

Role of abscisic acid in cadmium tolerance of rice (*Oryza sativa* L.) seedlings

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ABSTRACT

Changes in abscisic acid (ABA) contents in Cd-treated rice (*Oryza sativa* L.) seedlings of two cultivars were investigated. On treatment with CdCl₂, the ABA content rapidly increased in the leaves and roots of Cd-tolerant cultivar (cv. Tainung 67, TNG67) but not in the Cd-sensitive cultivar (cv. Taichung Native 1, TN1). The reduction of transpiration rate of TN1 caused by Cd was less than that of TNG67. Exogenous application of ABA reduced transpiration rate, decreased Cd content, and enhanced Cd tolerance of TN1 seedlings. Exogenous application of the ABA biosynthesis inhibitor, fluridone, reduced ABA accumulation, increased transpiration rate and Cd content, and decreased Cd tolerance of TNG67 seedlings. Fluridone effect on Cd toxicity of TNG67 seedlings was reversed by the application of ABA. The roles of endogenous ABA in Cd tolerance of rice seedlings are discussed and suggested.

Key-words: *Oryza sativa* L.; abscisic acid; cadmium tolerance; transpiration rate.

INTRODUCTION

The plant hormone abscisic acid (ABA) is a sesquiterpenoid synthesized from xanthophylls (Taylor, Burbidge & Thompson 2000; Seo & Koshihara 2002) and appears to influence several physiological and developmental events (Zeevaert & Creelman 1988; Kende & Zeevaert 1997; Busk & Pages 1998; Leung & Giraudat 1998; Seo & Koshihara 2002). The level of ABA in plants increases upon their exposure to environmental stress (Zeevaert & Creelman 1988; Xu *et al.* 1995), such as drought (Cowan, Turner & Botha 1995; Imai, Moses & Bray 1995; Zhu, Hasegawa & Bressan 1997), high temperature (Ward & Lawlor 1990), low temperature (Lee, Lur & Chu 1993; Ishitani *et al.* 1997), salinity (LaRosa *et al.* 1987), and flooding (Zhang & Zhang 1994). ABA significantly increases freezing (Guy 1990), chilling (Lee *et al.* 1993), drought (Zeevaert & Creelman 1988), and salt tolerance (LaRosa *et al.* 1987) in several plant species. It appears that ABA is a general endogenous

inducer of tolerance to environment stresses (Mantyla, Lang & Palva 1995).

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 effluent from industries, mining burning and leakage 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 & Kao 1995). Cd damages the photosynthetic apparatus (Krupa 1988; Siedlecka & Baszynski 1993), lowers chlorophyll content (Stobart *et al.* 1985; Larsson, Bordman & Asp 1998), and inhibits the stomatal opening (Barcelo & Poschenrieder 1990).

In Taiwan, inappropriate disposal of industrial waste has given rise to widespread Cd contamination of irrigated water (higher than 10 p.p.m.). Thus, there is urgent need to study the mechanism of Cd tolerance of rice plants. Although increased levels of ABA have been found in plants exposed to Cd, no correlation between ABA and Cd tolerance could be established (Barcelo, Cabot & Poschenrieder 1986; Poschenrieder, Gunse & Barcelo 1989). Our preliminary observations demonstrated that rice seedlings of cultivar Tainung 67 (TNG67) are more tolerant to Cd than those of cultivar Taichung Native 1 (TN1). It appears that these two cultivars of rice seedlings with different tolerance to Cd provide a good system to study mechanism of Cd tolerance of rice plants. In the present investigation, we shall examine the role of ABA in Cd tolerance of rice seedlings.

MATERIALS AND METHODS

Plant cultivation and treatment

Two rice (*Oryza sativa* L.) cultivars, an Indica type cultivar, Taichung Native 1 (TN1) and a Japonica cultivar, Tainung 67 (TNG67) were used in this study. 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 under dark condition. 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 & Lee 1989). The concentrations of N, P, K, S, Ca and Mg in half-strength Kimura B solution are 11.5, 2.9, 7.2, 15.0, 7.4 and 8.7 p.p.m.,

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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, ABA, and fluridone treatments, CdCl₂, ABA and fluridone 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% HNO₃ and 17.5% H₂O₂ 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 is expressed on the basis of dry weight (DW).

