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Cadmium toxicity of rice leaves is mediated through lipid peroxidation

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Abstract

Oxidative stress, in relation to toxicity of detached rice leaves, caused by excess cadmium was investigated. Cd content in $CdCl_2$-treated detached rice leaves increased with increasing duration of incubation in the light. Cd toxicity was followed by measuring the decrease in chlorophyll and protein. $CdCl_2$ was effective in inducing toxicity and increasing lipid peroxidation of detached rice leaves under both light and dark conditions. These effects were also observed in rice leaves treated with $CdSO_4$, indicating that the toxicity was indeed attributed to cadmium ions. Superoxide dismutase (SOD), ascorbate peroxidase (APOD), and glutathione reductase (GR) activities were reduced by excess $CdCl_2$ in the light. The changes in catalase and peroxidase activities were observed in $CdCl_2$-treated rice leaves after the occurrence of toxicity in the light. Free radical scavengers reduced $CdCl_2$-induced toxicity and at the same time reduced $CdCl_2$-induced lipid peroxidation and restored $CdCl_2$-decreased activities of SOD, APOD, and GR in the light. Metal chelators (2,2′-bipyridine and 1,10-phenanthroline) reduced $CdCl_2$ toxicity in rice leaves in the light. The reduction of $CdCl_2$ toxicity by 2,2′-bipyridine (BP) is closely associated with a decrease in lipid peroxidation and an increase in activities of antioxidative enzymes. Furthermore, BP-reduced toxicity of detached rice leaves, induced by $CdCl_2$, was reversed by adding Fe^{2+} or Cu^{2+}, but not by Mn^{2+} or Mg^{2+}. Reduction of $CdCl_2$ toxicity by BP is most likely mediated through chelation of iron. It seems that toxicity induced by $CdCl_2$ may require the participation of iron.

Abbreviations: APOD – ascorbate peroxidase, BP – 2,2′-bipyridine, CAT – catalase, GR – glutathione reductase, GSH – reduced glutathione, MDA – malondialdehyde, PA – 1,10-phenanthroline, SB – sodium benzoate, SOD – superoxide dismutase, TU – thiourea

Introduction

Cadmium is one of the most toxic heavy metals with no described biological function. It is supplied to soil, air and water mainly by effluent from industries, mining, burning and leakage of waste, and by fertilization with phosphate and sewage sludge. 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; Sidlecka and Baszynsky 1993), lowers chlorophyll content (Larsson et al. 1998; Stobart et al. 1985), and inhibits the stomatal opening (Barcelo and Poschenrieder 1990).

One possible mechanism by which excess heavy metals may damage plant tissues is the stimulation of free radical production, by imposing oxidative stress (Foyer et al. 1997). Plant cells are equipped with several free radical detoxifying enzymes to protect them against oxidative damage. These enzymes include superoxide dismutase (SOD), ascorbate peroxidase (APOD), glutathione reductase (GR), catalase (CAT) and peroxidase (POD) (Foyer et al. 1997; Nakano and Asada 1981; Polle and Rennenberg 1994; Smith et al. 1989).

Although Cd is a non redox active metal, it was found to produce oxidative stress in plant tissues (Chaoui et al. 1997; Gallego et al. 1996; Hendry et

al. 1992; Somashekaraiah et al. 1992; Stroinski and Kozlowska 1997; Stroinski and Zielezinska 1997). Cd can inhibit (and sometimes stimulate) the activities of several antioxidative enzymes. In *Phaseolus aureus* Cd produced lipid peroxidation, decrease in CAT activity and increase in POD and APOD activities (Shaw 1995). In *Helianthus annuus* leaves, Cd enhanced lipid peroxidation, increased lipoxygenase activity and decreased the activities of SOD, CAT, APOD, GR and dehydroascorbate reductase (Gallego et al. 1996). In *Phaseolus vulgaris* roots and leaves, 5 μM Cd enhanced activities of POD and APOD, and increased lipid peroxidation (Chaoui et al. 1997). Cd treatment significantly increased lipid peroxidation in pea plants (Lozano-Rodriguez et al. 1997), in *Helianthus annuus* (Gallego et al. 1996), and in *Festuca rubra* (Wong et al. 1997), whereas no peroxidation was found in Cd-exposed plants and hairy roots of *Daucus carota* (Sanita di Toppi et al. 1998, 1999). In this study, effects of Cd on the toxicity, lipid peroxidation, H_2O_2, and some free radical detoxifying enzymes in detached rice leaves were investigated.

