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Changes in ammonium ion content and glutamine synthetase activity in rice leaves caused by excess cadmium are a consequence of oxidative damage

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Abstract

Ammonium ion accumulation and the decrease in glutamine synthetase (GS) activity induced by $CdCl_2$ were investigated in relation to lipid peroxidation in detached rice leaves. $CdCl_2$ was effective in increasing ammonium ion content, decreasing GS activity and increasing lipid peroxidation. Free radical scavengers (glutathione, thiourea, sodium benzoate) and an iron chelator (2,2′-bipyridine) were able to inhibit the decrease in GS activity and ammonium ion accumulation caused by $CdCl_2$ and at the same time inhibit $CdCl_2$-induced lipid peroxidation. Paraquat, which is known to produce oxygen radicals, decreased GS activity, increased ammonium ion content, and increased lipid peroxidation. GS1 appears to be the predominant isoform present. Excess Cd caused a decrease in GS1 but not in GS2 in detached rice leaves. An increase in lipid peroxidation preceded ammonium ion accumulation and the decrease in GS1 activity. These results suggest that the decrease in GS activity and the accumulation of ammonium ions in detached rice leaves are a consequence of oxidative damage caused by excess Cd.

Abbreviations: BP – 2,2′-bipyridine, DW – dry weight, FW – fresh weight, GS – glutamine synthetase, GS1 – cytosolic form of GS, GS2 – chloroplastic form of GS, GSH – reduced glutathione, MDA – malondialdehyde, PQ – paraquat, SB – sodium bezoate, TU – thiourea

Introduction

Cadmium is one of the most toxic heavy metals with no known biological function. Although Cd is a non-redox active metal, it was found to produce oxidative stress in plant tissues. Cd-increased lipid peroxidation has been demonstrated in *Phaseolus aureus* seedlings (Shaw 1995), *Phaseolus vulgaris* roots and leaves (Chaoui et al. 1997), *Helianthus annuus* leaves (Gallego et al. 1996a, 1996b), *Pisum sativum* shoot and root tissues (Lozano-Rodriguez et al. 1997), *Solanum tuberosum* tubers (Stroinski and Zielezinska 1997), and *Festuca rubra* seedlings (Wang et al. 1997). However, no peroxidation was found in Cd-treated plants and hairy roots of *Daucus carota* (Sanita di Toppi et al. 1998, 1999).

Ammonium ions are a central intermediate of nitrogen metabolism (Miflin and Lea 1976). GS is the key enzyme in ammonia assimilation and catalyzes the ATP-dependent condensation of ammonium ions with glutamate to produce glutamine (Miflin and Lea 1976). A decline in GS activity has been shown in leaves subjected to water stress, when exposed to excess Cu, and during dark-induced senescence (Backer et al. 1986; Chen and Kao 1998; Chen et al. 1997; Lin and Kao 1998; Peeters and Van Laere 1992; Thomas 1978), which may result in, at least in part, an accumulation of ammonium ions in leaves. In fact, various investigators have been able to show the accumulation of ammonium ions in leaves during senescence and under water stress and excess Cu conditions (Chen and Kao 1998; Chen et al. 1997; Lin

and Kao 1998). It is generally believed that GS activity in plants is regulated at the transcriptional level (Edwards et al. 1990; Forde et al. 1989; Hirel et al. 1987; Roche et al. 1993; Sukanya et al. 1994; Walker and Cornzzi 1989). Aside from transcriptional regulation, GS activity in plants might also be regulated at the level of turnover. Oxidative modification of GS has been implicated as the first step in the turnover of GS in bacteria (Levine 1983; Rivett and Levine 1990). Stieger and Feller (1997) have shown that GS degradation in illuminated chloroplasts requires the function of the photosynthetic electron transport chain. Chloroplastic GS of wheat seedlings has been reported to be particularly prone to degradation under oxidative stress conditions (Palatnik et al. 1999). By incubating soybean root extracts enriched in GS in a metal-catalysed oxidation system to produce the hydroxyl radical, Ortega et al. (1999) have shown that GS is oxidised and that the oxidised GS is inactive and more susceptible to degradation than nonoxidised GS. It is clear that GS degradation requires the production of free radicals.

