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Changes in protein and amino acid contents in two cultivars of rice seedlings with different apparent tolerance to cadmium

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Abstract

Changes in protein and amino acid contents in Cd-treated rice (*Oryza sativa* L.) seedlings of two cultivars were investigated. By assessing the decrease in chlorophyll content in the second leaves as an indicator of Cd toxicity, it was seen that cv. Tainung 67 (TNG 67) seedlings were apparently more tolerant to Cd than cv. Taichung Native 1 (TN 1). Following treatment with $CdCl_2$, protein content decreased with a progressive and substantial increase of protease activity and total amino acids in TN 1, but not in TNG 67. The patterns of individual amino acids in Cd-treated leaves of both cultivars were examined and, only in cv. TN 1 a substantial increase in the content of all amino acids analysed, except for methione, was recorded. The role of these changes in endogenous amino acids in Cd toxicity of TN 1 leaves is discussed.

Abbreviations: FW – initial fresh weight, RWC – relative water content, TN 1 – Taichung Native 1, TNG 67 – Tainung 67

Introduction

Cd is an important environmental pollutant with high toxicity to animals and plants. It is released into the environment by traffic, metal-working industries, mining, as a by-product of mineral fertilizers, and from other sources (Nriagu and Pacyna 1988). Cd is readily taken up by plants, leading to toxic symptoms such as growth reduction (Chen and Kao 1995).

Boussama et al. (1999) demonstrated that total protein content in Cd-treated barley plants declined with a progressive and substantial increase of protease activity in the tissue. These changes, similar to those occurring during natural senescence of plants (Hortensteiner and Feller 2002), suggest that Cd may induce premature senescence (Vassilev et al. 1997). Measurements of chlorophyll content and lipid peroxidation also support the idea that a premature senescence due to Cd treatment takes place in pea leaves (McCarthy et al. 2001). Nevertheless, other symptoms, characteristic of an overall senescence programme, such as a lower protein content and a higher protease activity, were not observed in Cd-treated pea leaves (McCarthy et al. 2001).

Amino acids and other soluble nitrogenous compounds play an essential role in plant metabolism, being the primary products of inorganic nitrogen assimilation and precursors of proteins and nucleic acids. Because of the importance of soluble nitrogenous compounds, there has been much interest in the influence of environmental stress on their metabolism. A common response of plants to environmental stress is an accumulation of amino acids. Plants appear to preferentially accumulate proline (Aspinall and Paleg 1981) in response to environmental stress, but also other amino acids especially those derived from aspartic acid, including asparagine, isoleucine, leucine, methionine, and valine (Fukutoku and Yamada 1981; Handa et al. 1983; Munns et al. 1979). However, there is little information on the amino acid content of Cd-stressed plants. It has been shown that Cd induced proline accumulation in sunflower and mung

bean (Kastori et al. 1992; Schat et al. 1997; Zhang et al. 2000). However, Costa and Morel (1994) showed that Cd induced no accumulation of proline in lettuce but induced specific increases in the levels of asparagine, methionine and lysine.

In Taiwan, because Cd poses a serious problem for rice production, there is urgent need to study the mechanisms of Cd tolerance of rice plants. Our preliminary observation demonstrated that rice seedlings of cv. Tainung 67 (TNG 67) are apparently more tolerant to Cd than those of cv. Taichung Native 1 (TN 1). It seems that these two cultivars of rice seedlings with different apparent tolerance to Cd provide a model system to study mechanisms of Cd tolerance of rice plants. In the present investigation, we examine the effect of Cd on the changes in protein and amino acid contents in leaves of TN 1 and TNG 67 seedlings.

Materials and methods

Two rice (*Oryza sativa* L.) cultivars, an Indica type cultivar, TN 1 and a Japonica cultivar, TNG 67 were used in this study. Seeds were sterilised with 2.5% sodium hypochlorite for 15 min, washed extensively with distilled water and then germinated in Petri dishes containing 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 as described previously (Chu and Lee 1989). The hydroponically cultivated seedlings were grown in a Phytotron 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. $CdCl_2$ was added directly to the culture solution during the experiment. The experimental period was limited to avoid effects of senescence and a relatively high concentration (0.5 mM) was used.

