spd and Spm were effective in reducing Cd-induced lipid peroxidation (Fig. 6A) and H_2O_2 production (Figs. 3, 6B), Cd-increased antioxidative enzyme activities (Fig. 7), and Cd-decreased ASC and GSH contents (Fig. 8). Furthermore, detached rice leaves pretreated with Spd or Spm for 6 h in the dark had higher endogenous levels of Spd and Spm, and Spm, respectively, than those pretreated with water (Table 1). However, Put pretreatment had no effect on endogenous levels of Spd and Spm (Table 1).

Spd and Spm inhibit the uptake of Cd

To test if endogenous Spd and Spm affect Cd uptake, Cd content in detached rice leaves pretreated with Spd or Spm followed by treatment of $CdCl_2$ was determined. It was observed that Spd and Spm pretreatments resulted in a decrease (about 27%) in Cd content when compared with H_2O pretreatment (Fig. 9).

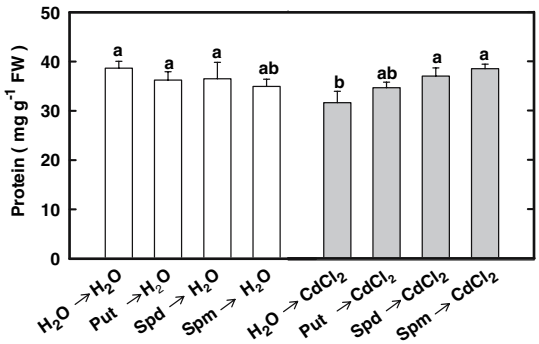

Fig. 5 Effect of pretreatments with polyamines on the content of protein in detached rice leaves in the presence or absence of $CdCl_2$. Detached rice leaves were pretreated with H_2O, 5 mM Put, 5 mM Spd, and 5 mM Spm, respectively, for 6 h in the dark and then treated with H_2O or 5 mM $CdCl_2$ for 18 h in the light. Values with the same letter are not significantly different at $P < 0.05$

Discussion

It has been shown that Cd increased ethylene production in detached rice leaves (Hou and Kao 1993). Here, we show that Cd induced H_2O_2

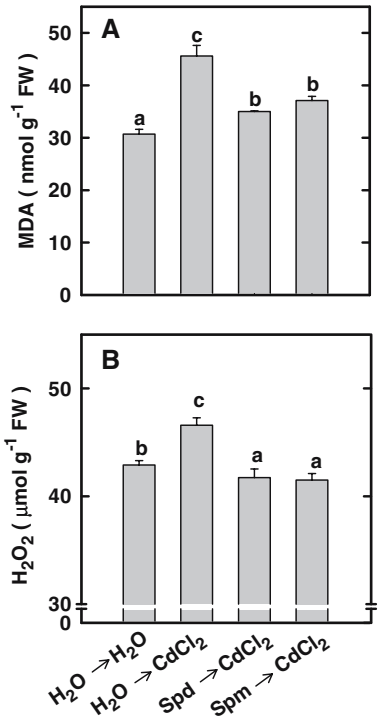

Fig. 6 Effect of pretreatments with Spd and Spm on the contents of MDA (A) and H_2O_2 (B) in detached rice leaves in the presence or absence of $CdCl_2$. Detached rice leaves were pretreated with H_2O, 5 mM Spd, and 5 mM Spm, respectively, for 6 h in the dark and then treated with H_2O or 5 mM $CdCl_2$ for 18 h in the light. Values with the same letter are not significantly different at $P < 0.05$

production in rice leaves (Fig. 2A, 3). Wounding is known to induce ethylene production (Yu and Yang 1980) and H_2O_2 generation (Orozco-Cárdenas et al. 2001). Ethylene biosynthesis shares a common precursor with Spd and Spm, thus wounding may induce a direct modification of the synthesis of Spd and Spm. When detached rice leaves are used to study H_2O_2, ethylene, polyamines, and senescence, 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, H_2O_2 and ethylene production of detached leaves induced by Cd is unlikely to be complicated by the wounding effect.

Cd is known to increase the production of H_2O_2 (Kuo and Kao 2004; Schützendübel et al. 2001; Olmos et al. 2003) and induce lipid peroxidation (Chien et al. 2002; Gallego et al. 1996; Kuo and Kao 2004). These results suggest that Cd treatment causes an oxidative stress in plants. Our results not only have shown that $CdCl_2$ increased the content of H_2O_2 (Figs. 2A, 3) and the activities of SOD, APX, GR, CAT, and POX (Figs. 2B–F), but also demonstrated that caused a decrease in GSH and ASC contents (Fig. 4). Meanwhile, protein loss (Fig. 1A) and lipid peroxidation (Fig. 1B) were observed in $CdCl_2$-treated rice leaves. All these results suggest that $CdCl_2$ causes an oxidative stress and that $CdCl_2$-induced toxicity in rice leaves is mediated through oxidative stress.

