

# Cadmium-induced ammonium ion accumulation of rice seedlings at high temperature is mediated through abscisic acid

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**Abstract** In this study, we examined interaction between cadmium (Cd) and temperature in rice seedlings. Effects of CdCl<sub>2</sub> (0.5 mM) simultaneously applied at high (35/30°C day/night), medium (25/20°C) and low (15/13°C) temperatures to rice seedlings were detected by measuring changes in biomass production and NH<sub>4</sub><sup>+</sup> content. Results indicated that Cd-induced biomass reduction and NH<sub>4</sub><sup>+</sup> accumulation increased in parallel with temperature increases. On treatment with CdCl<sub>2</sub>, the abscisic acid (ABA) content markedly increased in the leaves of seedlings grown at high temperature but not at medium and low temperatures. Exogenous application of ABA at medium temperature increased ABA and NH<sub>4</sub><sup>+</sup> contents in the leaves of rice seedlings. Fluridone (Flu) treatment, an inhibitor of carotenoid biosynthesis, reduced ABA content, as well as Cd-induced NH<sub>4</sub><sup>+</sup> accumulation in the leaves of rice seedlings grown at high temperature. These Flu effects can be reversed by application of ABA. Furthermore, Flu treatment did not reduce Cd content in leaves of seedlings grown at high temperature. All these results suggest that Cd-induced NH<sub>4</sub><sup>+</sup> accumulation at high temperature is mediated through ABA.

**Keywords** Abscisic acid · Ammonium ion · Cadmium · *Oryza sativa* · Temperature

## Abbreviations

ABA	Abscisic acid
DW	Dry weight
ELISA	Enzyme-linked immunosorbent assay
Flu	Fluridone
GS	Glutamine synthetase
NCED	9- <i>cis</i> -epoxycarotenoid dioxygenase
PAL	Phenylalanine ammonia-lyase

## Introduction

Cadmium (Cd) is a divalent heavy metal cation and is one of the most toxic heavy metals. It is supplied to soil, air, and water mainly by effluents from industries, mining, burning, and leakage waste, and by application of phosphate and sewage sludge. Cd is readily taken up by plants, leading to inhibition of physiological processes such as respiration, photosynthesis, cell elongation, plant-water relationships, nitrogen metabolism, and mineral nutrition (Sanità di Toppi and Gabbrielli 1999).

Global warming, accompanied by an increased frequency of periods with exceptionally high

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temperatures, is one of the most important characteristics of the accelerated climatic changes. Climatic models predict that global temperature will continue to increase in the future. Peng et al. (2004) analyzed weather data at the International Rice Research Institute Farm from 1979 to 2003 to examine temperature trends and reported that annual mean maximum and minimum temperatures have increased by 0.35°C and 1.13°C, respectively. Hence, plants will be more often exposed to high temperature conditions. It has been shown that Cd toxicity (the reduction of plant height, the decrease in chlorophyll content, and the accumulation of phenolic compounds and proline) of wheat seedlings increases in parallel to temperature increases (Öncel et al. 2000). Temperature is a key factor in altering ion accumulation (Chawla et al. 1991; Hooda and Alloway 1993; Macek et al. 1994; Mautsoe and Beckett 1996). The high toxicity of Cd induced by high temperature may be the results of much more Cd uptake from the medium.

$\text{NH}_4^+$  is a central intermediate of nitrogen metabolism (Mifflin and Lea 1976). It has been shown that on treatment with  $\text{CdCl}_2$ ,  $\text{NH}_4^+$  content increased in rice leaves (Hsu and Kao 2003). Glutamine synthetase (GS) is the key enzyme in  $\text{NH}_4^+$  assimilation and catalyzes the ATP-dependent condensation of  $\text{NH}_4^+$  with glutamate to produce glutamine (Mifflin and Lea 1976). Phenylalanine ammonia-lyase (PAL) catalyzes the elimination of  $\text{NH}_4^+$  from phenylalanine and produces *trans*-cinnamate (Hahlbrock and Grisebach 1979).  $\text{NH}_4^+$ , released from PAL reaction, is known to be trapped in the glutamine molecule by the action of GS (Razel et al. 1996; van Heerden et al. 1996). Sakurai et al. (2001) provided evidence to show that GS is partly coupled to the reaction of PAL in developing rice leaves. Cd-induced  $\text{NH}_4^+$  accumulation in rice leaves has been proved to be associated with the decrease in GS activity and the increase in PAL specific activity (Hsu and Kao 2004).

The plant hormone ABA is a sesquiterpenoid derived from xanthophyll (Nambara and Marion-Poll 2005) and appears to influence several physiological and developmental events (Seo and Koshiba 2002; Zeevaart and Creelman 1988). The level of ABA in plant tissues is known to increase

in response to exposure to heavy metals such as Cd, Ni, Zn, and Al (Fediuc et al. 2005; Foy 1998; Hollenbach et al. 1997; Hsu and Kao 2003; Poschenrieder et al. 1989; Rauser and Dumbroff 1981). Fediuc et al. (2005) demonstrated that Cd-induced ABA accumulation was observed in roots, but not shoots, of *Typha* and *Phragmites* plants. Hsu and Kao (2003) found that, on treatment with  $\text{CdCl}_2$ , the ABA content rapidly increased in the leaves and roots in Cd-tolerant rice cultivar (cv. Tainung 67) grown at normal growing temperature (30/25°C, day/night) but not in Cd-sensitive cultivar (cv. Taichung Native 1).