Chlorophyll was determined according to Wintermans and De Mots (1965) after extraction in 96% (v/v) ethanol. For protein determination, leaves were homogenised 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 of protein by the method of Bradford (1976) and for protease assay. Protease was assayed according to the method described by Sheoran and Garg (1978). One unit of protease activity was defined as a 0.01 increase of the absorbance at 280 nm per h.

Relative water content (RWC), defined as water content of leaf tissue as a percentage of that of the fully turgid tissue, was determined by the method of Weatherley (1950). For determination of amino acids, leaf samples were extracted with 2% sulfosalicylic acid and the homogenate was centrifuged at 15,000 *g* for 20 min. The supernatant was used directly for amino acid analysis (amino acid analyzer, Beckman 6300, Palo Alto, USA).

For ethylene production, the second leaves were excised and transferred to test tubes sealed with serum caps. After 3 h of incubation in the dark at 27 °C, a 1-mL gas sample was withdrawn from the headspace of the test tube. Ethylene was then assayed as described previously (Kao and Yang 1983). In an experiment with 1-methylcyclopropene, an inhibitor of ethylene action (Serek et al. 1994) rice seedlings were placed in glass chambers and exposed to 1 μL L^{-1} (v/v) of 1-mehtylcyclopropene for 24 h. After treatment, rice seedlings were given no further treatment or were treated with 0.5 mM $CdCl_2$. The effect of Cd toxicity was observed after 7 d of Cd treatment.

Chlorophyll, protein, amino acids and ethylene production were expressed on the basis of initial fresh weight (FW). Protease activity was expressed as units per mg protein. All experiments were repeated three times; within each experiment, treatments were replicated 4 times. The data reported here are averages of three independent experiments.

Results

In plants, the most apparent symptom of Cd toxicity is chlorosis of the leaves (Das et al. 1997). Figure 1 shows the effect of low concentrations of $CdCl_2$, in the range 5 to 50 μM, applied over a period of 6 d, on chlorophyll content of the second leaves of rice seedlings. It is clear that increasing concentration of $CdCl_2$ progressively decreased chlorophyll content in the second leaves of TN 1, but not in TNG 67 seedlings. When rice seedlings were treated with 0.5 mM $CdCl_2$ for 7 d, leaf chlorosis was also observed in TN 1 seedlings, but not in TNG 67 seedlings (data not shown). In short term (3 d) experiments, chlorosis was first observed in the second leaves of TN 1 seedlings. In the main experiment, in which 0.5 mM $CdCl_2$ was applied, the experimental period was limited to 3 d in order to avoid the effects of natural senescence. Figure 2 shows the changes in chlorophyll content in the second leaves of TN 1 and TNG 67

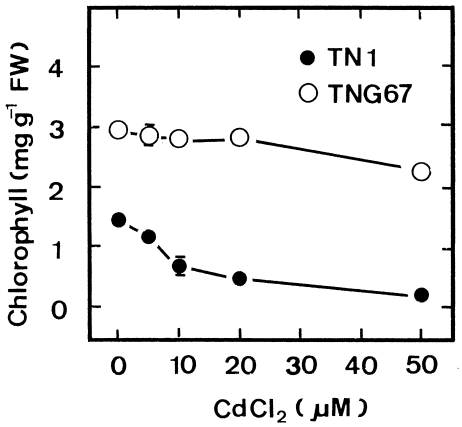

Figure 1. Effect of $CdCl_2$ concentrations on chlorophyll content in the second leaves of rice seedlings. Chlorophyll content was determined after 6 d of treatment. Vertical bars represent S.E. (n = 3).

seedlings, untreated or treated with $CdCl_2$. It is apparent that the decrease in chlorophyll was more pronounced in TN 1 leaves than in TNG 67 leaves, suggesting that TNG 67 seedlings are apparently more tolerant to Cd than TN 1 seedlings. The decrease in chlorophyll content in TN 1 leaves was evident after 2 d of Cd-treatment.