GSH functions as a direct antioxidant of ROS and is involved in the generation of ASC, which is utilized as a substrate for APX (Noctor and Foyer 1998). In the present study, we observed that the decrease in GSH content is one of the earliest steps in oxidative stress induced by $CdCl_2$ in rice leaves, which occurred at 4 h after treatment (Fig. 4B). It may be suspected that the decrease in GSH may favor the accumulation of ROS in Cd-treated rice leaves. In a review, Schützendübel and Polle (2002) also suggest that the depletion of GSH is apparently a critical step in Cd toxicity.

Cd induced a significant accumulation of H_2O_2 in rice leaves (Figs. 2A, 3). Accumulation of H_2O_2 has also been observed in Cd-treated pine and pea roots, pea leaves, and tobacco cells (Olmos et al. 2003; Romero-Puertas et al. 2003; 2004; Schützendübel et al. 2001). There are reports showing that NADPH oxidase was possibly involved in Cd-induced H_2O_2 production in pea leaves and tobacco cells (Olmos et al. 2003; Romero-Puertas et al. 2004). Our unpublished observations indicate that diphenyleneiodonium chloride and imidazole, inhibitors of NADPH oxidase, prevented Cd-induced H_2O_2 production in rice leaves.

Data from the present study indicate that Cd-induced oxidative damage in rice leaves is reduced by Spd and Spm. This conclusion is based on the observations that pretreatment with Spd and Spm prevented Cd-induced loss of protein (Fig. 5), increase in the contents of MDA (Fig. 6) and H_2O_2 (Figs. 3, 6B), decrease in the content of ASC and GSH (Fig. 8), and increase in the

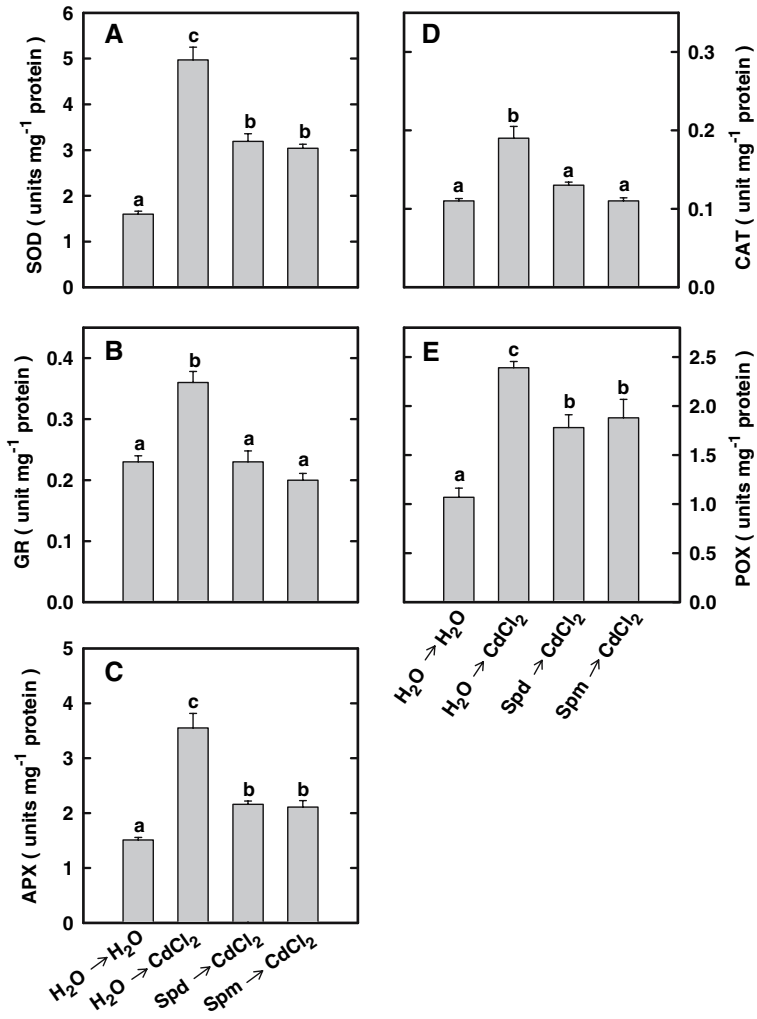

Fig. 7 Effect of pretreatments with Spd and Spm on the activities of antioxidative enzymes [SOD (A), GR (B), APX (C), CAT (D), and POX (E) in detached rice leaves in the presence or absence of $CdCl_2$. Detached rice leaves were pretreated with H_2O, 5 mM Spd, and 5 mM Spm, respectively, for 6 h in the dark and then treated with H_2O or 5 mM $CdCl_2$ for 18 h in the light. Values with the same letter are not significantly different at $P < 0.05$

activities of antioxidative enzymes (Fig. 7). Groppa et al. (2001) also demonstrated that Spm and Spd were effective in reducing Cd-caused lipid peroxidation in sunflower leaf discs. It is generally accepted that polyamines are highly protonated at physiological pH, which favors electrostatic binding of polyamines to negatively charged components of membranes, leading to membrane stabilization through ionic interactions (Slocum et al. 1984). The more pronounced protective effect of Spd and Spm could be accounted for by its longer chain and greater number of positive charges, which allows membrane stabilizing ability.