In this study, effects of Cd applied at low (15/13°C day/night), medium (25/20°C day/night), and high (35/30°C day/night) temperatures on the content of  $\text{NH}_4^+$  in leaves of rice seedlings were compared. Recently, we showed that ABA treatment resulted in an accumulation in  $\text{NH}_4^+$  in detached rice leaves (Hung and Kao 2005). We thus examined the possibility that ABA plays a role in mediating the Cd-induced  $\text{NH}_4^+$  accumulation in leaves of rice seedlings grown at high temperature.

## Materials and methods

### Plant material and treatments

Rice (*Oryza sativa* L., cv. Taichung Native 1) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. These seeds were then germinated in Petri-dishes with wetted filter papers at 37°C in the dark. After 48 h incubation, uniformly germinated seeds were selected and cultivated in a 250 ml beaker containing half-strength Kimura B solution containing the following macro- and micro-elements: 182.3  $\mu\text{M}$   $(\text{NH}_4)_2\text{SO}_4$ , 91.6  $\mu\text{M}$   $\text{KNO}_3$ , 273.9  $\mu\text{M}$   $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 91.1  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$ , 182.5  $\mu\text{M}$   $\text{Ca}(\text{NO}_3)_2$ , 30.6  $\mu\text{M}$  Fe-citrate, 0.25  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 0.2  $\mu\text{M}$   $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.2  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05  $\mu\text{M}$   $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , and 0.07  $\mu\text{M}$   $\text{H}_2\text{MoO}_4$ . The nutrient solutions (pH 4.7) were replaced every 3 days. The hydroponically cultivated seedlings were grown in a Phytotron (Agricultural Experimental Station, National Taiwan University, Taipei, Taiwan) with natural sunlight at 30/25°C day/night and 90% relative humidity.

Twelve-day-old seedlings with three leaves were grown in basic nutrient solution with or without CdCl<sub>2</sub> at high (35/30°C day/night), medium (25/20°C day/night), or low temperature (15/13°C day/night). Based on our experience from the experiments of Cd effect on rice seedlings, consistent Cd toxicity was observed in 2 days when 0.5 mM CdCl<sub>2</sub> was used for experiments. However, no consistent Cd toxicity was observed when lower CdCl<sub>2</sub> concentrations, in the range 5–50 mM, were applied over a longer period (6 days). For this reason in this study, 0.5 mM CdCl<sub>2</sub> was used and added directly to nutrient solution. For the experiments in which the effect of exogenous ABA was examined, various concentrations of ABA (5–40 mM, mixed isomers) were added directly to the culture solution for 2 days at medium temperature. In experiments to understand the role of endogenous ABA, 0.2 mM fluridone (Flu), which is known to block the conversion of phytoene to phytofluene in carotenoid biosynthesis pathway (Kowalczyk-Schröder and Sandmann 1992), was added directly to the nutrient solution for 2 days.

### Growth analysis

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

### Cd determination

For 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<sub>3</sub> and 17.5% H<sub>2</sub>O<sub>2</sub> 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). Amount of Cd is expressed on the basis of DW.

Determinations of protein, NH<sub>4</sub><sup>+</sup>, and abscisic acid

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 by the method of Bradford (1976). NH<sub>4</sub><sup>+</sup> was measured in the crude extract by the Berthelot reaction, modified according to Weatherburn (1967). The detailed procedure was described previously (Lin and Kao 1996). NH<sub>4</sub><sup>+</sup> contents are expressed on the basis of DW.

For 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 polyvinylpyrrolidone column and C18 (Sep-Pak Vac) cartridges (Waters, Milford, MA). 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). ABA immunoassay detection kit (PGR-1) was purchased from Sigma Chemical Co. (St. Louis, MO) is specific for (+)-ABA. By evaluating <sup>3</sup>H-ABA recovery, <sup>3</sup>H-ABA loss was (3% by the method described here. ABA content is expressed on the basis of DW.

### Enzyme assays

For extraction of GS, leaf samples were homogenized with 10 mM Tris-HCl buffer (pH 7.6, containing 1 mM MgCl<sub>2</sub>, 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 used 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<sub>4</sub>, and 16 μmol NH<sub>2</sub>OH; 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<sub>3</sub> and 5% (w/v) trichloroacetic acid in 1.5 M HCl. After centrifugation the absorbance of the supernatant was read at 540 nm. One unit of

GS activity is defined as 1  $\mu\text{mol}$  L-glutamate  $\gamma$ -monohydroxamate formed per min.

Phenylalanine ammonia-lyase (PAL) was extracted and determined according to Hyodo and Fujinami (1989). The calculation was based on the extinction coefficient ( $9500 \text{ M}^{-1} \text{ cm}^{-1}$ ) for *trans*-cinnamic acid. One unit of activity for PAL was defined as the amounts of enzyme which caused the formation of 1  $\mu\text{mol}$  *trans*-cinnamic acid per hour.

#### Transpiration rate

The transpiration rate was measured according to Greger and Johansson (1992). The weight of rice seedlings grown in hydroponic solution was determined at the beginning and the end of interval, respectively. The transpiration rate was calculated for the water loss during each interval and converted to a per day per seedling basis.