Figure 2 also shows the change in RWC in the second leaves of rice seedlings in the absence or present of 0.5 mM $CdCl_2$. RWC remained unchanged in TNG 67 leaves during the entire duration of Cd treatment. In contrast, a marked reduction of RWC was observed in TN 1 leaves after 3 d of Cd treatment.

When treated with $CdCl_2$, the protein content was markedly reduced in the second leaves of TN 1 seedlings but not in those of TNG 67 (Figure 3). The decrease in protein content in TN 1 leaves was already evident after 1 d of Cd treatment, indicating rapid protein degradation due to Cd treatment. This suggestion is supported by the observations that total amino acid content in Cd-treated TN 1 leaves increased with a progressive and substantial increase of protease activity in leaves (Figure 3).

Since total amino acid content increased in Cd-treated leaves of TN 1 but not in those of TNG 67, it was of great interest to identify the changes of individual amino acids, caused by Cd treatment. Consequently, we examined the changes of twenty amino acids in the second leaves of both cultivars (Figures 4, 5 and 6). Basically, Cd had no effect on the contents of amino acids or slightly increased amino acid contents (such as glutamine) in leaves of TNG 67 seedlings. Thus, the results from TN 1 leaves only are discussed the remainder of this section.

Plants assimilate inorganic nitrogen into glutamic acid, glutimine, aspartic acid, and asparagine. These compounds are used to transfer nitrogen from source organs to sink. The changes in these N-transport amino acids in Cd-treated leaves of TN 1 seedlings are presented in Figure 4. In general, the contents of these amino acids were higher in the leaves of treated seedlings throughout the experiments. The content of glutamic acid remained unchanged in Cd-treated TN 1 leaves, but was higher than that of leaves receiving no Cd. This indicates that glutamic acid is either less utilised or less transported in Cd-treated leaves of TN 1. The pattern of arginine change (Figure 5) was basically parallel to that of glutamic acid. In TN 1 leaves, the glutamine content increased during the entire duration of Cd treatment (Figure 4). The changes in the content of glycine, serine, histidine, tyrosine, phenylalanine, tryptophan, valine, leucine, isoleucine, lysine, and threonine (data not shown) closely resemble the variation in glutamine shown in Figure 4. Proline contents in Cd-treated TN 1 leaves remained unchanged during the first two days of Cd treatment but increased substantially after 3 d of Cd treatment (Figure 5). However, proline content in Cd-treated TN 1 leaves was still higher than that in control leaves (Figure 5) during the first two days of treatment, suggesting that proline in Cd-treated TN 1 leaves is either less transported or less utilised. The increase in ornithine content in TN 1 leaves was observed only after 3 d of Cd treatment (Figure 5). The change in alanine content in Cd-treated TN 1 leaves was quite similar to that of ornithine (Figure 5). Contrary to the changes in the contents of amino acids mentioned above, methionine content in TN 1 leaves was decreased by Cd treatment (Figure 6).

Methionine is known to be a precursor of ethylene biosynthesis (Yang and Hoffman 1984). The decrease in methionine content in TN 1 leaves may suggest that methionine was converted to ethylene. Thus, it was of great interest to determine the changes in ethylene production in rice seedlings untreated or treated with $CdCl_2$. Figure 7 shows that slightly higher ethylene production in Cd-treated TN 1 leaves than control leaves was observed only after 2 d of Cd treatment.