Superoxide radicals can serve as a source to produce more active hydroxyl radicals by Haber-Weiss and Fenton reactions (Naqui and Chance 1986). Transition metals, such as iron and copper, are able to accelerate Haber-Weiss and Fenton reactions (Gutteridge et al. 1981). Paraquat, also known as methyl viologen, is widely used herbicide in agriculture and has long been known to exert its phytotoxic effects by catalyzing the transfer of electrons from photosystem I of chloroplast membranes to molecular oxygen, producing oxygen radicals that cause lipid peroxidation and membrane damage (Calderbank 1968). It has been demonstrated that iron ions are essential mediator in paraquat toxicity in pea leaves (Zer et al. 1994) and in rice leaves (Chang and Kao 1997). It has been shown that methyl jasmonate-promoted senescence of detached rice leaves is mediated through free radical-induced lipid peroxidation (Hung and Kao 1998). Recently, we also demonstrated that iron played a major role in methyl jasmonate-promoted senescence (Fang and Kao 2000) It is not known whether iron plays a mediatory role in Cd-induced toxicity. Thus, the role of transition metals in Cd-induced toxicity in rice leaves was also examined.

Materials and methods

Rice (*Oryza sativa* cv. Taichung Native 1) was cultured as previously described (Larsson et al. 1998). The apical 3-cm segments excised from the third leaves of 12-day-old seedling were used. A group of 10 segments was floated in a Petri dish containing 10 ml of test solution. Incubation was done at 27 °C in the light (40 μmol $m^{-2} s^{-1}$) or in darkness.

For determination of Cd, detached rice 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 0.1 N HCl. Cd was then quantified using an atomic absorption spectrophotometer (Model AA-6800, Shimadzu, Kyoto). Chlorophyll was determined according to Wintermans and De Mots (1965) after extraction in 96% (v/v) ethanol. For protein extraction, leaf segments were homogenized in 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 (Bradford 1976) and for enzyme assays. Malondialdehyde (MDA) was extracted with 5% (w/v) trichloroacetic acid and determined according to Heath and Packer (1968). The H_2O_2 content was colorimetrically measured, as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenising 50 mg leaf tissue with 3 ml of phosphate buffer (50 mM, pH 6.5). The homogenate was centrifuged at 6,000 *g* for 25 min. To determine H_2O_2 contents, 3 ml of extracted solution was mixed with 1 ml of 0.1% titanium chloride in 20% (v/v) H_2SO_4. The mixture was then centrifuged at 6,000 *g* for 15 min. The intensity of the yellow colour of the supernatant was measured at 410 nm. H_2O_2 level was calculated using the extinction coefficient 0.28 $\mu mol^{-1} cm^{-1}$.

POD activity was measured using modification of the procedure of MacAdam et al. (1992). Activity was calculated using the extinction coefficient (26.6 $mM^{-1} cm^{-1}$ at 470 nm) for tetraguaiacol. 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 optical density at 240 nm, and activity was calculated using the extinction coefficient (40 $mM^{-1} cm^{-1}$ at 240 nm) for H_2O_2 (Somashekaraiah et al. 1992). SOD was determined according to Paoletti et al. (1986). APOD was determined according to Nakano and Asada (1981). The decrease in ascorbate concentration was followed as the decline in optical density 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 of Foster and Hess (1980). One unit of activity for CAT, POD, SOD, APOD, and GR were defined as the amount of enzyme which broke down 1 μmol of H_2O_2 per min, caused the formation of 1 μmol tetraguaiacol per min, inhibited 50% the rate of NADH oxidation observed in control, broke down 1 μmol of ascorbate per min, and decreased 1 A_{340} per min, respectively.

Cd content was expressed per g dry weight. Chlorophyll, protein, H_2O_2 and MDA levels and enzyme activities were expressed per g fresh weight. Absolute levels of each measurement varied among experiments because of seasonal effects. However, the patterns of responses to Cd were reproducible. For all measurements, each treatment was repeated four times. All experiments described here were repeated at least three times. Similar results and identical trends were obtained each time. The data reported here are from a single experiment.

Results

In the present paper, Cd toxicity in detached rice leaves caused by excess Cd was assessed by a decrease in chlorophyll and protein contents. Previously, we observed that increasing concentration of $CdCl_2$ from 0.1 to 5 mM progressively decreased chlorophyll and protein contents in detached rice leaves in the light and no further decrease was observed at 10 mM $CdCl_2$ (Chien and Kao 2000). Thus, 5 mM $CdCl_2$ was used in the present investigation. Figure 1 shows the time courses of chlorophyll and protein contents in detached rice leaves floating on water or $CdCl_2$ (5 mM) in the light. It is clear that the promotion of the loss of chlorophyll and protein (or toxicity) by $CdCl_2$ was evident 12 h after treatment. To be sure that the described toxicity was related to an increase in the leaf Cd content, Cd concentration was determined in detached rice leaves with either water or 5 mM $CdCl_2$ (Figure 1). Cd content in control leaves remained unchanged during 48 h of incubation in the light. However Cd content in $CdCl_2$-treated detached rice leaves increased with increasing duration of incubation. It was obvious that the increase in Cd content in $CdCl_2$-treated detached rice leaves was evident 12 h after treatment.

