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Alleviation by calcium of cadmium-induced root growth inhibition in *Arabidopsis* seedlings

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Abstract Cadmium (Cd) is a highly toxic element for living organisms. Its toxicity and the thereof by calcium (Ca) in 2-week-old *Arabidopsis* seedlings were studied. The seedlings were treated with Cd at concentrations of 50–500 μM for 24 h. Retardation of root growth was evident through increased cell death at the root elongation zone. Supplementing with 30 mM Ca restored root elongation in the presence of up to 200 μM Cd, resulting in up to a 1/3 decrease in Cd content in the seedlings compared to *Arabidopsis* treated with Cd alone. Ca was able to alleviate Cd toxicity, presumably through competition for metal ion influx. These results provide us with information regarding plant ion uptake systems and metal ion toxicities.

Key words: Cd toxicity, root elongation, metal ion influx.

Cd is a highly toxic element that is widespread in our environment as a result of industrial processes. It is a suspected carcinogen in humans and is toxic to living cells even at very low concentrations (Stohs et al. 2000). In plants, a low concentration (5 μM) of Cd reduced chlorophyll content and photochemical quantum yield of photosynthesis in *Brassica napus* (Baryla et al. 2001; Larsson et al. 1998). Accumulation and absorption of its element caused Fe(II) deficiency in the root due to the inhibition of root Fe(III) reductase (Alcantara et al. 1994). Cd also induced the generation of reactive oxygen species (ROS) and affected various toxicities in the cells, resulting in inhibition of plant growth and severely suppressed root elongation (Stohs and Bagchi 1995; Arduini et al. 1996; Arisi et al. 2000; Schutzendubel et al. 2001). However the behaviors and resultant effects of this element in plants are still unknown.

In general, Cd ions are first taken up by molecules in plant root. They then enter the root cells and injure the molecule machineries. Studies on metal uptake point out that uptake systems for essential cations such as Fe, Cu, and Zn also contribute to the uptake of Cd (Cohen et al. 1998; Hart et al. 1998; Clemens et al. 1998; Huang et al. 1994). For example, the plant transporter IRT1 was initially identified as a Fe transporter (Eide et al. 1996). However, this protein can also transport Mn, Zn and Cd (Connolly et al. 2002; Korshunova et al. 1999). The plant transporter LCA1 mediates the uptake of Ca and Cd in yeast (Clemens et al. 1998). CPx-ATPases, HMA4 supports a role in Zn and Cd transport (Bernard et al. 2004). Vacuolar Ca^{2+}/H^{+} antiporter CAX2 (calcium exchanger 2) in Arabidopsis is able to transport Ca, Cd, and Mn (Hirschi et al. 2000). Moreover, it was shown that Cd competes with Ca at both Ca channels (Nelson 1986) and intracellular Ca binding proteins (Rivetta et al. 1997). It has also been proposed that Cd uptake and toxicity to animals may be caused by an interaction with Ca (Hinkle et al. 1987).

In order to study the toxicities of Cd in plants, the effects of Cd and cell viabilities in relation to the influence of Ca were investigated. We show that Cd causes serious damage to the root tips in Arabidopsis, corroborating not only our previous data but also observations from other plant species (Schutzendubel et al. 2001). In addition, we found that Ca can greatly alleviate the toxicity of Cd, which is consistent with the competition theory among metal ions during influx. Studies on the relationship between Cd and Ca are thus useful for understanding Cd toxicities.

Materials and methods

Plant materials and chemical treatments

Seeds of *Arabidopsis thaliana* (var Columbia) were

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Abbreviations: Cd, cadmium; Ca, calcium; MS, Murashige-Skoog.