In recent studies, we found that $CdCl_2$ induced ammonium ion accumulation in detached rice leaves (Chien and Kao 2000). Evidence was also presented to show that $CdCl_2$-induced ammonium ion accumulation in detached rice leaves is attributed to a decrease in GS activity (Chien and Kao 2000). Since $CdCl_2$ is known to induce lipid peroxidation in detached rice leaves (Chien et al. 2001), we examined the possible involvement of lipid peroxidation (oxidative stress) in regulating the decline of GS activity and accumulation of ammonium ions in detached rice leaves.

Materials and methods

Rice (*Oryza sativa* cv. Taichung Native 1) was cultured as described previously (Chen and Kao 1998). Briefly, rice seedling were planted on a stainless net floating on half-strength Johnson's modified nutrient solution (pH 4.2) in a 500-ml beaker. The nutrient solution was replaced every 3 days. Rice plants were grown for 12 days in a greenhouse, where natural light was provided and the temperature was controlled at 30 °C during the day and at 25 °C at night. The apical 3 cm of the third leaf was used for the experiment. A group of ten segments was floated in a Petri dish containing 10 ml of test solutions. Unless otherwise indicated, 5 mM $CdCl_2$ was used. Incubation was carried out at 27 °C in the light (40 μmol m^{-2} s^{-1}) for the time indicated.

For determination of Cd, detached rice leaves were dried at 65 °C for 48 h. Dried materials was ashed at 550 °C for 20 h. Ash residue was incubated with 31% HNO_3 and 17.5% H_2O_2 at 70 °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). Malondialdehyde (MDA), routinely used as an indicator of lipid peroxidation, was extracted with 5% (w/v) trichloroacetic acid determined according to Heath and Packer (1968).

Ammonium ions were extracted by homogenising leaf segments in 0.3 mM sulphuric acid (pH 3.5). The homogenate was centrifuged for 10 min at 39 000 × *g* and the supernatant was used for determination of ammonium ions as described by Lin and Kao (1996).

For extraction of GS, leaf segments were homogenised with 10 mM Tris-HCl buffer (pH 7.6, containing 1 mM $MgCl_2$, 1 mM EDTA and 1 mM 2-mercaptoethanol) using a chilled pestle and mortar. The homogenate was centrifuged at 15 000 × *g* for 30 min and the resulting supernatant was use for determination of GS activity. The whole extraction procedure was carried out at 4 °C. GS was assayed by the method of Oaks et al. (1980). The reaction mixture contained in a final volume of 1 ml was 80 μmol Tris-HCl buffer, 40 μmol L-glutamic acid, 8 μmol ATP, 24 μmol $MgSO_4$, and 16 μmol NH_2OH; the final pH was 8.0. The reaction was started by addition of the enzyme extract and, after incubation for 30 min at 30 °C, was stopped by adding 2 ml 2.5% (w/v) $FeCl_3$ and 5% (w/v) trichloroacetic acid in 1.5 M HCl. After centrifugation the absorbance of the supernatant was read at 540 nm. The definition of 1 U of GS activity is defined as 1 μmol L-glutamate γ-monohydroxamate formed per min. In order to separate the activites of GS1 and GS2 isofroms in leaf extract, activities were also measured in the same conditions in the presence of 1 mM glucosamine-6-phosphate, a specific inhibitor of GS2 (Hirel and Gadal 1980).

Cd content was expressed per g dry weight (DW), ammonium and MDA contents and GS activity were expressed per g fresh weight. Absolute levels of each measurement varied among experiments because of seasonal effects. However, the patterns of responses to $CdCl_2$ or PQ were reproducible. For all measurements, each treatment was repeated four times. All

experiments were repeated at least three times and yielded results consistent with the data reported here.