#### Statistical analysis

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

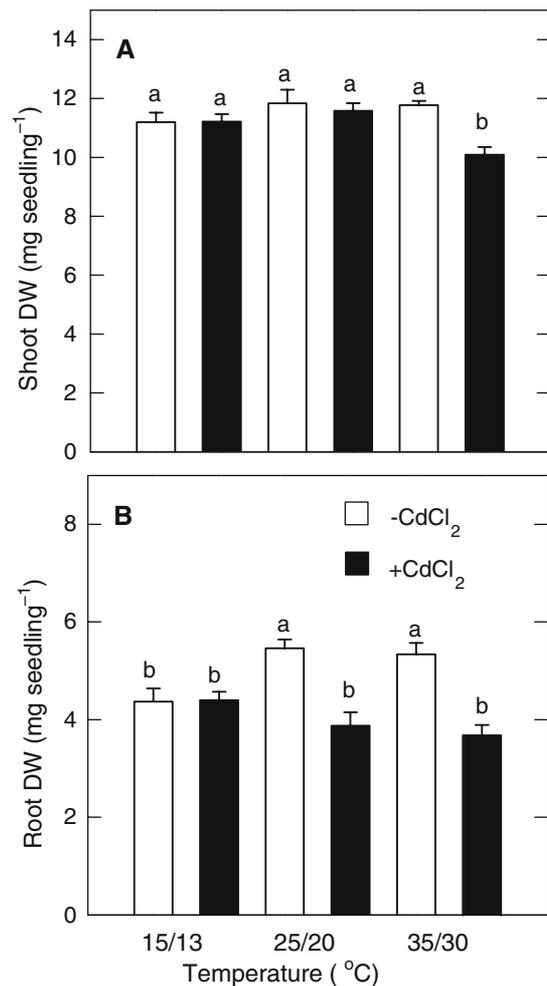
## Results

### Biomass production

In the absence of  $\text{CdCl}_2$ , no significant difference in shoot DW of rice seedlings treated with different temperatures was observed (Fig. 1A).  $\text{CdCl}_2$  treatment resulted in a 15% reduction of shoot DW at high temperature but not at low and medium temperatures (Fig. 1A). Low temperature treatment had lower root DW of rice seedlings in the absence of  $\text{CdCl}_2$  than medium and high temperatures (Fig. 1B).  $\text{CdCl}_2$  treatment reduced root DW to the level of 70% of the untreated seedlings at high and medium temperatures (Fig. 1B). However, Cd had no effect on root DW at low temperature (Fig. 1B).

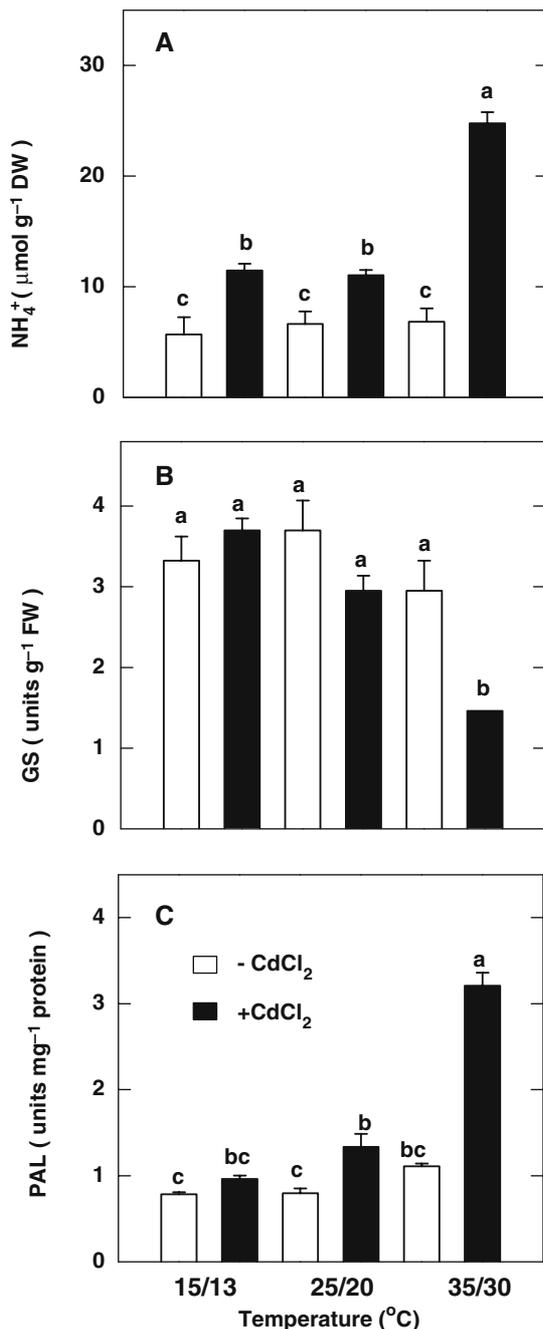
### Effect of temperatures on $\text{NH}_4^+$ content

$\text{NH}_4^+$  is a central intermediate of nitrogen metabolism in plants (Mifflin and Lea 1976). Here,