MDA content in $CdCl_2$-treated detached rice leaves was observed to be greater than that in water-

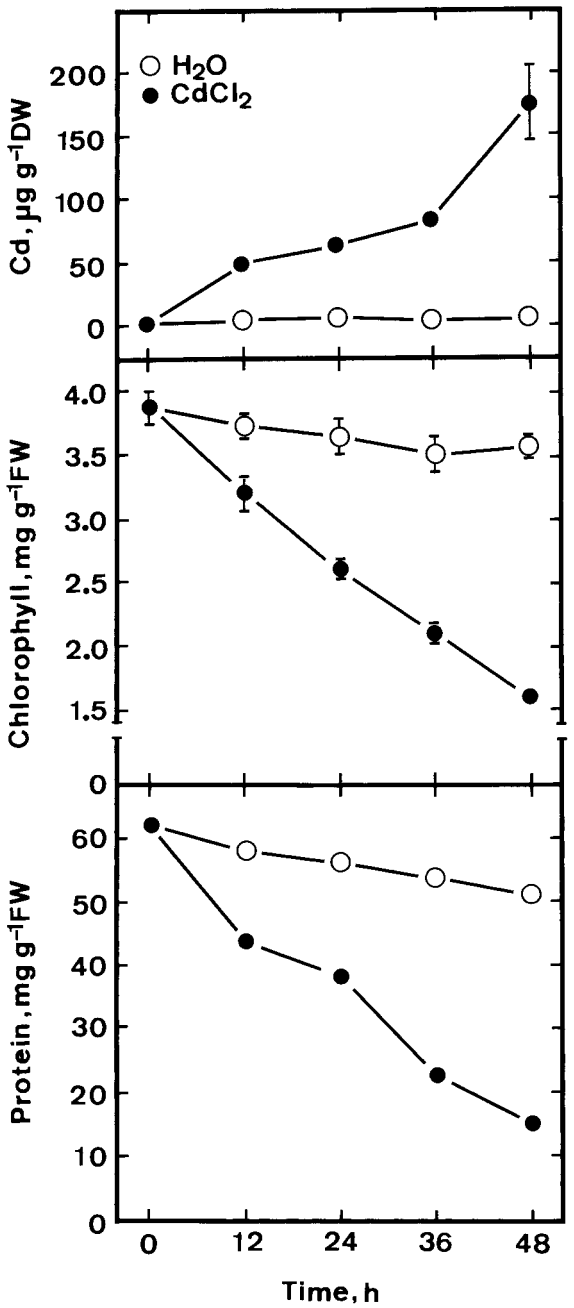

Figure 1. Changes in contents of Cd, chlorophyll, and protein in detached rice leaves treated with $CdCl_2$. Detached rice leaves were treated with either water or 5 mM $CdCl_2$ in the light. Vertical bars represent S.E. (n = 4).

treated controls, throughout the entire duration of incubation (Figure 2). This showed that $CdCl_2$ induced toxicity in detached rice leaves was linked to lipid peroxidation. Figure 2 also showed that H_2O_2 content increased significantly in detached rice leaves incu-