Results and discussion

Increasing concentration of $CdCl_2$ from 0.1 to 5 mM progressively increased ammonium ion content in detached rice leaves in the light (Figure 1C). No further increase was observed at 10 mM $CdCl_2$. Ammonium ion content increased ~2-fold in detached rice leaves treated with 5 mM $CdCl_2$ for 48 h in the light (Figure 1C). Ammonium ions are a central intermediate of nitrogen metabolism in plants (Miflin and Lea 1976). Ammonium ions are produced during nitrate assimilation, deamination of amino acids and photorespiration (Miflin and Lea 1976). The assimilation of ammonium ions requires GS (Miflin and Lea 1976). Recently, we reported that the decrease in GS activity but not the promotion of nitrate reduction is the source of Cd-induced ammonium ion accumulation in detached rice leaves (Chien and Kao 2000). We also showed that excess Cd increased lipid peroxidation in detached rice leaves (Chien et al. 2001). GS activity and MDA content were observed to decrease and increase, respectively, with the increase in $CdCl_2$ concentrations (Figure 1B and C). These results indicate that ammonium accumulation caused by $CdCl_2$ is attributed to the decline in GS activity and increase in lipid peroxidation. To be sure that the described changes in ammonium ion and MDA contents and GS activity were related to an increase in the leaf Cd content, Cd concentration was determined in detached rice leaves with various concentration of $CdCl_2$. Increasing concentration of $CdCl_2$ progressively increased leaf Cd content (Figure 1A).

Lipid peroxidation is a free radical mediated process (Hirel and Gadal 1980). If lipid peroxidation is important in regulating the decrease in GS activity and the increase in ammonium ion content, then free radical scavengers, such as reduced glutathione (GSH), sodium benzoate (SB) and thiourea (TU), are expected to decrease MDA and ammonium ion contents and to increase GS activity. The results reported in Figure 2 demonstrated that this is, in fact, correct. Altough free radical scavengers are effective in reducing ammonium ion content induced by $CdCl_2$, ammonium ion contents are still high (Figure 2B). This is probably due to the fact that free radical scavengers are less effective in reducing lipid peroxidation (Figure 2A) in detached rice leaves.

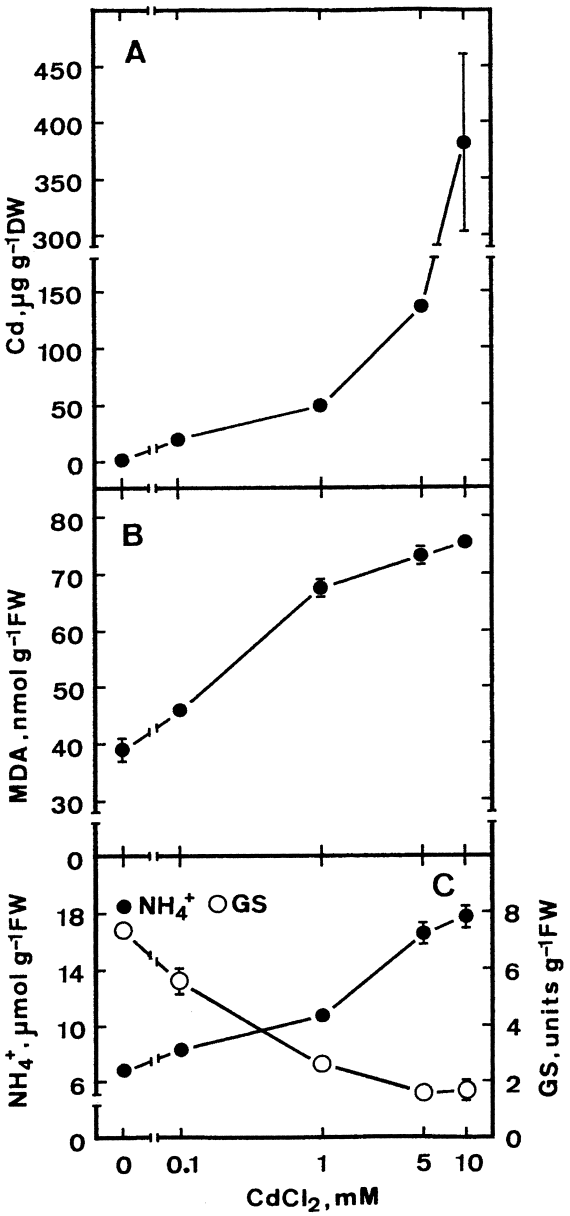

Figure 1. Effect of $CdCl_2$ on the contents of Cd, malondialdehyde (MDA) and ammonium ions and the activity of glutamine synthetase (GS) in detached rice leaves in the light. Detached rice leaves were incubated in solutions containing 0–10 mM $CdCl_2$. All measurements were made 48 h after treatment. Vertical bars represent standard errors (n = 4). Only these standard errors larger than the symbol are shown.