**Fig. 1** Effect of temperatures on the dry weight of shoot (A) and roots (B) of rice seedlings treated with or without  $\text{CdCl}_2$  (0.5 mM). All measurements were made two days after treatment. Bars indicate standard error ( $n = 4$ ). Values with the same letter are not significantly different at  $P < 0.05$

we show that on treatment with  $\text{CdCl}_2$ ,  $\text{NH}_4^+$  content increases about 3-fold in the second leaves of rice seedlings grown at high temperature, but 80% at medium and low temperatures (Fig. 2A). At high temperature,  $\text{CdCl}_2$  treatment resulted in a 50% decrease in GS activity in the second leaves (Fig. 2B). However, there was no decrease in GS activity in the second leaves of rice seedlings treated with  $\text{CdCl}_2$  at medium and low temperatures (Fig. 2B).  $\text{CdCl}_2$  treatment resulted in about 3-fold increase in PAL specific activity in leaves of rice seedlings grown at high



**Fig. 2** Effect of temperatures on the contents of  $\text{NH}_4^+$  (A), and the activities of GS (B), and the specific activities of PAL (C) in the second leaves of rice seedlings treated with or without  $\text{CdCl}_2$  (0.5 mM). All measurements were made two days after treatment. Bars indicate standard error ( $n = 4$ ). Values with the same letter are not significantly different at  $P < 0.05$

temperature (Fig. 2C). However, slight (30%) and no increase in PAL specific activity in the

second leaves of rice seedlings treated with  $\text{CdCl}_2$  at medium and low temperatures, respectively (Fig. 2C).

#### Effect of temperatures on Cd content

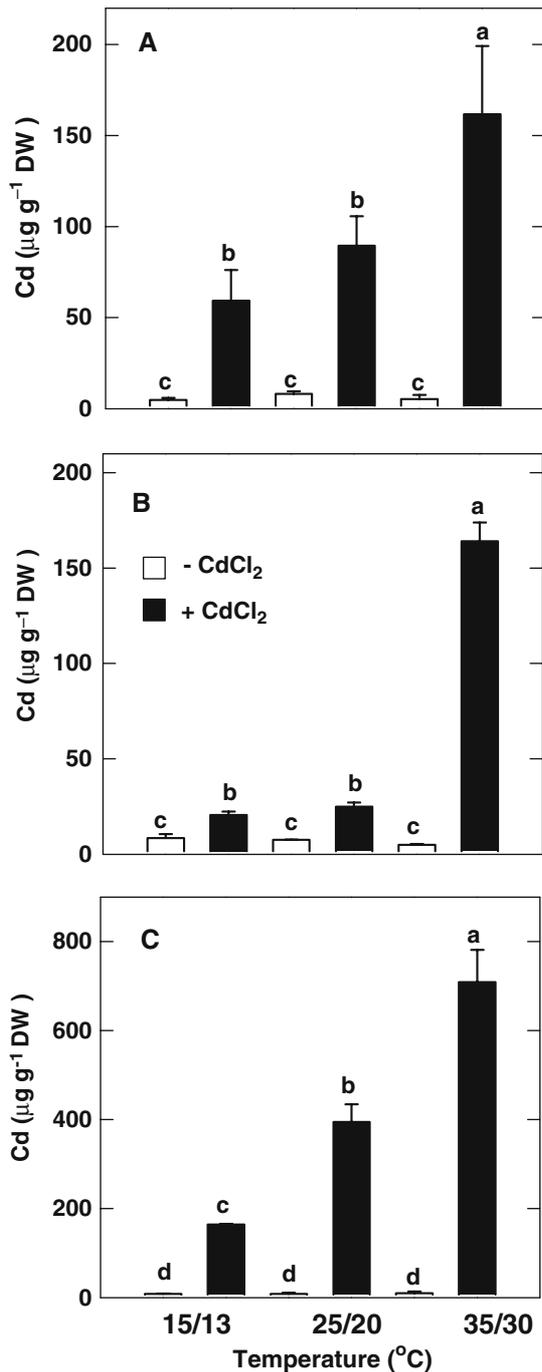
$\text{CdCl}_2$  treatment resulted in 30-, 11-, and 13-fold increase in Cd content in the third leaves (Fig. 3A), 32-, 3-, and 2.4-fold increase in the second leaves (Fig. 3B), and 69-, 48-, and 19-fold increase in roots (Fig. 3C) at high, medium, and low temperatures, respectively. The results in Figs. 3A–C are consistent with the idea that the amount of Cd reaching the shoot is usually lower than the amount in the roots (Javis et al. 1976). Figure 4 demonstrated that the increase in Cd content in the second leaves was evident 8, 24, and 36 h after  $\text{CdCl}_2$  treatment at high, medium, and low temperatures, respectively.

