Distinct roles of abscisic acid in rice seedlings during cadmium stress at high temperature

Yi Ting HSU and Ching Huei KAO*

Department of Agronomy, National Taiwan University, Taipei, Taiwan, Republic of China

(Received November 16, 2007; Accepted May 7, 2008)

ABSTRACT. Cd toxicity was judged by the decrease in chlorophyll and protein contents. Twelve-day-old seedlings of rice cultivars [Taichung Native 1 (TN1) and Taichung Native 1 (TN1)] were treated with or without CdCl₂ at high temperature (35/30°C day/night). The results indicated that at high temperature, TNG 67 seedlings are a Cd-tolerant cultivar while TN1 seedlings are Cd-sensitive. On treatment with CdCl₂, the abscisic acid (ABA) contents increased in the leaves of both TNG67 and TN1 seedlings grown at high temperature. Fluridone (Flu), an inhibitor of carotenoid biosynthesis, treatment, reduced ABA accumulation, increased transpiration rate and Cd content, and decreased Cd tolerance of TNG67 seedlings grown at high temperature. Flu’s effect on Cd toxicity of TNG 67 seedlings was reversed by the application of ABA. For TN1 rice seedlings grown at high temperature, Flu treatment resulted in less Cd-induced ABA accumulation, as well as toxicity. These Flu effects were reversible by application of ABA. However, Flu treatment did not reduce Cd content in the leaves of TN1 seedlings grown at high temperature. Exogenous application of ABA at high temperature provoked chlorosis, a symptom of Cd toxicity, in the leaves of TN1, but not in TNG67 seedlings. Suggested roles for endogenous ABA in Cd tolerance of TNG67 seedlings and Cd toxicity of TN1 seedlings are discussed.

Keywords: Abscisic acid; Cadmium; High temperature; Oryza sativa.

INTRODUCTION

Cadmium (Cd) is a pollutant and its presence in the environment is essentially due to anthropogenic activities (Sanitá di Toppi and Gabbirelli, 1999). Major sources of Cd pollution are industrial processes and phosphate fertilizers (Pinot et al., 2000). Because of its long biological half-life, Cd, which belongs to the group of non-essential transition metals, is highly toxic. Taken up in excess by plants, Cd directly or indirectly inhibits physiological processes, such as respiration, photosynthesis, cell elongation, plant-water-relationship, nitrogen metabolism, and mineral nutrition, resulting in poor growth and low biomass (Sanitá di Toppi and Gabbirelli, 1999; Kuo and Kao, 2004; Sun et al., 2007).

Global warming, accompanied an increased frequency of periods with exceptionally high temperature, is one of the most important characteristics of the accelerated climatic changes. Global surface temperatures are projected to increase by 1.4 to 5.8°C by 2100, in relation to rising concentrations of greenhouse gases in the atmosphere (Houghton et al., 2001; Cui et al., 2006). 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 to 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) to wheat seedlings increases parallel to temperature increases (Oncel 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 Backett, 1996). The high toxicity of Cd induced by high temperature may be a result of much more Cd uptake from the medium.

The plant hormone abscisic acid (ABA) is a sesquiterpenoid derived from xanthophyll (Seo and Koshiba, 2002; Hsu and Kao, 2004; Nambara and Marion-Poll, 2005; Zhang et al., 2005) and appears to influence several physiological and developmental events (Zeevaart and Creelman, 1988; Seo and Koshiba, 2002). Heavy metals such as Cd, Ni, Zn, and Al (Raiser and Dumbroff, 1981; Poschenrieder et al., 1989; Hollenbach et al., 1997; Foy, 1998; Fediuc et al., 2005) have been shown to increase ABA contents in plants. Fediuc et al. (2005) demonstrated that Cd-induced ABA accumulation was observed in roots, but not in shoots, of Typha and Phragmites plants.
Plants grown in the field may encounter several abiotic stresses, rather than a single stress. Recently, Mittler (2006) emphasized the importance of focusing the research programs on the response of plants to a combination of two different abiotic stresses. Previously, we have shown that, on treatment with CdCl₂, the ABA content increased in the leaves of the rice seedling leaves of the Tainung 67 cultivar (TNG67) but not in Taichung Native 1 (TN1) grown at normal temperature (30/25°C, day/night) (Hsu and Kao, 2003). Our recent work demonstrated that Cd treatment resulted in an increase in ABA content in the leaves of TN1 seedlings grown at high temperature (35/30 °C, day/night) (Hsu et al., 2006). It is not known whether CdCl₂ also increases ABA content in TNG67 leaves at high temperature. In this work, we shall examine the roles of endogenous ABA in TN1 and TNG67 seedlings during Cd stress at high temperature.