Determination of chlorophyll, protein, and proline

Chlorophyll content was determined according to Wintermans & 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 supernatant were used for determination by the method of Bradford (1976). Proline was extracted and its concentration determined by the method of Bates, Waldren & Teare (1973). Chlorophyll, protein, and proline contents are expressed on the basis of initial fresh weight (FW).

ABA determination

For extraction of ABA, leaves or roots were homogenized with a mortar and pestle 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 cartridges. The eluates were concentrated to dryness by vacuum-evaporation and re-suspended 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, USA) is specific for (+)-ABA. By evaluating ³H-ABA recovery, ABA loss was less than 3% by the method described here. ABA content is expressed on the basis of initial FW.

Transpiration rate

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

RESULTS

Evaluation of Cd tolerance

In plants, the most general symptom of Cd toxicity is chlorosis (Das, Samantaray & Rout 1997). When seedlings were treated with 0.5 mM CdCl₂ for 7 d, chlorosis was observed in TN1 seedlings, but not in TNG67 seedlings (data not shown). In short-term (3 d) experiments, chlorosis is first observed in the second leaf of TN1 seedlings. Thus, Cd toxicity in the second leaf caused by excess Cd was assessed by a decrease in chlorophyll and protein contents. Increasing concentration of CdCl₂ from 0.5 to 1.5 mM progressively decreased chlorophyll and protein contents in leaves of TN1 seedlings (Fig. 1). However, the decrease in chlorophyll and protein contents in leaves of TNG67 seedlings was less pronounced than those of TN1 seedlings (Fig. 1). Figure 2 shows the time courses of chlorophyll and protein contents in leaves of TN1 and TNG67 seedlings treated with or without CdCl₂ (0.5 mM). It is apparent that the decrease in chlorophyll and protein contents in TN1 leaves is more pronounced than that in TNG67 leaves. All these results suggest that TNG67 seedlings are Cd-tolerant cultivar. When the effect of Cd on biomass production was considered (Fig. 3), TNG67 seedlings are also more tolerant to Cd than TN1.

Water and proline contents

Proline is known to accumulate in leaves under water deficit (Hanson & Hitz 1982). Figure 4 shows the changes in

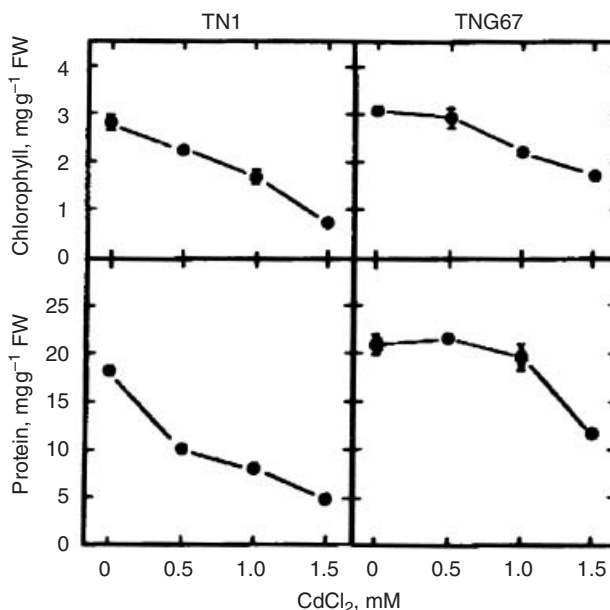


Figure 1. Effect of CdCl₂ on chlorophyll and protein contents in the second leaf of rice seedlings. Chlorophyll and protein contents were measured after 2 d of treatment. Data are means (± SE) of four replicates of a single typical experiment. Three series of independent experiments were carried out giving reproducible results.