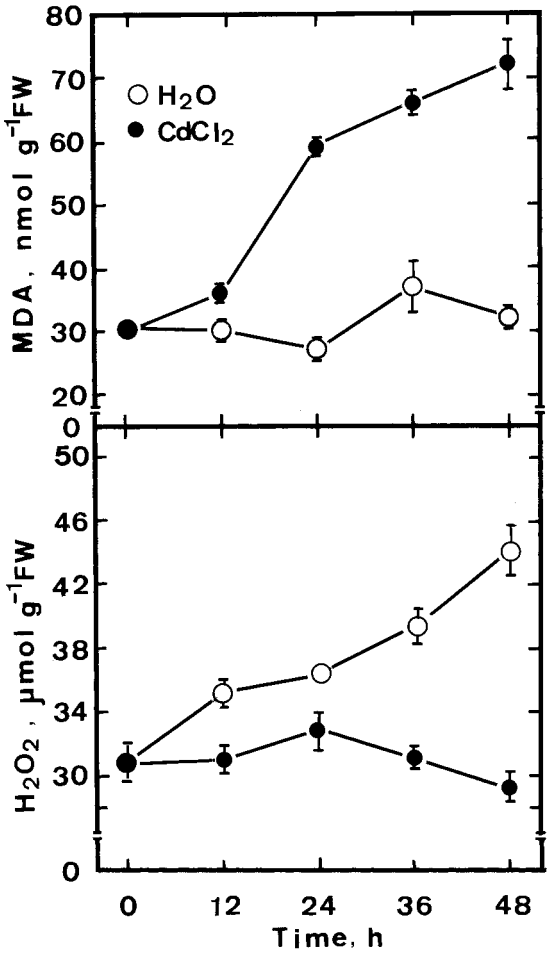

Figure 2. Changes in contents of malondialdehyde (MDA) and H_2O_2 in detached rice leaves treated with $CdCl_2$. Detached rice leaves were treated with either water or 5 mM $CdCl_2$ in the light. Vertical bars represent S.E. (n = 4).

bated in water. However, H_2O_2 content remained unchanged in $CdCl_2$-treated rice leaves throughout the entire duration of incubation.

When the effects of $CdSO_4$ on toxicity and lipid peroxidation of detached rice leaves were compared with those of $CdCl_2$, it was found that $CdSO_4$ and $CdCl_2$ were equally effective in inducing toxicity and lipid peroxidation under light or dark conditions (Figure 3), indicating that toxicity and lipid peroxidation are induced by Cd ions rather than by SO_4^{2-} or Cl^-.

Lipid peroxidation is a free radical mediated process (Foyer et al. 1997; Nakano and Asada 1981; Polle and Rennenberg 1994; Smith et al. 1989). The striking increase in lipid peroxidation in $CdCl_2$-treated detached rice leaves may be a reflection of the decline of antioxidative enzymes. As shown in Fig-

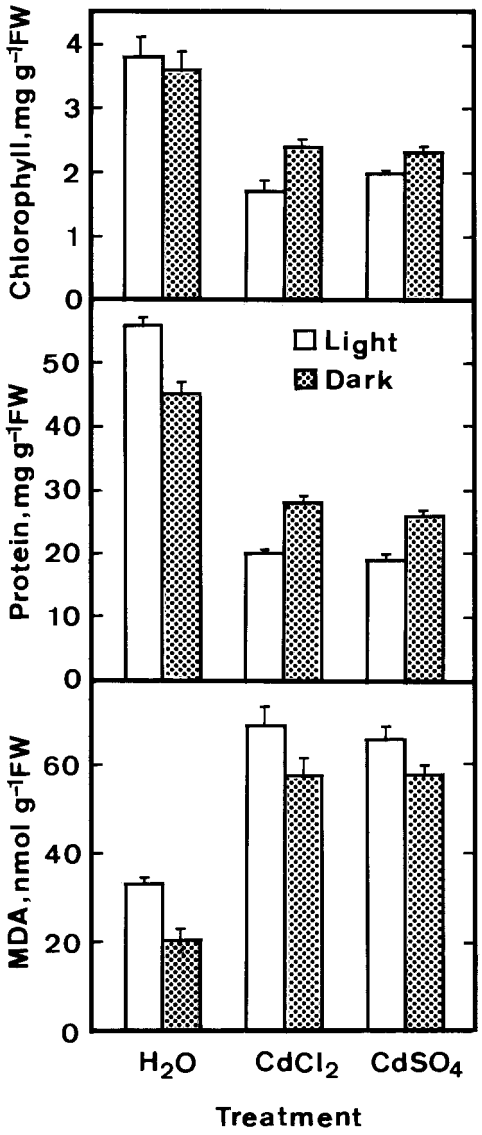

Figure 3. Effects of light and dark on contents of chlorophyll, protein, and malondialdehyde (MDA) in detached rice leaves were treated with $CdCl_2$ or $CdSO_4$. Detached rice leaves were treated with water, 5 m*M* $CdCl_2$, or 5 mM $CdSO_4$. Chlorophyll, protein, and MDA were determined after 48 h in the light or in the dark. Vertical bars represent S.E. (n = 4).

ure 4, $CdCl_2$-treated detached rice leaves had reduced activities of SOD, APOD, and GR than the controls. CAT and POD activities decreased and increased, respectively, significantly in detached rice leaves treated with $CdCl_2$ and water (Figure 5). However, the effects were more pronounced in $CdCl_2$-treated rice leaves than in water-treated controls after the occurrence of toxicity (24 h after treatment).