The decrease in ASC and GSH contents in rice leaves treated with $CdCl_2$ suggests that ASC and GSH contents may be regulated by the synthesis and oxidation. GSH is the precursor of phytochelatins, cysteine-rich peptides, synthesized via phytochelatin synthase (Cobbett and Goldsbrough 2002). A severe depletion of GSH is a common response to Cd caused by an increased consumption of GSH for phytochelatin production (Schützendübel and Polle 2002). Thus, the sharp decline in GSH content in $CdCl_2$-treated rice leaves may also be due to phytochelatin biosynthesis.

The present results indicated that Spd and Spm reduced Cd-decreased ASC and GSH contents (Fig. 8). Theses observations suggest that the capacity of Spd and Spm to scavenge H_2O_2 might

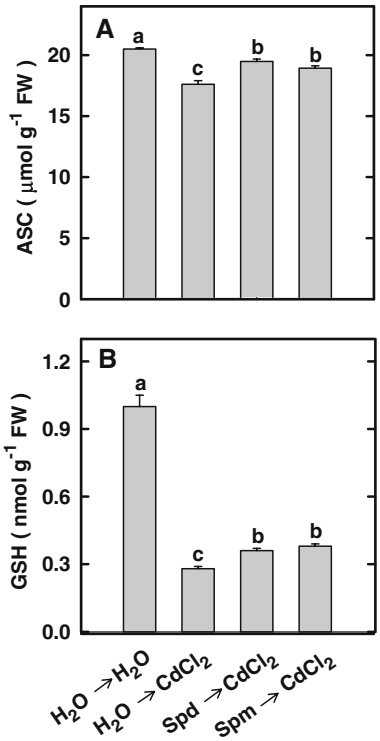

Fig. 8 Effect of pretreatments with Spd and Spm on the contents of ASC (A) and GSH (B) in detached rice leaves in the presence or absence of $CdCl_2$. Detached rice leaves were pretreated with H_2O, 5 mM Spd, and 5 mM Spm, respectively, for 6 h in the dark and then treated with H_2O or 5 mM $CdCl_2$ for 18 h in the light. Values with the same letter are not significantly different at $P < 0.05$

Table 1 Effect of polyamines on the contents of endogenous polyamines in detached rice leaves

Treatment	Put (nmol g^{-1} FW)	Spd (nmol g^{-1} FW)	Spm (nmol g^{-1} FW)
H_2O	182.9 ± 9.6^b	121.83 ± 6.6^b	89.9 ± 8.9^c
Put	222.8 ± 3.0^a	142.8 ± 7.0^b	74.1 ± 2.2^c
Spd	149.9 ± 5.8^c	168.91 ± 5.5^a	184.1 ± 15.1^b
Spm	145.9 ± 13.7^c	136.38 ± 11.5^b	341.1 ± 33.9^a

Detached rice leaves were treated with either water, Put, Spd, or Spm (5 mM) for 6 h in dark. Values with the same letter in each column are not significantly different at $P < 0.05$

increase in rice leaves that were pretreated with Spd or Spm followed by treatment of $CdCl_2$ (Fig. 6B).

In considering a possible mechanism for the reduction of Cd-induced oxidative damage by

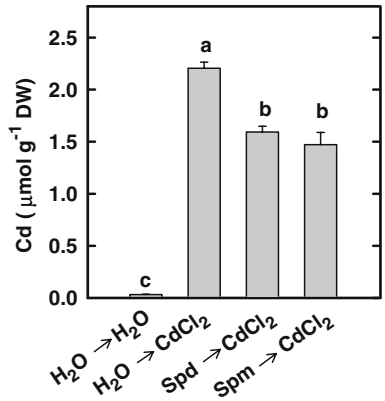

Fig. 9 Effect of pretreatments with Spd and Spm on the content of Cd in detached rice leaves in the presence or absence of $CdCl_2$. Detached rice leaves were pretreated with H_2O, 5 mM Spd, and 5 mM Spm, respectively, for 6 h in the dark and then treated with H_2O or 5 mM $CdCl_2$ for 18 h in the light. Values with the same letter are not significantly different at $P < 0.05$

polyamines, we speculated that Spd and Spm might inhibit Cd uptake from the medium. Here, we show that Cd content in detached rice leaves pretreated with Spd and Spm followed by treatment of $CdCl_2$ was lower that those pretreated with H_2O. Our findings suggest that increase in endogenous Spd or Spm may block, though slightly (27%), the uptake of Cd (Fig. 9). In the present study, pretreatment of detached rice leaves with exogenous Spd or Spm was found to reverse almost completely the Cd-induced H_2O_2 generation and lipid peroxidation (Fig. 6). It appears that Spd and Spm were able to protect Cd-induced oxidative damage and this protection was related to the avoidance of H_2O_2 generation and the reduction of Cd uptake.

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