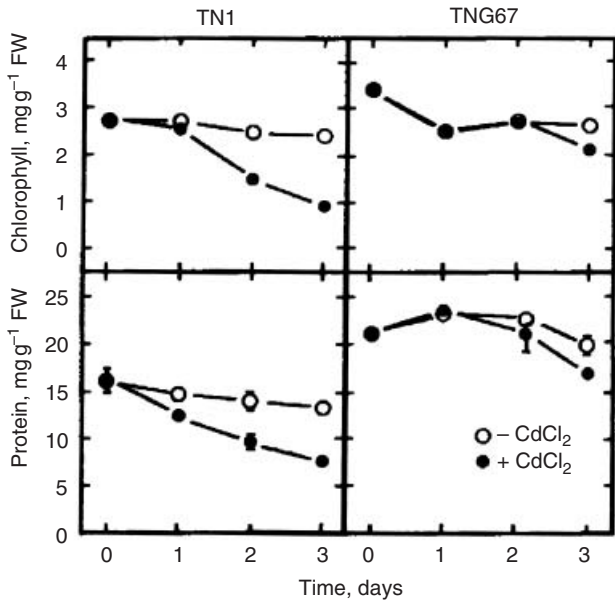


Figure 2. Changes in chlorophyll and protein contents in the second leaf of rice seedlings treated with or without CdCl₂ (0.5 mM). Data are means (± SE) of four replicates of a single typical experiment. Three series of independent experiments were carried out giving reproducible results.

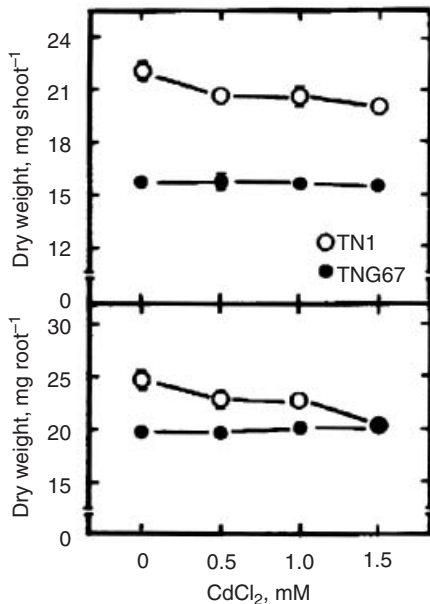


Figure 3. Effect of CdCl₂ on dry weight of shoot and root of rice seedlings. Dry weight was measured after 2 d of treatment. Data are means (± SE) of four replicates of a single typical experiment. Three series of independent experiments were carried out giving reproducible results.

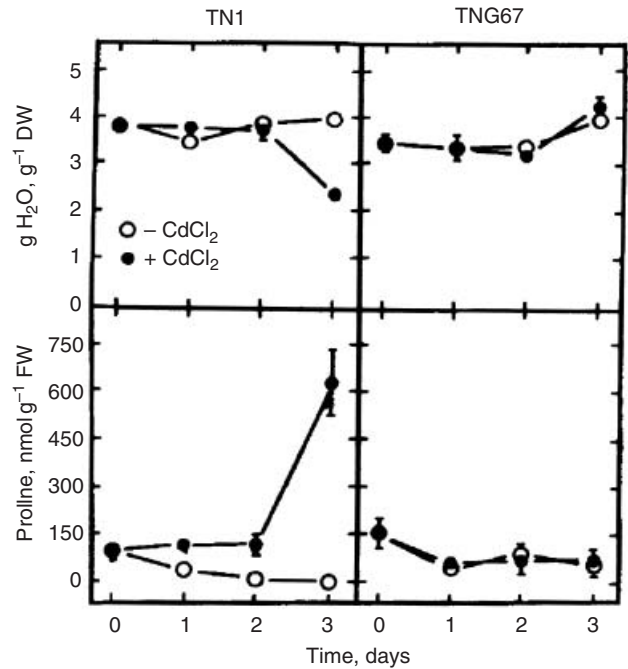


Figure 4. Changes in water and proline contents in the second leaf of rice seedlings treated with or without CdCl₂ (0.5 mM). Data are means (± SE) of four replicates of a single typical experiment. Three series of independent experiments were carried out giving reproducible results.

water and proline contents in leaves of Cd-treated seedlings. Water and proline contents in TNG67 leaves remained unchanged after Cd treatment. In contrast, a marked reduction of water content and an accumulation of proline were observed in TN1 leaves after 3 d of Cd treatment.

ABA accumulation in leaves and roots

Changes in endogenous ABA in leaves and roots of seedlings treated with or without CdCl₂ are shown in Fig. 5. In TNG67 leaves, ABA content increased with the increase of Cd treatment duration. ABA content in leaves of TN1 seedlings remained unchanged during the first two days of Cd treatment and slightly increased subsequently. A more pronounced ABA increase caused by CdCl₂ was also observed in TNG67 roots than in TN1 roots. It is evident that ABA accumulates more rapidly and is greater in TNG67 seedlings than in TN1 seedlings in response to CdCl₂.