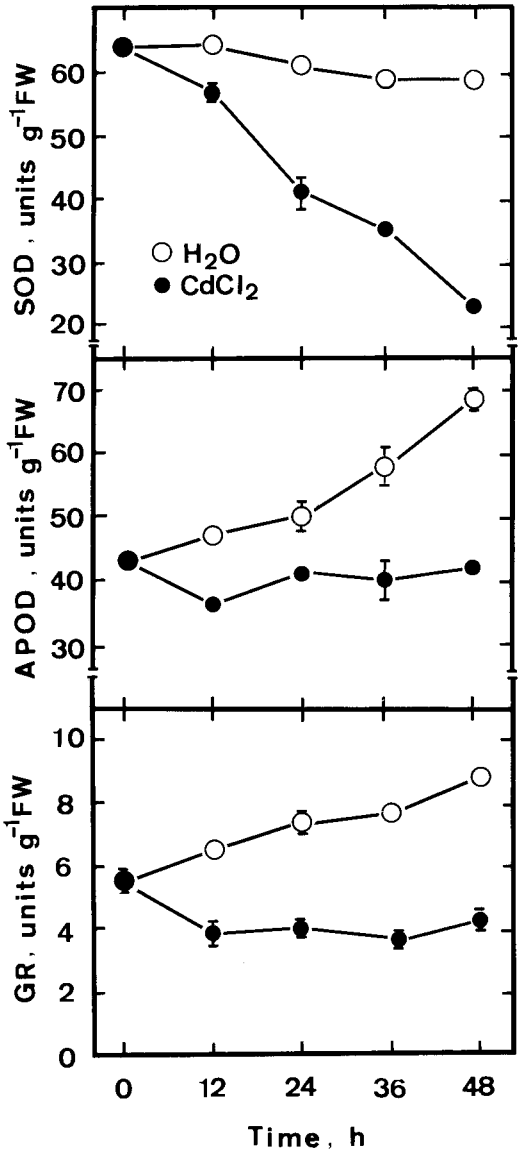

Figure 4. Changes in activities of superoxide dismutase (SOD), ascorbate peroxidase (APOD), and glutathione reductase (GR) in detached rice leaves treated with $CdCl_2$. Detached rice leaves were treated with either water or 5 mM $CdCl_2$ in the light. Vertical bars represent S.E. (n = 4).

When free radical scavengers such as thiourea (TU), sodium benzoate (SB), and reduced glutathione (GSH) were treated together with $CdCl_2$, it was found that they partially prevented the decrease in chlorophyll and protein contents as well as the increase in lipid peroxidation (Table 1). Similarly, TU, SB, and GSH also restored SOD, APOD, and GR activities (Table 2).

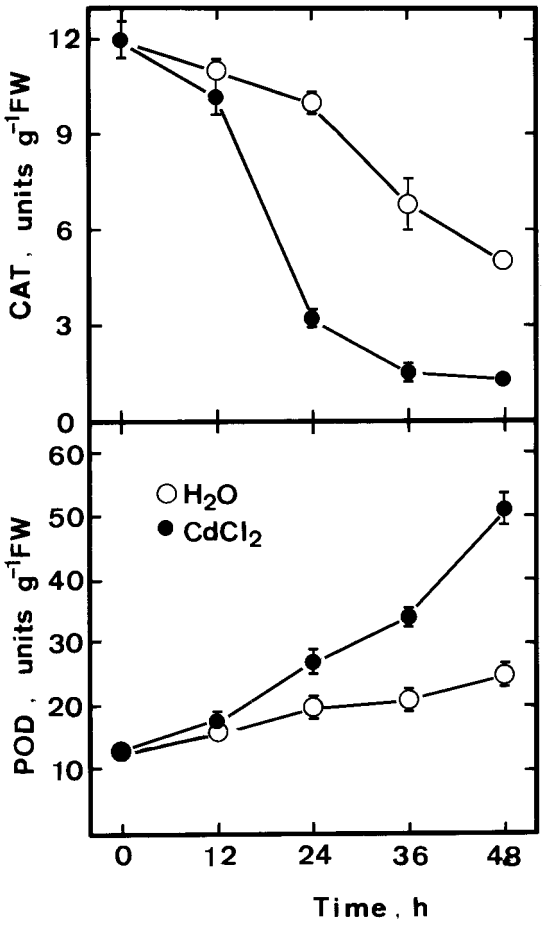

Figure 5. Changes in activities of catalase (CAT) and peroxidase (POD) in detached rice leaves treated with $CdCl_2$. Detached rice leaves were treated with either water or 5 mM $CdCl_2$ in the light. Vertical bars represent S.E. (n = 4).