#### Effect of temperatures on transpiration rate and ABA content

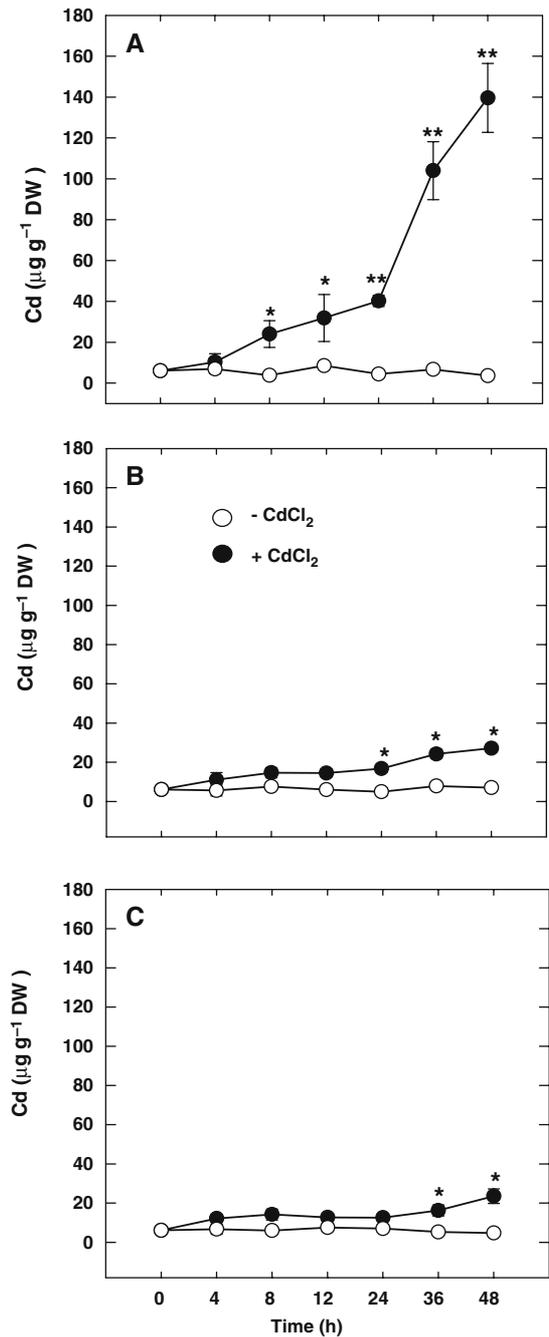
Cd has been shown to decrease transpiration rate in several plants (Hagemeyer et al. 1986; Kirkham 1978; Lamoreaux and Chaney 1978; Schlegel et al. 1987). We also observed that Cd decreased transpiration rate in rice seedlings (Fig. 5A).  $\text{CdCl}_2$  treatment reduced transpiration rate of rice seedlings grown at low, medium, and high temperatures, respectively, to 53%, 29%, and 13% of the control values (Fig. 5A). At high temperature,  $\text{CdCl}_2$  treatment resulted in about 3-fold increase in endogenous ABA in the second leaves (Fig. 5B). However, 25% and no increase in ABA content in the second leaves of rice seedlings treated with  $\text{CdCl}_2$  at medium and low temperatures, respectively (Fig. 5B).

#### Exogenous application of ABA

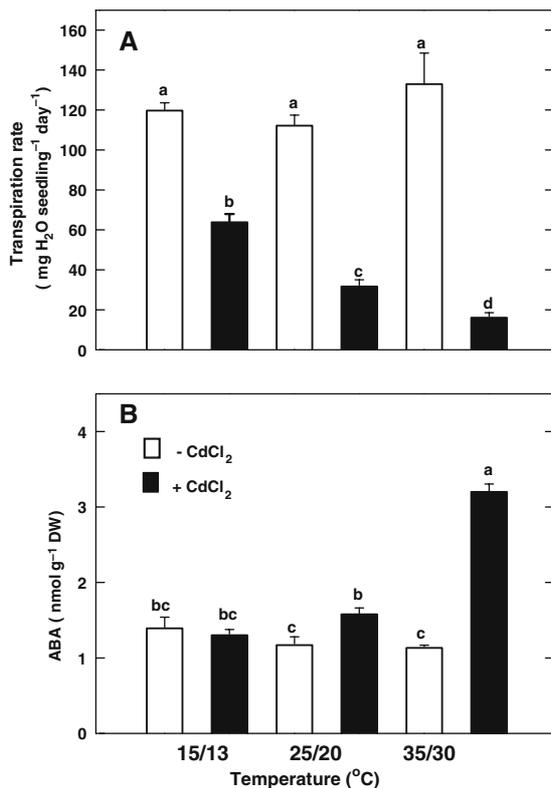
To study the effect of exogenous ABA, various concentrations of ABA (5–40  $\mu\text{M}$ ) were added directly to the culture solution at medium temperature. The results indicated that exogenous ABA treatment increased endogenous ABA and  $\text{NH}_4^+$  contents in the second leaves of rice seedlings grown at medium temperature (Figs. 6A, B).



**Fig. 3** Effect of temperatures on Cd concentrations in the third leaves (A), the second leaves (B), and roots (C) of rice seedlings treated with or without CdCl<sub>2</sub> (0.5 mM). Cd concentrations were determined 2 days after treatment. Bars indicate standard error ( $n = 4$ ). Values with the same letter are not significantly different at  $P < 0.05$



**Fig. 4** Changes in Cd concentrations in the second leaves of rice seedlings treated with or without CdCl<sub>2</sub> (0.5 mM) at 35/30°C (A), 25/20°C (B), and 15/13°C (C). Bars indicate standard error ( $n = 4$ ). Asterisks represent values that are significant between -CdCl<sub>2</sub> and + CdCl<sub>2</sub> treatments at  $P < 0.05$



**Fig. 5** Effect of temperatures on transpiration rates (A) and (B) ABA contents in the second leaves of rice seedlings treated with or without CdCl<sub>2</sub> (0.5 mM). ABA contents were determined 2 days after treatment. Bars indicate standard error ( $n = 4$ ). Values with the same letter are not significantly different at  $P < 0.05$