Pretreatment with ABA

If ABA plays an important role in Cd tolerance, then pretreatment of TN1 seedlings with ABA is expected to counteract Cd-induced toxicity. Since some chlorosis symptom was visually observed in the second leaf of TN1 seedlings treated with ABA for 2 d, the third leaf was used to evaluate the effect of ABA pretreatment on the Cd toxicity of TN1 seedlings. Endogenous ABA content significantly

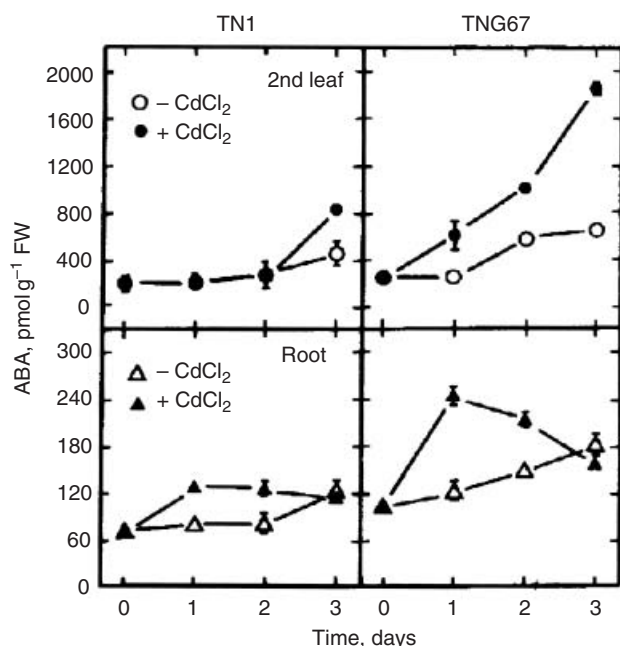


Figure 5. Changes in ABA content in the second leaf and roots of rice seedlings treated with or without CdCl_2 (0.5 mM). Data are means (\pm SE) of four replicates of a single typical experiment. Three series of independent experiments were carried out giving reproducible results.

increased in the third leaf, shoot and roots of TN1 seedlings treated with ABA (Fig. 6). When these ABA-pretreated TN1 seedlings were transferred to nutrient solution containing 1.5 mM CdCl_2 , Cd toxicity in the third leaf was significantly reduced (Fig. 7). However, ABA pretreatment had no effect on Cd toxicity in the third leaf of TNG67 over the time course of these experiments (data not shown).

Fluridone treatment

The role of ABA in Cd tolerance was further tested by using an inhibitor of ABA biosynthesis, fluridone, which blocks the conversion of phytoene to phytofluene in carotenoid biosynthesis pathway (Kowalczyk-Schröder & Sandmann 1992). Fluridone was observed to inhibit the increase in ABA content and enhance Cd toxicity in leaves of TNG67 seedlings (Fig. 8). Table 1 also shows that the effect of fluridone on Cd toxicity in leaves of TNG67 seedlings can be rescued by the application of ABA. These results further strengthened the role of ABA on Cd tolerance of rice seedlings. Fluridone treatment, however, did not enhance Cd toxicity in leaves of TN1 seedlings (data not shown).

Cd content

Figure 9 shows the changes in Cd content in rice seedlings treated with CdCl_2 . The Cd content in TNG67 leaves remained unchanged after Cd treatment. In contrast, a