Superoxide can serve as a source to generate more active hydroxyl radicals by Haber-Weiss and Fenton reactions (Foyer et al. 1997). Transition metals, such as iron and copper, are able to accelerate Haber-Weiss and Fenton reactions (Naqni and Chance 1986). In a

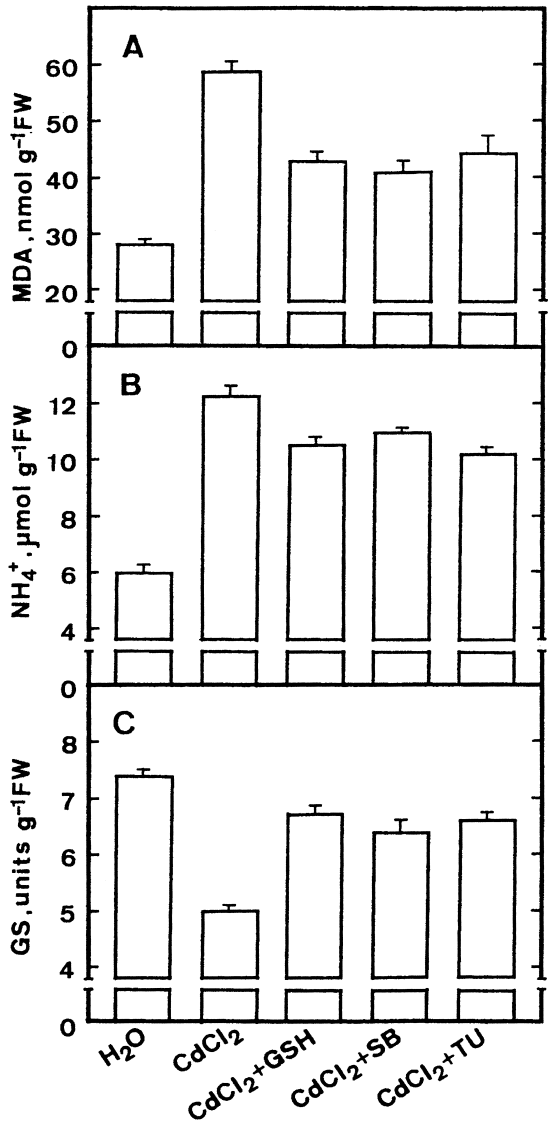

Figure 2. Effect of free radical scavengers on the content of malondialdehyde (MDA) and ammonium ions and the activity of glutamine synthetase (GS) in detached rice leaves in the light. The concentrations of $CdCl_2$, thiourea (TU), sodium benzoate (SB) and reduced glutathione (GSH) were 5 mM. All measurements were made 24 h after treatment. Vertical bars represent standard errors (n = 4).