Superoxide can serve as a source to generate more active hydroxyl radicals by Haber-Weiss and Fenton reactions (Naqui and Chance 1986). Transition metals, such as iron and copper, are able to accelerate Haber-Weiss and Fenton reactions (Gutteridge et al. 1981). Thus, it is of great interest to know whether transition metals are required in $CdCl_2$-induced toxicity in detached rice leaves. We investigated this by using the metal chelators BP and PA. Pretreatment of BP or PA caused a reduction in $CdCl_2$-induced increase in toxicity and MDA content (Figure 6). Table 3 shows the protection of SOD, APOD, and GR by BP against the $CdCl_2$-induced loss of their activities. All these results indicate that BP-reduced $CdCl_2$ toxicity is closely associated with the decrease in lipid peroxidation and increase in protective enzyme activities in rice leaves.

Table 1. Effects of oxygen radical scavengers on chlorophyll, protein, and malondialdehyde (MDA) contents in detached rice leaves treated with $CdCl_2$. The concentrations of $CdCl_2$, thiourea (TU), sodium benzoate (SB), and reduced glutathione (GSH) were 5 mM. Chlorophyll, protein, and MDA contents were determined after 24 h in the light. The data represent mean values ± S.E. (n = 4).

Treatment	Chlorophyll, mg g^{-1} FW	Protein, mg g^{-1} FW	MDA, nmol g^{-1} FW
H_2O	4.2 ± 0.1	43.5 ± 0.9	28.3 ± 0.3
$CdCl_2$	2.9 ± 0.1	22.1 ± 1.2	58.7 ± 1.7
$CdCl_2$ + TU	3.4 ± 0.1	28.1 ± 0.9	44.0 ± 3.5
$CdCl_2$ + SB	3.8 ± 0.1	32.2 ± 0.9	40.8 ± 1.7
$CdCl_2$ + GSH	3.9 ± 0.1	33.4 ± 2.0	42.4 ± 1.9

Table 2. Effects of oxygen radical scavengers on superoxide dismutase (SOD), ascorbate peroxidase (APOD), and glutathione reductase (GR) activities in detached rice leaves treated with $CdCl_2$. The concentrations of $CdCl_2$, thiourea (TU), sodium benzoate (SB), and reduced glutathione (GSH) were 5 mM. Enzyme activities were determined after 24 h in the light. The data represent mean values ± S.E. (n = 4).

Treatment	Enzyme activity, units g^{-1} FW		
	SOD	APOD	GR
H_2O	64.8 ± 4.2	48.5 ± 1.1	10.3 ± 0.5
$CdCl_2$	44.6 ± 0.6	42.9 ± 1.8	5.9 ± 0.2
$CdCl_2$ + TU	52.5 ± 3.9	56.8 ± 1.2	8.6 ± 0.1
$CdCl_2$ + SB	53.9 ± 4.4	48.2 ± 1.9	7.0 ± 0.1
$CdCl_2$ + GSH	50.2 ± 3.4	56.9 ± 2.0	8.4 ± 0.5

The reversal of the protective effect of BP against $CdCl_2$ toxicity was studied using divalent metal ions such as Fe^{2+}, Cu^{2+}, Mn^{2+}, and Mg^{2+} (Figure 7). The effect of BP could be reversed by Fe^{2+} and Cu^{2+}. However, Mn^{2+} and Mg^{2+} had no effect. These results indicated that BP can protect leaf cells against $CdCl_2$ toxicity by chelation of Fe^{2+} or Cu^{2+}.

Discussion

It has been demonstrated that excess Cd treatment notably increases lipid peroxidation in plant tissues (Chaoui et al. 1997; Gallego et al. 1996, 1996; Hendry et al. 1992; Lozano-Rodriguez et al. 1997; Shaw 1995; Somashekaraiah et al. 1992; Wong et al. 1997). However, no increase in lipid peroxidation by Cd has also been reported (Sanita di Toppi et al. 1998, 1999). In the present investigation, the addition of excess Cd to the incubation medium, under light or dark condition, induced toxicity (Figure 1) and increased the lipid peroxidation (Figure 2) in detached rice leaves. Figure 3 shows that $CdCl_2$ or $CdSO_4$ induces more lipid peroxidation and, at the same time, more pronounced toxicity in the light than in the dark. These results support the possibility that $CdCl_2$-induced toxicity is mediated through oxidative stress. This conclusion is supported further by the observations that free radical scavengers were able to reduce the toxicity caused by $CdCl_2$ and at the same time reduce $CdCl_2$-induced MDA content (Table 1). The effects of $CdCl_2$ on the loss of chlorophyll and protein could have resulted from the effect of free radicals produced by the treatment with Cd ions.