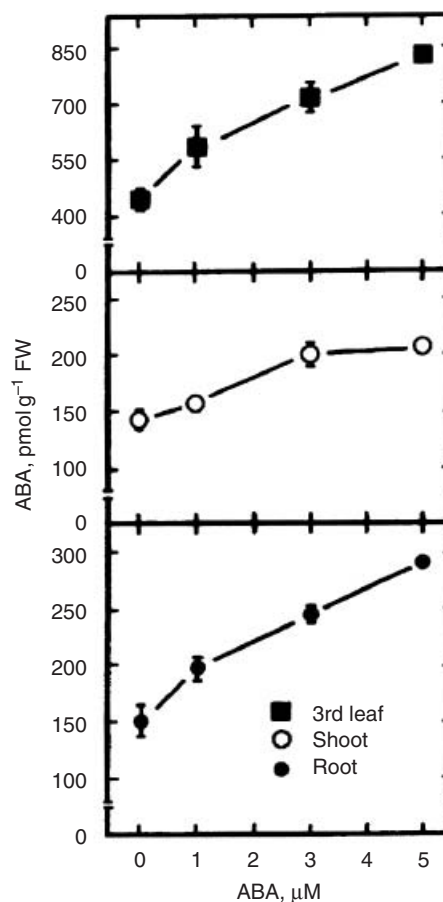


Figure 6. Effect of exogenously applied ABA on the content of endogenous ABA in the third leaf, shoot and roots of TN1 rice seedlings. ABA content was measured after 2 d of treatment. Data are means (\pm SE) of four replicates of a single typical experiment. Three series of independent experiments were carried out giving reproducible results.

Table 1. Effect of fluridone (Flu, 0.2 mM) on chlorophyll and protein contents in the second leaf of TNG67 rice seedlings treated with CdCl_2 (0.5 mM) and various concentrations of ABA

Treatment			Chlorophyll (mg g^{-1} FW)	Protein (mg g^{-1} FW)
CdCl_2	Flu	ABA (μM)		
-	-	0	2.51 ± 0.08	19.0 ± 0.8
-	+	0	2.51 ± 0.03	20.5 ± 1.1
+	-	0	2.47 ± 0.05	18.3 ± 0.5
+	+	0	1.58 ± 0.08	11.1 ± 0.5
+	+	2	2.09 ± 0.13	13.4 ± 0.8
+	+	4	2.62 ± 0.03	17.8 ± 1.5
+	+	6	2.73 ± 0.06	20.8 ± 1.0
+	+	8	2.64 ± 0.07	19.2 ± 1.0
+	+	10	2.74 ± 0.10	17.5 ± 1.8

Chlorophyll and protein contents were measured after 2 d of treatment. Data are means (\pm SE) of four replicates of a single typical experiment. Three series of independent experiments were carried out giving reproducible results.

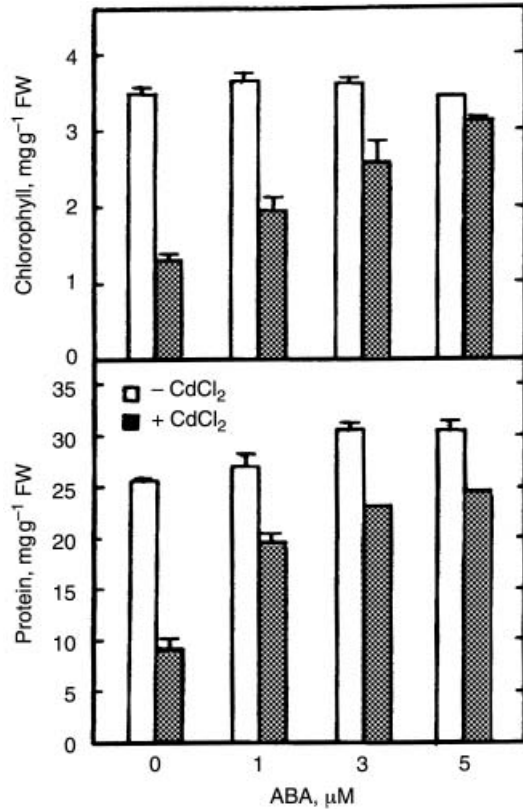


Figure 7. Effect of ABA-pretreatment on chlorophyll and protein contents in the third leaf of TN1 rice seedlings treated with or without CdCl₂ (1.5 mM). TN1 rice seedlings were pretreated with ABA for 2 d and then treated with or without CdCl₂ (1.5 mM) for 2 d. Data are means (\pm SE) of four replicates of a single typical experiment. Three series of independent experiments were carried out giving reproducible results.

marked increase in Cd content in Cd-treated TN1 leaves was observed. The increase in Cd content was greater in TN1 roots than in TNG67 roots. ABA pretreatment resulted in a decrease in Cd content in TN1 seedlings (Table 2) and fluridone caused an increase in Cd content in TNG67 seedlings (Table 3).