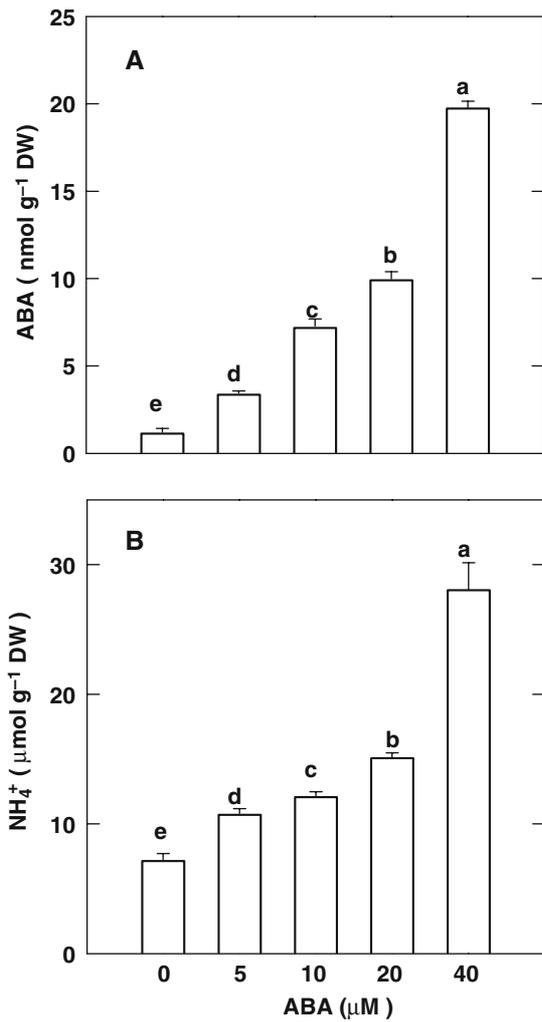
#### Fluridone treatment

The role of ABA in Cd-induced increase in NH<sub>4</sub><sup>+</sup> content at high temperature was tested further by using Flu, which blocks the conversion of phytoene to phytofluene in carotenoid biosynthesis pathway (Kowalczyk-Schröder and Sandmann 1992). Flu treatment was observed to inhibit the increase in ABA content caused by CdCl<sub>2</sub> (Fig. 7A) and reduce Cd-induced accumulation of NH<sub>4</sub><sup>+</sup> (Fig. 8A), decrease in GS activity (Fig. 8B), and increase in PAL specific activity (Fig. 8C) in the second leaves of rice seedlings grown at high temperature. The effects of Flu on reducing Cd-induced increase in NH<sub>4</sub><sup>+</sup> content and PAL specific activity, and the decrease in GS activity can be reversed by the application of ABA (Figs. 8A–C). ABA alone was also found to be

able to increase NH<sub>4</sub><sup>+</sup> content and PAL specific activity and to decrease GS activity in the second leaves of rice seedlings grown at high temperature when compared with untreated control (Figs. 8A–C). Figs. 7B, C also show that Cd content in the second leaves and transpiration rate of rice seedlings treated with Flu plus CdCl<sub>2</sub> at high temperature are similar to those with CdCl<sub>2</sub> alone.