surface sterilized by using a 10-min incubation in 70% ethanol and by a 20-min incubation in 5% (w/v) sodium hypochlorite (active chlorine 8.5–13.5%, Nacalai Tesque, Kyoto, Japan) containing 0.05% (v/v) Tween-20. After three washes with distilled water, seeds (10–15 per bottle) were cultivated in 10 ml half-strength Murashige-Skoog (MS) medium supplemented with 1% sucrose in a greenhouse at 23°C under continuous light. For treatments with chemicals, seedlings were hydroponically cultivated for one week in 10 ml half strength MS medium supplemented with 1% sucrose in a greenhouse at 23°C under continuous light. To determine the effect of Cd toxicity, plants were transferred to 1% agarose plate containing half strength MS medium and 1% sucrose with or without 100–500 μM $CdCl_2$. The effects of Ca were determined by culturing plants on an agar plate containing half strength MS medium, 30 mM $CaCl_2$ and 200 μM $CdCl_2$. After 1 to 2 weeks, root growth was observed.

Quantification of Cd

Extraction and measurement of Cd was performed as previously described (Harada et al. 2001). Two-week-old *Arabidopsis* seedlings, which were cultivated in 10 ml half strength MS medium supplemented with 1% sucrose, were treated with 500 μM $CdCl_2$ in the presence or absence of 30 mM $CaCl_2$ for 24 h. At first, the sample was washed twice with distilled water, and then five volumes of precooled Tris-HCl (50 mmol/L, pH 7.2) buffer and sea sand C (Nakalai Tesque) were added. Tissues were homogenized with sea sand C and the crude extract was centrifuged at 12,500×g for 15 min. The supernatant was diluted with 19 volumes of the buffer solution and the concentration of Cd was measured with an atomic absorption/film emission spectrophotometer (AA-6500S, Shimadzu, Kyoto, Japan) as described (Harada et al. 2001).

Histochemical staining

One-week-old *Arabidopsis* root were used. Plants were cultivated under similar conditions of Cd toxicity and Ca effect assay. Roots were stained for 10 min in half-strength MS medium supplemented with 0.05% (w/v) evans blue (Wako) or fluorecein diacetate (Wako). Root tissue for analysis of anatomical changes was fixed in 5% glutaraldehyde (Wako) and 0.16 M K-PO_4 buffer (pH 6.8) for 2 h. The root was then separated and washed twice with 0.16 M K-PO_4 buffer and rinsed 3 times with distilled water. The sample was submerged in a solution of 25% ethanol/75% H_2O, which was replaced every 5 min for a total of 4 times. The percentage of ethanol in the replacement solution was raised at each step to 50, 70, 90 and 100%, respectively. After a 5 min incubation in 100% ethanol, the solution was replaced with liquid resin (Technovit 7100, Heraeus Kulzer, Wehrheim, Germany). The tissue was infiltrated with resin over 12 h at room temperature then polymerized and sectioned to 1.2 μm thickness. The resultant sections were stained with 0.2% safranin for 5 min followed by 0.5% fast green for 25 min. All samples were observed by using a microscope (PROVIS AX70, Olympus, Tokyo, Japan) equipped with a fluorescence module. Fluorescent images were captured separately using a CCD camera (monochrome images, CoolSNAP-HQ, Photometrics; color images, DP12, Olympus).

Results

Effects of Cd on root growth

Plants hydroponically cultured for a week were transferred to solid medium containing varying concentrations Cd^{2+}. Root growth was strongly suppressed by the treatment of 100–200 μM Cd. Samples died when treated with 500 μM Cd^{2+} for one week (Figure 1). When samples were exposed to 200 μM Cd^{2+}, root elongation was almost completely retarded, while the shoot survived but grew slowly.

Inhibition of root growth and effect of Ca

Root tip growth is seriously inhibited under the presence of Cd (200 μM). Since Cd uptake was suggested to rely on essential metal ion, especially Ca transporters, 1-week-old seedlings were transferred to solid medium containing both Ca and Cd, and observed for 2 weeks. Results showed an addition of 30 mM Ca to be effective for improved root elongation (Figure 2). Under this condition, plants were able to live on a plate containing Cd of over 500 μM (data not shown).

Effect of Ca on Cd influx

To investigate the reason why Ca in the incubation medium was able to reduce the Cd-induced inhibition of plants, the amount of endogenous Cd was estimated by atomic absorption spectrophotometer. During the first 24 h incubation, the content of Cd per gram fresh weight was reduced from 46.7 μg in the absence of Ca to 17.4 μg in the presence of Ca (Figure 3). Though the content of Zn was measured at the same time, no significant difference was observed (data not shown).