Table 2. Effect of ABA-pretreatment of Cd content in the third leaf and roots of TN1 rice seedlings treated with CdCl₂ (1.5 mM)

Pretreatment ABA	Treatment CdCl ₂	Cd (mg g ⁻¹ DW)	
		Third leaf	Roots
-	-	0.20 \pm 0.02	2.04 \pm 0.25
-	+	8.58 \pm 0.96	743.1 \pm 97.8
+	+	1.46 \pm 0.93	415.7 \pm 54.3

TN1 rice seedlings were pretreated with ABA (5 μ M) for 2 d and then treated with CdCl₂ (1.5 mM). Cd content was measured after 2 d of treatment. Data are means (\pm SE) of four replicates of a single typical experiment. Three series of independent experiments were carried out giving reproducible results.

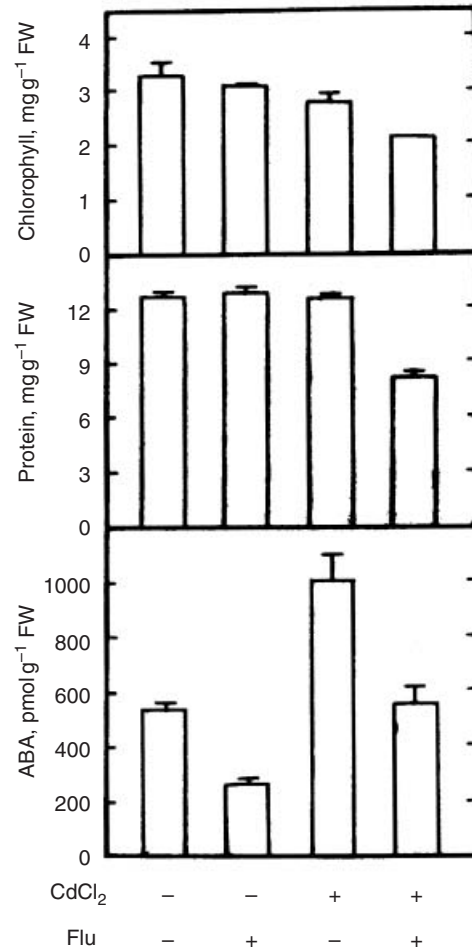


Figure 8. Effect of fluridone (Flu, 0.2 mM) on the contents of ABA, chlorophyll, and protein in the second leaf of TNG67 rice seedlings treated with or without CdCl₂ (0.5 mM). The contents of ABA, chlorophyll, and protein were measured after 2 d of treatments. Data are means (\pm SE) of four replicates of a single typical experiment. Three series of independent experiments were carried out giving reproducible results.

Transpiration rate

Transpiration rate of TN1 seedlings was observed to be higher than that of TNG67 seedlings (Table 4). Cd treatment decreased transpiration rate in both TN1 and TNG67 seedlings (Table 4). However, the decrease of transpiration rate in response to CdCl₂ was less pronounced in TN1 than TNG67 seedlings (Table 4). Fluridone treatment resulted in an increase in transpiration rate in Cd-treated TNG67, whereas ABA pretreatment caused a decrease in transpiration rate in Cd-treated TN1 (Table 4).

Effect of lower CdCl₂ concentrations

The concentrations of CdCl₂ used in the aforementioned study were ranged from 0.5 to 1.5 mM CdCl₂. We also conducted experiments with lower CdCl₂ concentrations, in the range 5–50 μ M, applied over a longer period (6 d). In-