**MATERIALS AND METHODS**

**Plant materials and treatments**

Rice (*Oryza sativa* L., cv. TN1 and TNG67) 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 μM (NH₄)₂SO₄, 91.6 μM KNO₃, 273.9 μM MgSO₄·7H₂O, 91.1 μM KH₂PO₄, 182.5 μM Ca(NO₃)₂, 30.6 μM Fe·citrate, 0.25 μM H₂BO₃, 0.2 μM MnSO₄·H₂O, 0.2 μM ZnSO₄·7H₂O, 0.05 μM CuSO₄·5H₂O, and 0.07 μM H₂MoO₄. Kimura B solution contains the desired nutrient elements (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 μg ml⁻¹) and has been widely used for growing rice plants. Since young rice seedlings were used for the present study, the nutrient solution contained no silicon, although silicon is essential for rice. 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 moved to a Phytotron with temperature controlled at 35/30°C (day/night) and grown in the Kimura B solution with or without 0.5 mM or 30 μM CdCl₂. For the experiments in which the effect of exogenous ABA was examined, various concentrations of ABA (mixed isomers, 5-40 μM) were added directly to the Kimura B solution. In experiments to understand the role of endogenous ABA, 0.2 mM fludione (Flu), which is known to block the conversion of phytoene to phytofluene in the carotenoid biosynthesis pathway (Kowalczyk-Schröder and Sandmann, 1992), was added directly to the Kimura B solution.

**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% HNO₃ and 17.5% H₂O₂ 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). Cd amounts are expressed on a dry weight (DW) basis.

**Determination of chlorophyll, protein, and ABA**

Chlorophyll content was determined according to Wintermans and De Mots (1965) after extraction in 96% (v/v) ethanol. For protein determination, leaves were homogenized in a 50 mM sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,600 g for 20 min, and the supernatants were used for determination by the method of Bradford (1976). Chlorophyll and protein contents are expressed on the basis of initial fresh weight (FW).

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). The ABA immunoassay detection kit (PGR-1) purchased from Sigma Chemical Co. (St. Louis, MO) is specific for (+)-ABA. By evaluating ³H-ABA recovery, ³H-ABA loss was less than 3% by the method described here. ABA content is expressed on the basis of FW.

**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 end of the 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**

Absolute levels of each measurement varied among experiments because of seasonal effects. However, the patterns of responses to CdCl₂ were reproducible. For all measurements, each treatment was performed four times. All experiments were performed at least thrice. Similar results and identical trends were obtained each time. The data reported here are from a single experiment. Statistical differences between measurements (n = 4) on different treatments or on different times were analyzed following LSD test.
RESULTS

Evaluation of Cd toxicity

In plants, the most general symptom of Cd toxicity is chlorosis (Das et al., 1997). In our previous work, we observed that chlorosis first occurred in the second leaves of TN1 seedlings treated with CdCl$_2$ (Hsu and Kao, 2003). Thus, in the present study, Cd toxicity in the second leaves by 0.5 mM CdCl$_2$ was assessed by decreases in chlorophyll and protein contents. A marked decrease in chlorophyll and protein was observed in TN1 seedlings after CdCl$_2$ treatment at high temperature (Figure 1A, B). However, Cd had a slight effect on reducing chlorophyll and protein contents in the second leaves of TNG67 seedlings at high temperature (Figure 1D, E).

ABA accumulation

CdCl$_2$ treatment resulted in a 4-fold increase in ABA content in the second leaves of both TN1 and TNG67 seedlings grown at high temperature (Figure 1C, F).

Fluridone effect

Fluridone (Flu) is known to block the conversion of phytoene to phytofluene in the carotenoid biosynthesis pathway (Kowalczyk-Schröder and Sandmann, 1992). When Flu was added to the nutrient solutions, reduction of Cd-induced ABA accumulation in the second leaves, and Cd tolerance of TNG67 seedlings grown at high temperature was observed (Figures 2B and 3B, D). The effect of Flu on the reduction of Cd tolerance of TNG67 seedlings at high temperature was reversed by exogenously applied ABA (Figure 3B, D). ABA per se did not affect chlorophyll and protein contents in TNG67 leaves at high temperature (Figure 3B, D).