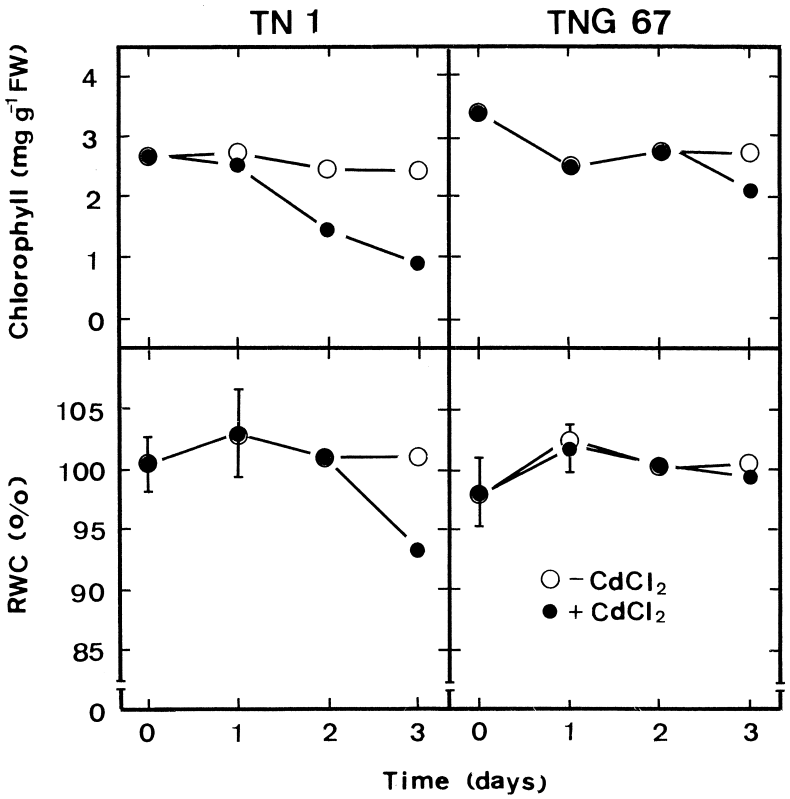

Figure 2. Changes in chlorophyll contents and RWC in the second leaves of rice seedlings untreated or treated with$CdCl_2$ (0.5 mM). Vertical bars represent S.E. (n = 3).

Discussion

Based on the change in chlorophyll content, we show that leaves of TN 1 seedlings were more sensitive to Cd than those of TNG 67 (Figures 1 and 2). Evidence is also provided to show that total protein content in Cd-treated TN 1 leaves decreased with progressive increase of protease activity in the leaves (Figure 3), suggesting that Cd may induce premature senescence of TN 1 leaves.

Metals are known to disrupt plant water relations. It has been shown that Cd reduces RWC of lettuce seedlings (Costa and Morel 1994). In the present investigation, reduction of RWC by Cd in TN 1 leaves occurred only after the appearance of Cd toxicity (Figure 2). Thus, Cd toxicity in TN 1 leaves is unlikely to be caused by water deficit.

It has been suggested that proline accumulation in plants in response to Cd is induced by a Cd-imposed increase in water deficit, rather than by a toxic Cd accumulation *per se* (Costa and Morel 1994; Schat et al. 1997). However, Kastori et al. (1992) observed proline accumulation in Cd-treated leaf disc and suggested that this accumulation was attributable to Cd uptake *per se*. In our work, we observed that proline accumulation in Cd-treated TN 1 leaves occurred at the time when RWC was reduced (Figures 2 and 5). Furthermore, our unpublished data show that the increase in Cd content occurred long before proline accumulation in Cd-treated TN 1 leaves. Thus, proline accumulation in response to Cd in TN 1 leaves is most likely due to water deficit. Costa and Morel (1994) have suggested that the functional significance of Cd-induced proline accumulation would lie in its contribution to water balance maintenance and that proline-mediated alleviation of water deficit could substantially contribute to the Cd tolerance of the plants. However, in our work, no proline accumulation was observed in Cd-tolerant TNG 67 leaves (Figure 5). Thus, the functional role of proline accumulation in Cd-treated TN 1 leaves remains to be elucidated.