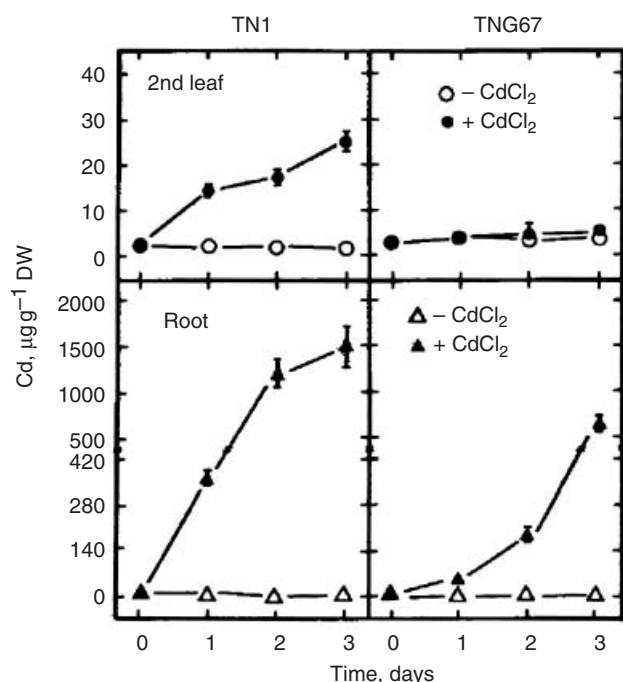


Figure 9. Changes in Cd content in the second leaf and roots of rice seedlings treated with or without CdCl₂ (0.5 mM). Data are means (\pm SE) of four replicates of a single typical experiment. Three series of independent experiments were carried out giving reproducible results.

ing concentrations of CdCl₂ from 5 to 50 μ M progressively decreased chlorophyll and protein contents in the second leaf of TN1 but not in TNG67 seedlings (Fig. 10). Compared with TNG67 seedlings, higher Cd content and lower ABA content in the second leaf of TN1 seedlings occurred between 5 and 50 μ M CdCl₂ (Fig. 10). Thus, the responses to lower CdCl₂ concentrations are basically in accordance with those to higher CdCl₂ concentrations.

DISCUSSION

The present study indicated that ABA is involved in Cd

Table 3. Effect of fluridone (Flu, 0.2 mM) on Cd content in the second leaf and roots of TNG67 rice seedlings treated with or without CdCl₂ (0.5 mM)

Treatment		Cd (μ g g ⁻¹ DW)	
CdCl ₂	Flu	Second leaf	Roots
-	-	1.38 \pm 0.28	1.36 \pm 0.23
-	+	5.24 \pm 0.52	4.28 \pm 1.43
+	-	8.51 \pm 1.35	107.0 \pm 6.8
+	+	67.2 \pm 10.0	132.6 \pm 6.0

Cd content was measured after 2 d of treatment. Data are means (\pm SE) of four replicates of a single typical experiment. Three series of independent experiments were carried out giving reproducible results.

Table 4. Effect of CdCl₂, fluridone (Flu, 0.2 mM) and ABA pretreatment on transpiration rate of rice seedlings

Cultivar	Pretreatment ABA	Treatment		Treatment rate (g H ₂ O d ⁻¹ seedling ⁻¹)
		Flu	CdCl ₂	
TN1	-	-	-	0.64 \pm 0.01
TN1	-	-	+	0.34 \pm 0.02
TNG67	-	-	-	0.44 \pm 0.02
TNG67	-	-	+	0.15 \pm 0.01
TN1	-	+	+	0.23 \pm 0.02
TN1	-	-	+	0.56 \pm 0.02
TN1	+	-	+	0.11 \pm 0.02

For CdCl₂ and fluridone experiments, CdCl₂ (0.5 mM) was applied with or without Flu. For the experiments of ABA pretreatment, TN1 rice seedlings were pretreated with ABA (5 μ M) for 2 d and then treated with CdCl₂. Transpiration rate was measured after 1 d of treatment. Data are means (\pm SE) of four replicates of a single typical experiment. Three series of independent experiments were carried out giving reproducible results.

tolerance of rice seedlings. This conclusion was based on observations that (a) the increase in endogenous ABA content in Cd-tolerant cultivar (TNG67) was more pronounced than that in Cd-sensitive cultivar (TN1) (Figs 5 & 10); (b) exogenous application of ABA caused an increase in Cd tolerance of TN1 (Fig. 7); (c) fluridone treatment reduced ABA content, as well as Cd tolerance of TNG 67 seedlings (Fig. 8); and (d) the effect of fluridone on Cd toxicity of TNG 67 seedlings can be rescued by the application of ABA (Table 1). The results suggest that the regulation of endogenous ABA biosynthesis under Cd stress is causally correlated to the tolerance of rice seedlings. Since