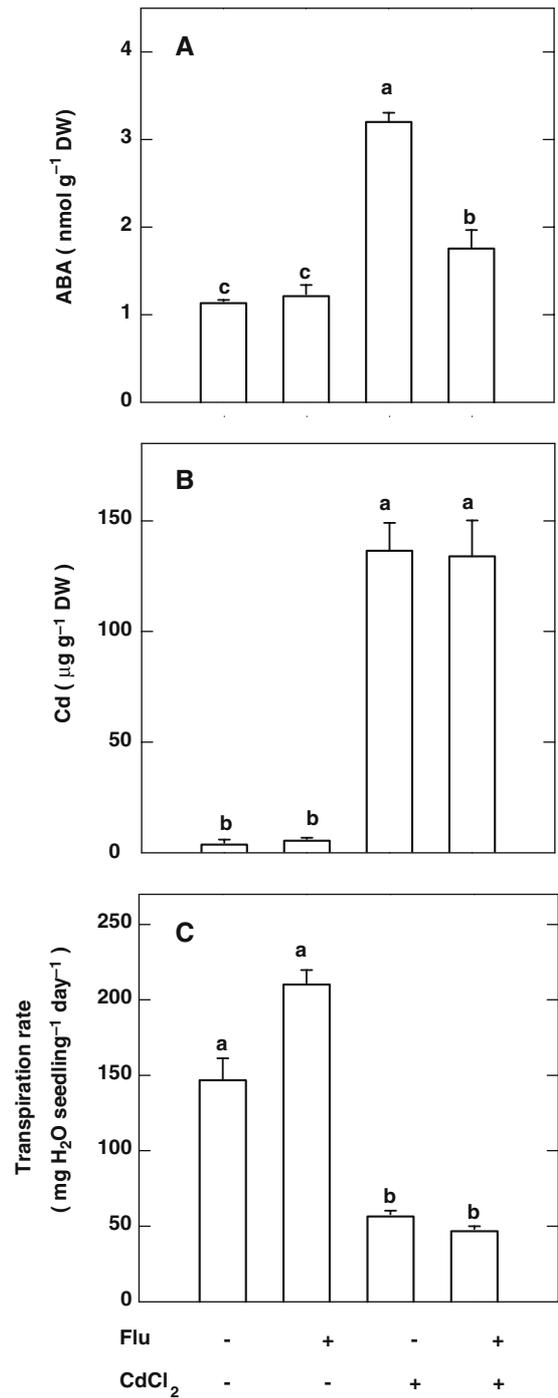
#### Discussion

The interactive effects of temperature and heavy metal stress (Cd and Pb) on the growth and some biochemical compounds have been studied in wheat seedlings (Öncel et al. 2000). In general, Cd toxicity (reduction of plant height, decrease in chlorophyll content, and accumulation of phenolic compounds) of wheat seedlings increases in parallel to temperature increases (Öncel et al. 2000). In rice seedlings, Cd-induced biomass reduction and NH<sub>4</sub><sup>+</sup> accumulation are also in parallel to temperature increases (Figs. 1A, B and 2A). The high biomass reduction and NH<sub>4</sub><sup>+</sup> accumulation caused by CdCl<sub>2</sub> at high temperature seem to be a result of more and rapid Cd uptake from the medium (Figs. 3A–C and 4A–C). It has been shown that enhanced temperature increased the uptake of Cd in *Solanum nigrum* (Macek et al. 1994), *Lemna minor* (Chawla et al. 1991), *Lolium perenne* (Hooda and Alloway 1993), and mosses (Mautsoe and Beckett 1996). The massive difference (7-fold) in Cd uptake in the leaves of rice seedlings grown at high and medium temperatures suggests that the uptake of Cd in rice seedlings is also temperature-dependent. In the present study, rice seedlings were exposed to different air temperatures. In our system, high air temperature treatment resulted in high root temperature in about 1 h after treatment (unpublished data). A great body of evidence has demonstrated that Cd is taken by the roots via essential metal transporters (Cohen et al. 1998; Korshunova et al. 1999; Lasat et al. 2000; Pence et al. 2000; Salt et al. 1995). It is not known whether high root temperature increases the activities of metal transporters or Cd uptake in rice roots in the presence of Cd. Further research in this direction is likely to be highly rewarding.



**Fig. 6** Effect of ABA concentration on the content of ABA (**A**) and  $\text{NH}_4^+$  (**B**) in the second leaves of rice seedlings grown at medium temperature. All measurements were made two days after treatment. Bars indicate standard error ( $n = 4$ ). Values with the same letter are not significantly different at  $P < 0.05$

It has been shown that the decrease in GS activity and the increase on PAL activity are responsible for  $\text{NH}_4^+$  accumulation in Cd-treated rice leaves (Hsu and Kao 2004). Here, we also show that on treatment with  $\text{CdCl}_2$ , the decrease in GS activity and the increase in PAL specific activity and  $\text{NH}_4^+$  content are more pronounced in the leaves of rice seedlings grown at high temperature than those at medium and low temperatures (Figs. 2B, C).



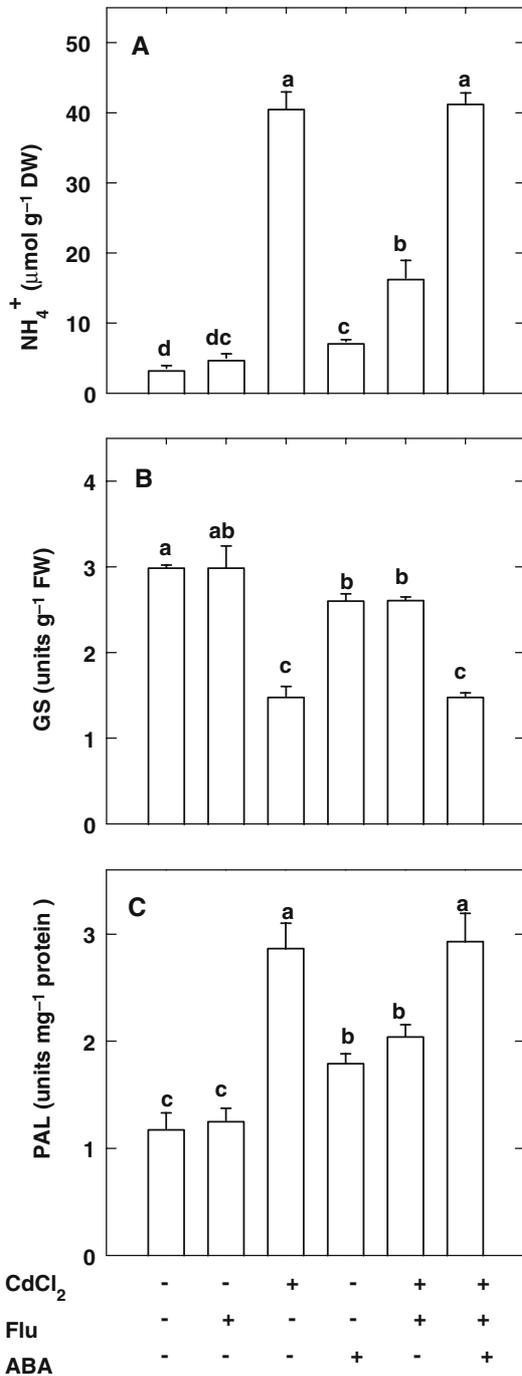
**Fig. 7** Effect of fluridone (Flu, 0.2 mM) on the contents of ABA (**A**) and Cd (**B**) in the second leaves of rice seedlings, and the transpiration rates (**C**) of the rice seedlings treated with or without  $\text{CdCl}_2$  (0.5 mM) at 35/30°C. All measurements were made 2 days after treatment. Bars indicate standard error ( $n = 4$ ). Values with the same letter are not significantly different at  $P < 0.05$

At low temperature,  $\text{CdCl}_2$  treatment resulted in an increase in  $\text{NH}_4^+$  content, but had no effect on GS activity and PAL specific activity in the second leaves (Figs. 2A–C). Thus, Cd-induced  $\text{NH}_4^+$  accumulation in rice seedlings at low

temperature cannot be explained by GS and PAL. Ammonium ion is produced during nitrate assimilation, deamination of amino acids, and photorespiration (Mifflin and Lea 1976). Thus, the possibilities that nitrate reduction, deamination of amino acid, and photorespiration may be responsible for Cd-induced  $\text{NH}_4^+$  accumulation at low temperature cannot be excluded.