SOD, APOD, and GR are important antioxidative enzymes in plant leaves (Nakano and Asada 1981; Polle and Rennenberg 1994; Smith et al. 1989). It has been reported that excess Cd increased the activity of SOD (Chaoui et al. 1997; Okamoto et al. 1996; Schickler and Caspi 1999), decreased the activity of SOD (Kato and Shimizu 1987), and decreased the activities of APOD and GR (Gallego et al. 1996; Schickler and Caspi 1999). However, the results that we obtained with detached rice leaves in the light showed that SOD, APOD, and GR activities in $CdCl_2$-treated rice leaves were less than those in water-treated controls (Figure 4). The decreases in these antioxidative enzyme activities caused by $CdCl_2$ were partially restored by addition of the free radical scavengers such as TU, SB, and GSH (Table 2), indicating that $CdCl_2$ treatment resulted in oxidative damage in detached rice leaves. The changes in CAT and POD activities were observed in detached rice leaves after the occurrence of toxicity caused by $CdCl_2$ (Figure 5). Thus, CAT and POD may play minor, if any, role in regulating $CdCl_2$-induced toxicity in detached rice leaves.

The decrease in SOD activity in leaves treated with $CdCl_2$ would result in an accumulation in the superoxide radicals. The susperoxide radicals can react with H_2O_2 through Fe- or Cu-catalyzed Haber-Weiss and Fenton reactions to produce hydroxyl radicals (Gutteridge et al. 1981; Naqui and Chance 1986). Short-term treatment of potato tuber discs with $CdCl_2$

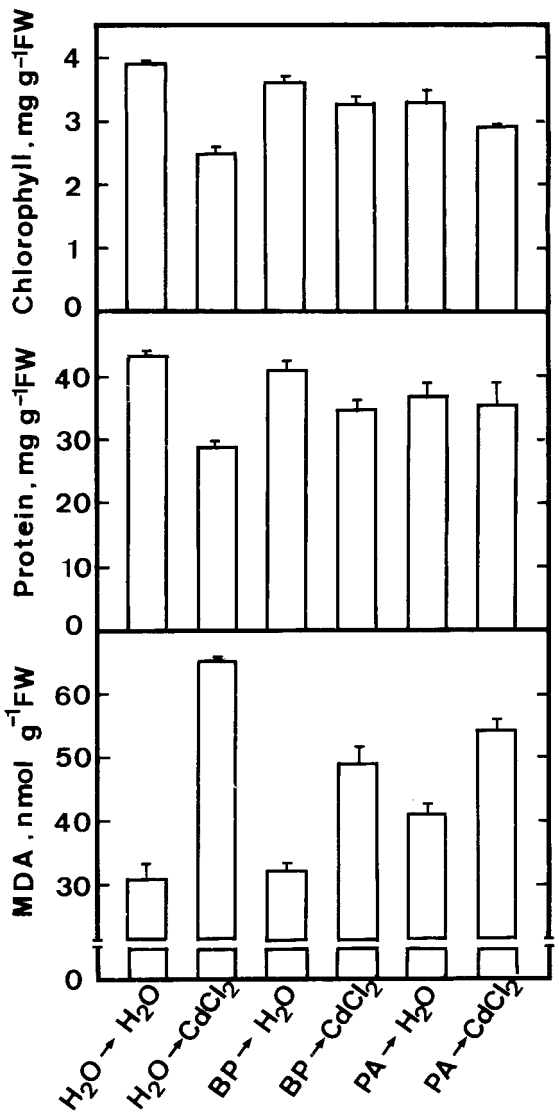

Figure 6. Effects of 2,2′-bipyridine (BP) and 1, 10-phenanthroline (PA) on contents of chlorophyll, protein, and malondialdehyde (MDA) in detached rice leaves were treated with $CdCl_2$. Detached rice leaves were pretreated with either water, 5 mM BP, or 5 mM PA for 6 h in the light and then treated with either water or 5 mM $CdCl_2$ for 24 h in the light. Vertical bars represent S.E. (n = 4).