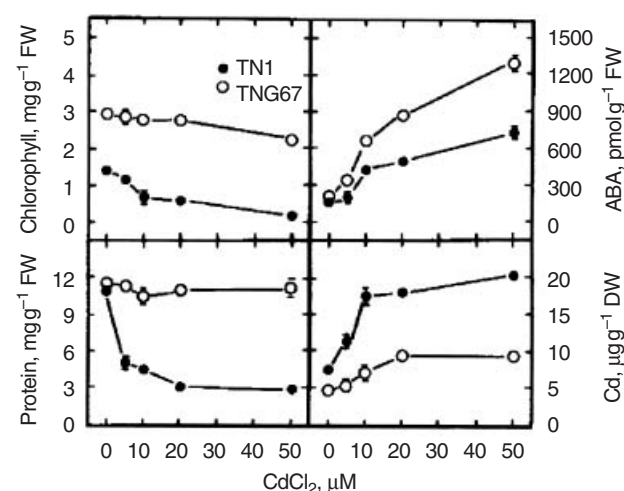


Figure 10. Effect of lower CdCl₂ concentrations on chlorophyll, protein, ABA, and Cd contents in the second leaf of rice seedlings. All measurements were made after 6 d of treatment. Data are means (\pm SE) of four replicates of a single typical experiment. Three series of independent experiments were carried out giving reproducible results.

fluridone is an inhibitor of ABA biosynthesis through the carotenoid pathway (Kowalczyk-Schröder & Sandmann 1992), the effects of this inhibitor on TNG67 may imply that ABA biosynthetic pathway in response to Cd appears to be the same as that established in other stress conditions (Zeevaart & Creelman 1988; Seo & Koshiha 2002). In addition, the defect of ABA accumulation in TN1 may account for the Cd intolerance of the cultivar. ABA is known to increase rapidly under water deficit (Hsiao 1973). Since no water deficit was observed in leaves of TNG67 treated with Cd (Fig. 4), it is unlikely that Cd-induced ABA accumulation in leaves of TNG67 is caused by water deficit.

There was an increase in ABA content in Cd-treated TNG67 leaves, which did not result in proline accumulation, conversely in Cd-treated TN1 leaves proline increased but not ABA (Figs 4 & 5). This is in contrast with the generally accepted idea that ABA triggers proline accumulation (Aspinall & Paleg 1981). On treatment with CdCl₂, protein content decreased with a progressive and substantial increase in protease activity and total amino acids in TN1 leaves but not in TNG67 (data not shown). It appears that protein degradation, less proline utilization and/or less proline translocation may contribute to the accumulation of proline in Cd-treated TN1 leaves. Since Cd toxicity in TN1 seedlings preceded the decrease in water content and the increase in proline content (Figs 2 & 4), it is unlikely that Cd toxicity observed in TN1 is mediated through water deficit.

Plants have a range of potential mechanisms at the cellular level that might be involved in the 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 the damaging effects (Hall 2002). In this connection, a reduced translocation of Cd to the shoot appears to be the possible mechanism of Cd tolerance in the shoot. Cd translocation to shoot has been suggested to be driven by transpiration (Salt *et al.* 1995). TN1 seedlings have significantly larger leaves and more stomata per unit leaf area than TNG67 seedlings (Lin, Lur & Chu 2001), which may lead to higher transpiration rate in TN1 leaves (Table 4). Cd has been shown to decrease transpiration rate in several plants (Kirkham 1978; Lamoreaux & Chaney 1978; Hagemeyer, Kahle & Breckle 1986; Schlegel, Godbold & Hütermann 1987). We also observed that Cd decreased transpiration rate of both cultivars (Table 4). Cd treatment reduced transpiration rate in TN1 and TNG67 to about 45 and 65% of the control value, respectively (Table 4). Thus, the reduction of transpiration rate of TN1 seedlings caused by Cd, which is unable to accumulate ABA, was less than that of TNG67 seedlings, which accumulate ABA, and consequently resulted in higher Cd content in TN1 than in TNG67 seedlings (Table 4, Figs 9 & 10). Exogenous ABA application could result in the decrease in transpiration rate (Table 4), and Cd content (Table 2), and increase in Cd tolerance of TN1 seedlings (Fig. 7). The fluridone effects on the tolerant cultivar further indicated that, not only was ABA biosynthesis

blocked, but that transpiration rate and Cd content were increased (Tables 3 & 4).

In conclusion, we provide evidence that the increase of endogenous ABA content is closely related to Cd tolerance of rice seedlings. ABA may exert its regulatory effect on transpiration rate, which reduces the translocation of Cd to the shoot.

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