Flu was also observed to inhibit Cd-induced ABA accumulation in the second leaves of TN1 seedlings at high temperature (Figure 3B, D).
high temperature (Figure 2A). However, Flu reduced Cd toxicity of TN1 seedlings at high temperature (Figure 3A, C). The effect of Flu on the reduction of Cd toxicity of TN1 seedlings grown at high temperature was reversed by adding ABA (Figure 3A, C). ABA treatment alone also clearly decreases chlorophyll and protein contents in leaves of TN1 seedlings at high temperature (Figure 3A, C). On treatment with CdCl$_2$, ABA content significantly increased in the leaves of both Cd-tolerant cultivar (TNG67) and Cd-sensitive cultivar (TN1) at high temperature (Figure 1C, F). Thus, seedlings treated with CdCl$_2$ + ABA were not included in experiments of Figure 3 or subsequent experiments (Figures 4 and 5).

**Cd concentration and transpiration rate**

Cd concentration in the second leaves of TN1 seedlings treated with Flu plus CdCl$_2$ at high temperature is similar to that with CdCl$_2$ alone (Figure 4A). In contrast, Flu caused an increase in Cd concentration in Cd-treated TNG67 seedlings (Figure 4B). The Flu effect on the increase in Cd concentration in leaves of TNG67 seedlings at high temperature was reversed by adding ABA (Figure 4B).

Cd has been shown to decrease transpiration rate in several plants (Kirkham, 1978; Lamoreaux and Chaney, 1978; Hagemeyer et al., 1986; Schlegel et al., 1987). We also observed that Cd decreased the transpiration rate of TN1 and TNG67 seedlings at high temperature (Figure 4C, D). Flu treatment resulted in an increase in transpiration rate in CdCl$_2$-treated TNG67 seedlings (Figure 4D). The Flu effect on the increase in transpiration rate at high temperature was reversed by exogenously applied ABA (Figure 4D). However, transpiration rate of TN1 seedlings treated with Flu plus CdCl$_2$ at high temperature is similar to that with CdCl$_2$ alone (Figure 4C).

**Effect of lower CdCl$_2$ concentrations**

The concentration of CdCl$_2$ used in the aforementioned study was 0.5 mM. We also conducted experiments with lower CdCl$_2$ (30 µM) applied over a longer period (6 days). At high temperature, CdCl$_2$ (30 µM) treatment resulted in an approximately 2-fold increase in ABA content in the second leaves of TN1 and TNG67 seedlings (Figure 5A, B). When Flu was added to the nutrient solutions, reduction of Cd-induced ABA accumulation in the second leaves and Cd tolerance of TNG67 seedlings at high temperature was observed (Figure 5B, D, F). The effect of Flu on the reduction of Cd tolerance of TNG67 seedlings to high temperature was reversed by exogenously applied

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**Figure 4.** Effect of fluridone (Flu, 0.2 mM) and ABA (5 µM) on Cd concentrations in the second leaves and the transpiration rate of TN1 (A, C) and TNG67 (B, D) rice seedlings treated with or without CdCl$_2$ (0.5 mM) at high temperature (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 $P < 0.05$.

**Figure 5.** Effects of fluridone (Flu, 0.2 mM) on the contents of ABA (A, B) and of fluridone and ABA (5 µM) on the contents of chlorophyll (C, D) and protein (E, F) in the second leaves of TN1 and TNG67 rice seedlings treated with or without low concentration of CdCl$_2$ (30 µM) for 6 days at high temperature (35/30°C). Bars indicate standard error ($n=4$). Values with the same letter are not significantly different at $P < 0.05$. 
ABA (Figure 5D, F). Flu was also observed to inhibit Cd-induced ABA accumulation in the second leaves of TN1 seedlings at high temperature (Figure 5A). However, it was observed to reduce Cd toxicity in leaves of TN1 seedlings at high temperature (Figure 5C, E). The effect of Flu on the reduction of Cd toxicity in the second leaves of TN1 seedlings grown at high temperature was reversed by adding ABA (Figure 5C, E). The contents of chlorophyll and protein were observed to be decreased by ABA alone (Figure 5C, E). Thus, the responses to lower CdCl$_2$ concentrations are basically in accordance with responses to higher ones.