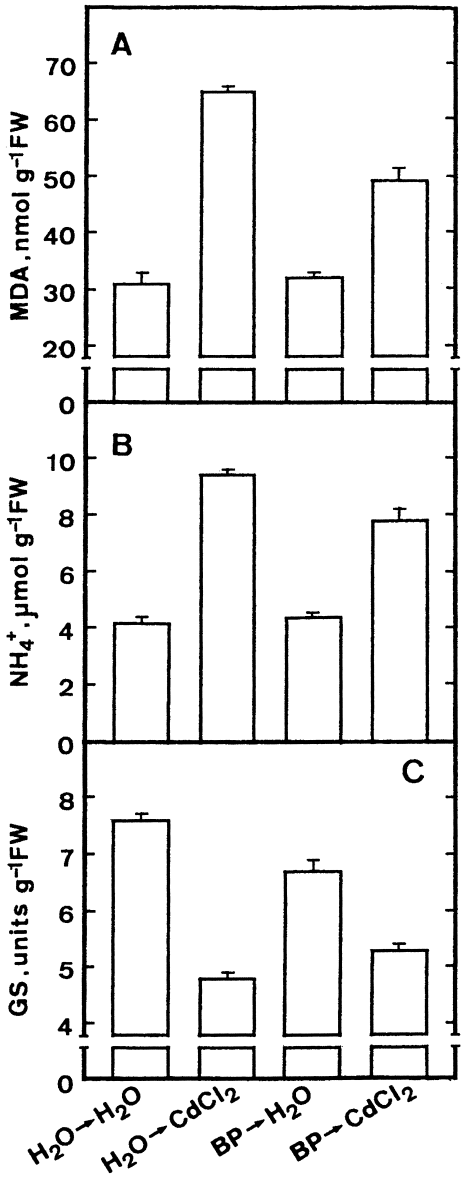

Figure 3. Effect of 2,2′-bipyridine (BP) on the contents of malondialdehyde (MDA) and ammonium ions and the activity of glutamine synthetase (GS) in detached rice leaves. Detached rice leaves were pretreated with either water or 5 mM BP for 6 h in the light and then treated with either water or 5 mM $CdCl_2$ for 24 h in the light. Vertical bars represent standard errors (n = 4).

recent study (Chien et al. 2001), pretreatment of leaf segments with 2,2′-bipyridine (BP) or 1,10-phenanthroline, iron chelators, caused a reduction of $CdCl_2$-induced increase in toxicity and MDA content. If lipid peroxidation plays a significant role in regulating ammonium ion accumulation and decline in GS activity, we also expect that pretreatment of leaf segments with BP would reduce the increase in ammonium ion content and the decrease in GS activity caused by $CdCl_2$. BP-pretreatment resulted in a reduction of $CdCl_2$-induced lipid peroxidation (Figure 3A) and ammonium ion accumulation (Figure 3B) and $CdCl_2$-induced decrease in GS activity (Figure 3C) in detached rice leaves. Thus, free radical generation is most likely involved in regulating ammonium ion ac-

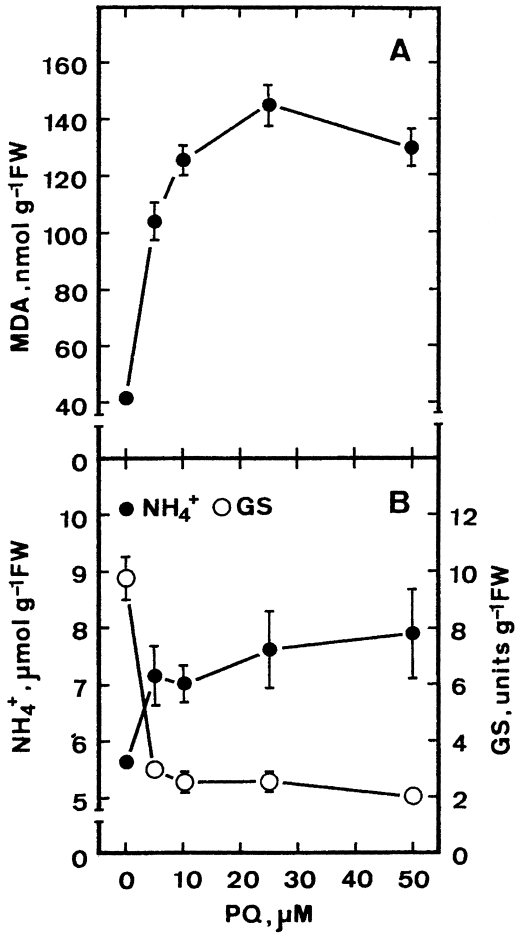

Figure 4. Effect of paraquat (PQ) on the contents of malondialdehyde (MDA) and ammonium ions and the activity of glutamine synthetase (GS) in detached rice leaves in the light. Detached rice leaves were incubated in solutions containing 0–50 μM PQ. All measurements were made at 48 h after treatment. Vertical bars represent standard errors (n = 4). Only these standard errors larger than the symbol are shown.

cumulation and GS activity. To test further this possibility, detached rice leaves were treated with a well known free-radical-generating chemical, paraquat (PQ). PQ treatment was observed to increase MDA content (Figure 4A) and at the same time increase ammonium ion content (Figure 4B) and decrease GS activity (Figure 4B) in detached rice leaves.