Cd-induced proteolysis and accumulation of amino acids was observed to occur prior to Cd toxicity

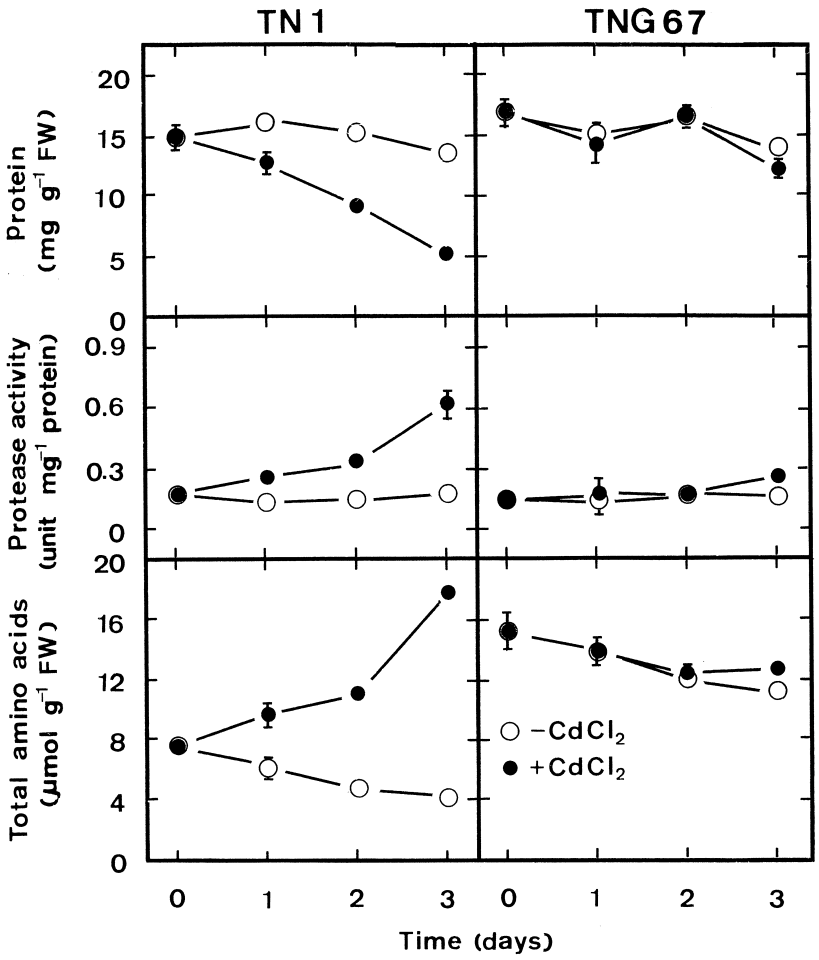

Figure 3. Changes in the contents of protein and total amino acids, and the activity of protease in the second leaves of rice seedlings untreated or treated with $CdCl_2$ (0.5 mM). Vertical bars represent S.E. (n = 3).

(chlorophyll degradation) in TN 1 leaves (Figures 2 and 3), suggesting that proteolysis or accumulation of amino acid might play some role in regulating Cd toxicity. Shibaoka and Thimann (1970) observed that a methanol soluble fraction from the roots of pea seedlings could promote chlorophyll degradation (senescence) in detached oat leaves. Since the active fraction was ninhydrin positive, it was thought to consist of one or more amino acids. Of the twenty-amino acids tested for their ability to promote chlorophyll degradation of oat leaves, serine was found most active whereas alanine, glycine, cysteine and phenylalanine had similar but weaker effects (Martin and Thimann 1972; Shibaoka and Thimann 1970). Using detached rice leaves, we also reported that exogenously applied glutamic acid, glutamine, aspartic acid, asparagines, glycine, serine, histidine, tyrosine, phyenylalanine, tryptophan, valine, leucine, isoleucine, lysine, and threonine promoted chlorophyll degradation (Kao 1980). In the present investigation, we observed that the contents of all these amino acids were higher in TN 1 leaves during the entire duration of Cd treatments (Figure 4 and unpublished data). It is suggestive that higher contents of these amino acids might be involved in regulating Cd toxicity in TN 1 leaves. On the other hand, the increase in ornithine and alanine contents in TN 1 leaves was observed only after 3 d of Cd treatment (Figure 5), indicating that the increase in these two amino acids is a consequence rather than a cause of Cd toxicity.