**Exogenous application of ABA**

Figure 6 shows the effect of ABA concentrations, in the range from 5 to 40 μM, applied over a period of 2 days, on the chlorosis of the second leaves of rice seedlings at high temperature. It is clear that increasing concentration of ABA progressively promotes chlorosis of the second leaves of TN1, but not of TNG67 seedlings at high temperature. It appears that in terms of leaf chlorosis, TNG67 is ABA-insensitive and TN1 is ABA-sensitive.

**DISCUSSION**

The Cd concentration in the shoot increased at high temperature with increasing light intensity (Greger, 1999). In the present study, rice seedlings were grown in a Phytotron with natural sunlight at 30/25°C or 35/30°C. Under natural sunlight conditions, light intensity varied daily or seasonally. Based on our experience from the experiments of Cd effect on rice seedlings, consistent Cd toxicity was observed in 2 and 6 days, respectively, when 0.5 mM and 30 μM CdCl$_2$ were used. However, no consistent Cd toxicity of rice seedlings exposed to CdCl$_2$ concentrations lower than 30 μM was observed. For this reason in this study, 0.5 mM or 30 μM CdCl$_2$ were added directly to nutrient solution. In a study on 64 soils (urban, forest and agricultural soils) containing various levels of Cd contamination, free dissolved Cd concentrations ranged from 0.1 to 2,000 nM (Sauvé et al., 2000). Thus, the concentrations of Cd used in our experiments can be considered very high. Basically, rice seedlings in the present study were considered to be suffering from acute Cd toxicity.

In the absence of CdCl$_2$, the DW of shoot and roots of TN1 or TNG67 seedlings grown at normal temperatures for 2 days was similar to that at high temperatures (data not shown). High temperature per se does not seem to exert stress effects on TN1 or TNG67 seedlings.

In the present study, we evaluated Cd toxicity by the decrease in chlorophyll and protein contents. On the basis of these criteria, we demonstrated that at high temperature, TNG67 seedlings are a Cd-tolerant cultivar while TN1

![Figure 6](image.png)

**Figure 6.** Effect of ABA on chlorosis of the second leaves of TN1 and TNG67 rice seedlings grown at high temperature (35/30°C). Arrows indicated the second leaves, and pictures were taken 2 days after treatment.
seedlings are Cd-sensitive (Figure 1). It has also been described previously that TNG67 seedlings grown at normal temperature are more tolerant to Cd than TN1 (Hsu and Kao, 2003).

Plants have several 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 a build-up of toxic concentrations at sensitive sites within the cell and thus preventing damage (Hall, 2002). In this connection, a reduced translocation of Cd to the shoot appears to be possible mechanism of Cd tolerance in the shoot. Some hold this translocation to be driven by transpiration (Salt et al., 1995). The mechanism of Cd tolerance of TNG67 seedlings grown at high temperature is basically related to the inhibitory effect of ABA on transpiration rate and Cd uptake. This conclusion was based on observations: (i) ABA accumulated in the second leaves of TNG67 seedlings under Cd stress at high temperature (Figure 1F); (ii) Flu treatment led to a decrease in the ABA content (Figure 2B), an increase in transpiration rate and Cd concentration (Figure 5B, D), and decrease in Cd tolerance of TNG67 seedlings treated with CdCl₂ at high temperature (Figure 3B, D); and (iii) the effects of Flu on the transpiration, Cd concentration, and Cd toxicity of TNG67 seedlings grown at high temperature were reversed by application of ABA (Figures 3B, D and 4B, D). Cd tolerance of TNG67 seedlings grown at high temperature appears to be mediated through ABA-induced inhibition of Cd uptake.

In contrast, we show that ABA is involved in Cd toxicity of TN1 seedlings grown at high temperature. This conclusion was based on observations that (i) the increase in endogenous ABA content in response to Cd in leaves of rice seedlings was observed at high temperature (Figure 1C); (ii) exogenous application of ABA to TN1 seedlings at high temperature increased ABA content (Hsu et al., 2006) and resulted in chlorophyll loss (Figures 3A and 5C) and chlorosis (Figure 6); (iii) Flu treatment reduced the ABA content, as well as the Cd toxicity to TN1 seedlings grown at high temperature (Figures 2A, 3A, C, and 5C, E); and (iv) the effect of Flu on the Cd toxicity of TN1 seedlings grown at high temperature can be reversed by the application of ABA (Figures 3A, C and 5C, E).