In green tissues of angiosperms the occurrence of two GS isoenzymes, GS1 and GS2, has been demonstrated. GS1 catalyses glutamine biosynthesis in the cytosol whereas GS2 is confined to the chloroplast (Lancien et al. 2000). Thus, it is of great interest to know the effect of excess Cd on the activities of GS1 and GS2 in detached rice leaves. By including 1 mM

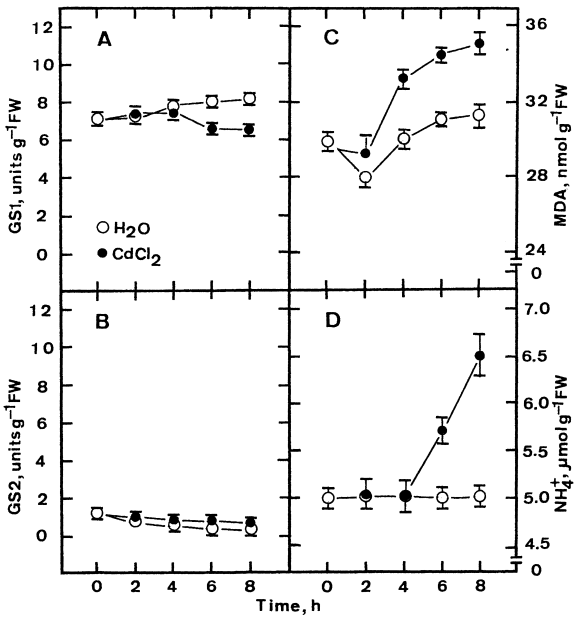

Figure 5. Time courses of the $CdCl_2$ effect on GS1 and GS2 activities, MDA contents, and ammonium ion contents in detached rice leaves. Detached rice leaves were incubated in water or 5 mM $CdCl_2$. Vertical bars represent standard errors (n = 4).

glucosamine-6-phosphate, a specific inhibitor of the chloroplastic GS2 isoform (Hirel and Gadal 1980) in the assay medium, discrimination between the activities of the two GS isoforms present in detached rice leaves was possible. It was found that GS1 appears to be the predominant isoform in detached rice leaves of the test variety (Figure 5A and B). It was also found that excess Cd (5 mM) caused a decrease in GS1 (Figure 5A) but had no effect on GS2 (Figure 5B), suggesting that the excess Cd-induced decrease in extracted total GS activity was mainly due to the GS1 cytosolic isoform. Lutts et al. (1999) also observed that GS1 was the predominant isoform in leaves of rice (cv. I Kong Pao and Nona Bokra).

To test the causal relationship between lipid peroxidation, ammonium ion level, and GS1 activity, detached rice leaves were incubated in distilled water and $CdCl_2$ (5 mM), respectively, for 2, 4, 6, and 8 h. Changes in MDA content, ammonium ion content, and GS1 activity were monitored. An increase in MDA content was observed at 4 h after $CdCl_2$ treatment (Figure 5C), whereas ammonium ion accumulation (Figure 5D) and the decrease in GS1 activity (Figure 5A) occurred at 6 h after $CdCl_2$ treatment. These results indicate that an increase in lipid peroxidation precedes ammonium ion accumulation and the decrease in GS1 activity. Clearly, the links between

$CdCl_2$ treatment, lipid peroxidation, ammonium ion accumulation and GS activity are well established. The results reported here suggest that the decreases in GS activity and the increases in ammonium ion content demonstrated in detached rice leaves are a consequence of oxidative damage caused by excess Cd. Our results are in agreement with the suggestion that GS is prone to degradation under oxidative stress conditions (Palatnik et al. 1999).

Acknowledgements

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