The fact that exogenously applied methionine retarded chlorophyll degradation in detached rice leaves (Kao 1980) and that the endogenous content of methionine decreased in Cd-treated TN 1 leaves (Fig-

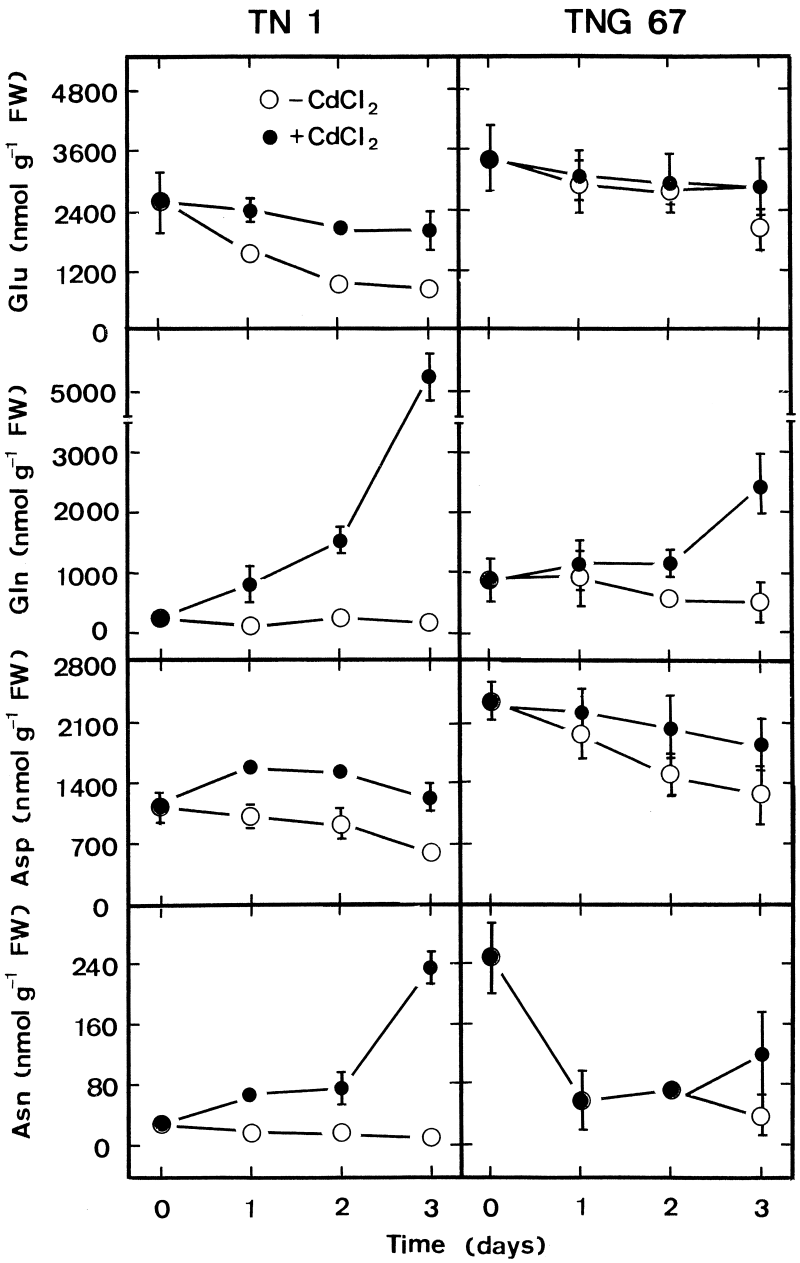

Figure 4. Changes in the contents of glutamic acid (Glu), glutamine (Gln), aspartic acid (Asp), and asparagine (Asn) in the second leaves of rice seedlings untreated or treated with $CdCl_2$ (0.5 mM). Vertical bars represent S.E. (n = 3).