The fact that Flu treatment reduced ABA content, but did not reduce Cd content in the second leaves and transpiration rate of TN1 seedlings in response to CdCl₂ at high temperature (Figures 1A and 4A) suggests that Flu effect on the reduction of Cd toxicity is attributable to reduction of ABA but not Cd concentration in the second leaves and transpiration rate of TN1 seedlings grown at high temperature. These results strengthen further our conclusion that Cd toxicity of TN1 seedlings at high temperature is mediated through ABA accumulation. 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 the case of Arabidopsis growth (root length and seedling fresh weight), the magnitude of Cd-induced inhibition in ABA-insensitive mutant was generally comparable to that in the wild type (Sharma and Kumar, 2002).

Cd is known to inhibit the transpiration rate of several plants (Lamoreaux and Chaney, 1978; Hagemeyer et al., 1986; Schlegel et al., 1987). The fact that Flu treatment had no effect on the transpiration rate of TN1 seedlings in response to CdCl₂ at high temperature suggests that the decrease in transpiration rate caused by CdCl₂ is not attributable to increases in ABA but to increases in Cd concentration (Figure 5A).

At high temperature, ABA increased at about the same magnitude (about 4-fold) in the leaves of Cd-tolerant TNG67 and Cd-sensitive TN1 seedlings in response to Cd (Figure 1C, F). Clearly, ABA may exert distinct roles during Cd stress. In terms of chlorophyll and protein loss and chlorosis, we observed that rice seedlings of cultivar TN1 are ABA-sensitive, and those of cultivar TNG67 are ABA-insensitive (Figures 3A-D and 6). This would explain why ABA accumulating in leaves of TNG67 seedlings grown at high temperature plays a protective role against Cd stress, while the leaves of TN1 seedlings show toxic effects of Cd stress. In our previous work, we showed that at normal temperatures CdCl₂ treatment resulted in ABA content increases in leaves of TNG67 but not in TN1 (Hsu and Kao, 2003). The data of the present study together with previous work (Hsu and Kao, 2003) clearly indicate that ABA could participate in regulation of Cd toxicity/tolerance of rice seedlings as diagrammatically shown in Figure 7.

It is well established that ABA is derived from xanthophylls (Nambara and Marion-Poll, 2005). Here, we show that ABA accumulation in rice leaves of both TN1 and TNG67 seedlings is induced by Cd at high temperature (Figure 1C, F). ABA in high plants is derived from C₄₀-carotenoid (Nambara and Marion-Poll, 2005). As Flu

![Figure 7. Regulation of Cd tolerance/toxicity by endogenous ABA in rice seedlings grown at normal and high temperatures.](image_url)
is an inhibitor of carotenoid biosynthesis (Kowalczyk-Schröder and Sandmann, 1992), the effect of this inhibitor on Cd-induced ABA accumulation in the second leaves of TN1 and TNG67 seedlings at high temperature may imply that ABA biosynthesis pathway in response to Cd at high temperature appears to be the same as that established in other stress conditions (Zeevaart and Creelman, 1988; Nambara and Poll, 2005).

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

LITERATURE CITED


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高溫下脫落酸在水稻幼苗鎘逆境所扮演之功能

許奕婷 高景輝

國立臺灣大學 農藝學系

氯化鎘在高溫（35/30℃）下處理中在來 1 號（TN1）與台農 67 號（TNG67）水稻幼苗，鎘毒害以葉綠素與蛋白質含量降低程度為指標。結果顯示 TN1 是不耐鎘品種，而 TNG67 是耐鎘品種。氯化鎘在高溫下處理 TN1 與 TNG67 幼苗，脫落酸含量明顯增加。類胡蘿蔔素合成抑制劑 Fluridone (Flu) 處理 TNG67 幼苗可降低鎘造成之脫落酸累積，增加蒸散速率與鎘含量以及增加鎘之毒害。外加脫落酸處理可克服 Flu 所增加之鎘毒害。而在高溫下 Flu 處理 TN1 幼苗可降低鎘所引起脫落酸之累積，以及鎘毒害。這些 Flu 之效應可被外加脫落酸所抵消。高溫下外加脫落酸處理可引起 TN1 幼苗之黃化現象（類似鎘毒害），但對 TNG67 幼苗不會造成黃化現象。文中對脫落酸在高溫鎘逆境下所扮演的角色做一詳細的討論。

關鍵詞：脫落酸；鎘；水稻；高溫。

Botanical Studies, Vol. 49, 2008