ABA is a sesquiterpenoid derived from xanthophylls (Nambara and Marion-Poll 2005). The level of ABA in plants increases upon their exposure to Cd stress (Fediuc et al. 2005; Hollenbach et al. 1997; Hsu and Kao 2003; Poschenrieder et al. 1989). Here, we show that ABA accumulation in rice leaves is induced by Cd at high temperature (Fig. 5B). However, high temperature itself does not cause ABA accumulation in rice seedlings leaves (Fig. 5B). It is now well established that ABA in higher plants is derived from  $\text{C}_{40}$ -carotenoids (Nambara and Morion-Poll, 2005). As Flu is an inhibitor of carotenoid biosynthesis (Kowalczyk-Schröder and Sandmann 1992), the effect of this inhibitor on Cd-induced ABA in the leaves of rice seedlings at high temperature may imply that ABA biosynthetic pathway in response to Cd at high temperature appears to be the same as that established in other stress conditions (Nambara and Marion-Poll 2005; Zeevaart and Creelman 1988).

The present study indicated that ABA is involved in Cd-induced  $\text{NH}_4^+$  accumulation in the leaves of rice seedlings grown at high temperature. This conclusion was based on observations that (a) the increase in endogenous ABA content in response to Cd in leaves of rice seedlings grown at high temperature was more pronounced than that at medium and low temperatures (Fig. 5B); (b) exogenous application of ABA at medium temperature increased ABA and  $\text{NH}_4^+$  contents in the second leaves (Figs. 6A, B); (c) Flu treatment



◀ **Fig. 8** Effect of fluridone (Flu, 0.2 mM) and ABA (5 μM) on the contents of  $\text{NH}_4^+$  (A), and the activities of GS (B) and the specific activities PAL (C) in the second leaves of rice seedlings treated with or without  $\text{CdCl}_2$  (0.5 mM) at 35/30°C. All measurements were made 2 days after treatment. Bars indicate standard error ( $n = 4$ ). Values with the same letter are not significantly different at  $P < 0.05$

reduced ABA content, as well as Cd-induced accumulation of  $\text{NH}_4^+$  and changes of the activities of enzymes responsible for  $\text{NH}_4^+$  accumulation in the leaves of rice seedlings grown at high temperature (Figs. 7A and 8A–C); and (d) the effects of Flu on Cd-induced  $\text{NH}_4^+$  accumulation and enzyme activities related to  $\text{NH}_4^+$  accumulation of rice seedlings grown at high temperature can be reversed by the application of ABA (Figs. 8A–C).

The fact that Flu treatment reduced ABA content, but did not reduce Cd content in leaves of rice seedlings in response to  $\text{CdCl}_2$  at high temperature (Figs. 7A, B) suggests that Flu effect on the reduction of Cd-induced  $\text{NH}_4^+$  accumulation is attributable to reduction of ABA but not Cd content in leaves of rice seedlings grown at high temperature. These results strengthen further our conclusion that Cd-induced  $\text{NH}_4^+$  accumulation at high temperature is mediated through ABA. Recently, Fediuc et al. (2005) reported that ABA mediated the Cd-induced stimulation of *O*-acetylserine (thiol) lyase (OASTL), the enzyme responsible for cysteine biosynthesis. However, in case of growth of *Arabidopsis* (root length and seedlings fresh weight), the magnitude of Cd-induced inhibition in ABA-insensitive mutants was generally comparable to that in the wild type (Sharma and Kumar 2002). Accumulation of proline in response to Cd has been reported in wheat and *Silene vulgaris* (Öncel 2002; Schat et al. 1997). We also reported that  $\text{NH}_4^+$  accumulation in dark-treated and water-stressed rice seedlings is associated with proline accumulation (Yang and Kao 2000). Our unpublished data indicated that Cd-induced proline accumulation in leaves of rice seedlings grown at high temperature is also mediated through ABA accumulation.

Cd is known to inhibit transpiration rate of several plants (Hagemeyer et al. 1986; Lamoireaux and Chaney 1978; Schlegel et al. 1987). We also found that  $\text{CdCl}_2$  treatment reduced transpiration rate of rice seedlings (Fig. 5A). Reduction of transpiration rate and increase in Cd content caused by  $\text{CdCl}_2$  in seedlings grown at high temperature were more pronounced than those at medium and low temperatures (Figs. 3B and 5A). Since Flu treatment had no effect on transpiration rate of rice seedlings in response to  $\text{CdCl}_2$  at high temperature (Fig. 7C). It appears

that the decrease of transpiration rate caused by  $\text{CdCl}_2$  is not attributable to increase in ABA content, but to increase in Cd content.

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