ure 6) suggests that the decrease of methionine also plays a role in regulating Cd toxicity. It has been shown that methionine lowers protease activity in rice leaves (Kao 1980). It seems that Cd-induced toxicity is likely mediated through the increase in protease activity. Methionine is known to be a precursor of ethylene biosynthesis (Yang and Hoffman 1984). Ethylene has been shown to promote chlorophyll loss of rice leaves (Kao and Yang 1983). The decrease in methionine content in Cd-treated TN 1 leaves possibly resulted from its conversion to ethylene, which in turn induced Cd toxicity. However, this possibility seems unlikely, because Cd had no obvious effect on ethylene production in TN 1 leaves (Figure 7). If a change in ethylene production is excluded as an explanation for the Cd toxicity of TN 1 leaves, a change

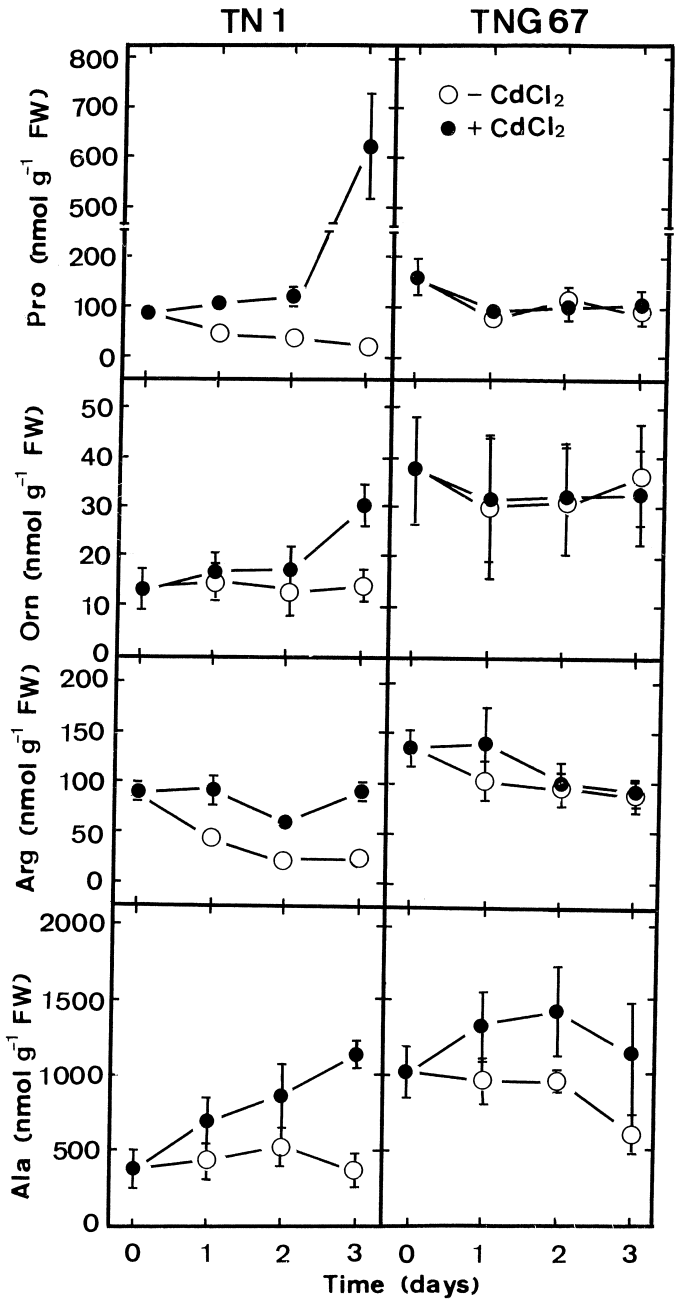

Figure 5. Changes in the contents of proline (Pro), ornithine (Orn), arginine (Arg), and alanine (Ala) in the second leaves of rice seedlings untreated or treated with $CdCl_2$ (0.5 mM). Vertical bars represent S.E. (n = 3).

is sensitivity to ethylene is an alternative possibility. However, 1-methylcyclopene, an inhibitor of ethylene action (Serek et al. 1994), was ineffective in inhibiting Cd toxicity of TN 1 leaves (data not shown). Thus, it seems that ethylene is not involved in regulating the toxic effects of $CdCl_2$ on rice leaves.