induced a greater H_2O_2 content (Stroinski and Zielezinska 1997). However, in the present work, H_2O_2 did not accumulate in $CdCl_2$-treated detached rice leaves (Figure 3). One of the possible explanation is that H_2O_2 is being utilised in Fe- or Cu-catalysed reactions in $CdCl_2$-treated rice leaves. This suggestion was supported by the observation that metal chelators such as BP and PA were effective in reducing $CdCl_2$-induced toxicity, in reducing $CdCl_2$-induced increases in MDA content (Figure 6), and in reducing $CdCl_2$-induced decreases in SOD, APOD and GR activities (Table 3). Since detached rice leaves were treated with BP and $CdCl_2$ separately, the protective effect of BP against $CdCl_2$ toxicity is unlikely to have been caused by the blockage of $CdCl_2$ uptake by BP. Furthermore, BP-reduced toxicity of detached rice

Table 3. Effects of 2,2′-bipyridine (BP) on superoxide dismutase (SOD), ascorbate peroxidase (APOD), and glutathione reductase (GR) activities in detached rice leaves treated with $CdCl_2$. Detached rice leaves were pre-treated with either water or 5 mM BP for 6 h in the light and then treated with either water or 5 mM $CdCl_2$ for 24 h in the light. The data represent mean values ± S.E. (n = 4).

Treatment	Enzyme activity, units g^{-1} FW		
	SOD	APOD	GR
H_2O, 6 h → H_2O, 24 h	63.5 ± 0.8	55.9 ± 0.4	5.8 ± 0.2
H_2O, 6 h → $CdCl_2$, 24 h	48.8 ± 3.7	39.8 ± 0.1	2.7 ± 0.2
BP, 6 h → H_2O, 24 h	65.2 ± 1.9	47.8 ± 0.9	4.6 ± 0.3
BP, 6 h → $CdCl_2$, 24 h	55.1 ± 2.2	44.6 ± 1.0	3.1 ± 0.1

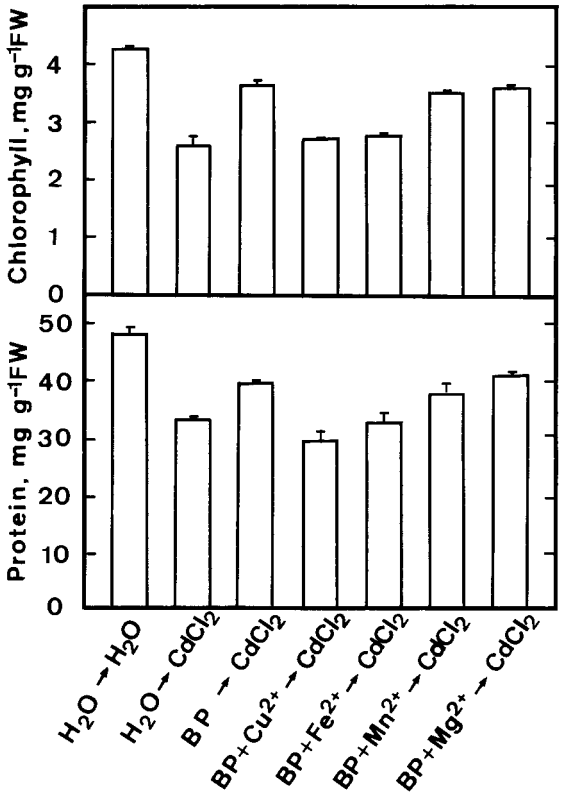

Figure 7. Reversal of BP-reduced $CdCl_2$ toxicity by metals. Detached rice leaves were pretreated with either water, 5 mM BP, or 5 mM BP plus 1 mM metal (sulfate salt) for 6 h in the light and then treated with either water or 5 mM $CdCl_2$ for 24 h in the light. Vertical bars represent S.E. (n = 4).

leaves, induced by excess Cd, was reversed by adding Fe^{2+} or Cu^{2+}, but not by Mn^{2+} or Mg^{2+}. The results of this study indicate that the protection of BP is mediated through chelation of iron or copper, suggesting that iron or copper play a major role in $CdCl_2$ toxicity in rice leaves. It is currently accepted that in vivo copper is unlikely to initiate the Haber-Weiss reaction in the way that has been demonstrated for iron (Halliwell and Gutteridge 1988). Thus, Cd toxicity in detached rice leaves may require the participation of iron.

It is generally considered that in intact plants most Cd taken up is retained in the roots (Javis et al. 1996). For the rice cultivar used in the present investigation, more than 50% of Cd taken up is retained in the roots (unpublished data). In the present investigation, detached rice leaves were used to study Cd-induced oxidative stress. It is not known whether Cd also induces oxidative stress in intact rice leaves. Thus, Cd-induced oxidative stress in intact leaves of rice seedlings merits further investigation.

Acknowledgements

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