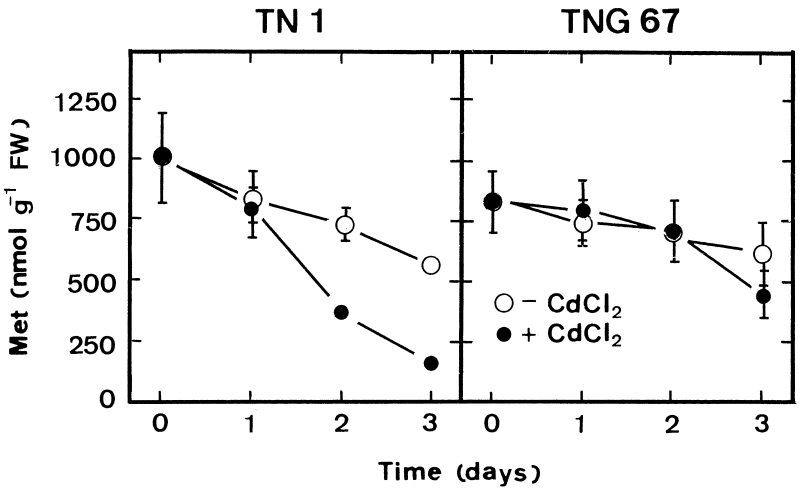

Figure 6. Changes in the content of methionine (Met) in the second leaves of rice seedlings untreated or treated with $CdCl_2$ (0.5 mM). Vertical bars represent S.E. (n = 3).

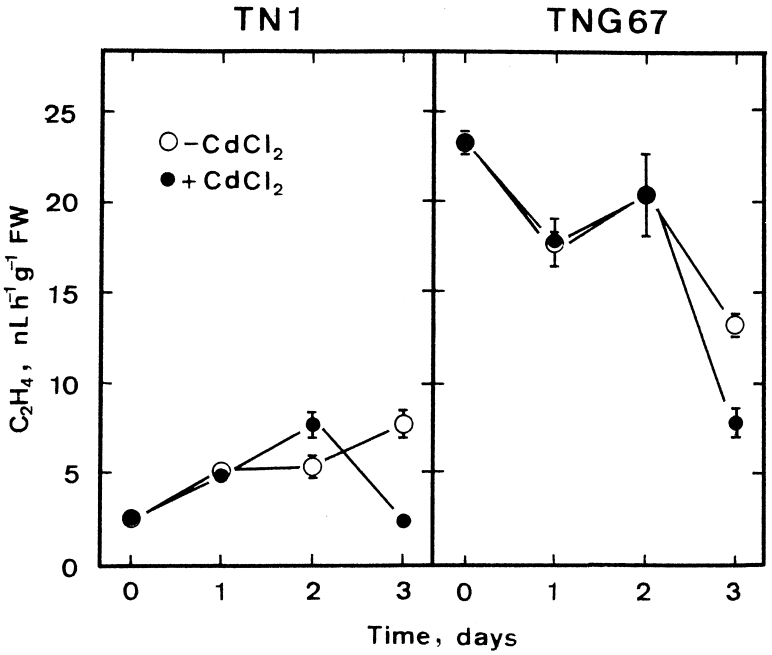

Figure 7. Changes in ethylene production in the second leaves of ric seedlings untreated or treated with $CdCl_2$ (0.5 mM). Vertical bars represent S.E. (n = 3).

Acknowledgements

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