Effects of Cd on root

In order to identify physiological changes caused by Cd on root tissues, evans blue and fluorecein diacetate were used. Dead cells are stained with evans blue while green fluorescence was observed in viable cells with fluorecein diacetate. Root tissues were microscopically examined and cell death was monitored by staining from dyes. When plants were exposed to Cd, the root tip changed to a brownish color and adjacent areas to the brown root was stained with evans blue (Figure 4A, B, C). An

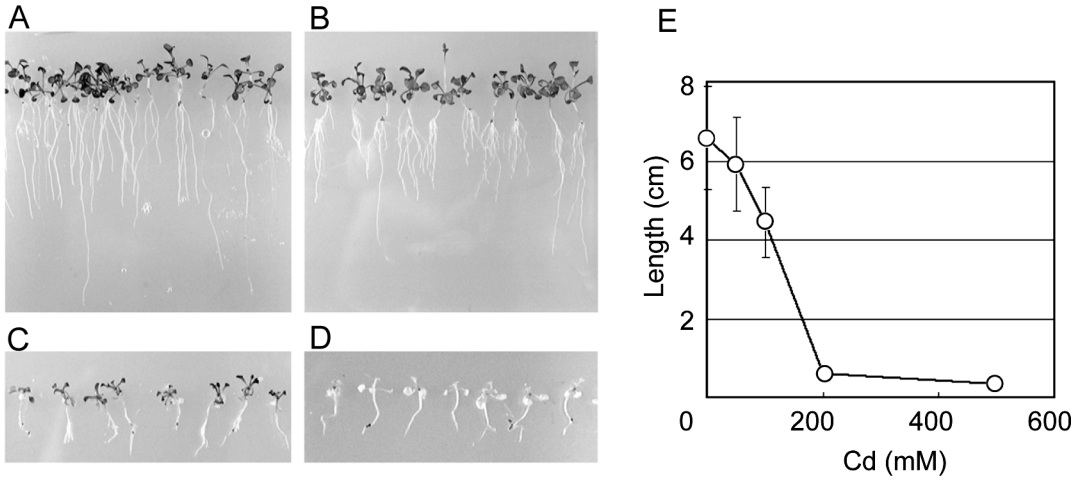

Figure 1. The effect of Cd on root growth of A. *thaliana*. 1-week-old plant was transferred to solid medium containing $CdCl_2$ (A, 0 μM; B, 100 μM; C, 200 μM; D, 500 μM). After 1 week, root growth of samples was observed. The elongation of each concentration (0, 50, 100, 200, 500 μM) was measured and plotted (E). 10 samples were used to calculate the value.

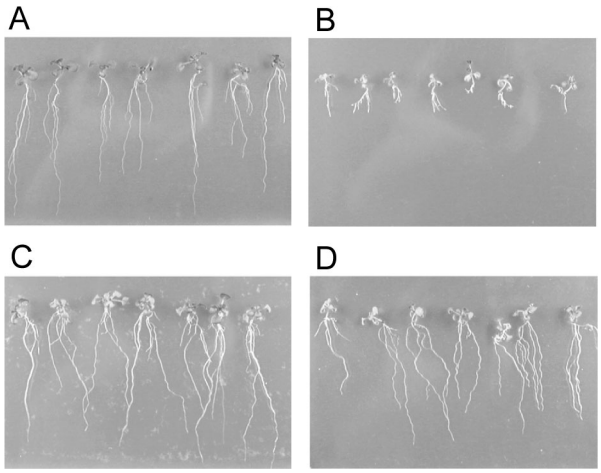

Figure 2. Influence of Ca on Cd toxicity. 1-week-old plants were transferred to solid medium. Plants were grown on half strength MS medium (A, control) and half strength MS medium containing 200 μM Cd (B), 30 mM Ca (C) and both Cd and Ca (D). After 1 week, root growth of samples was observed.

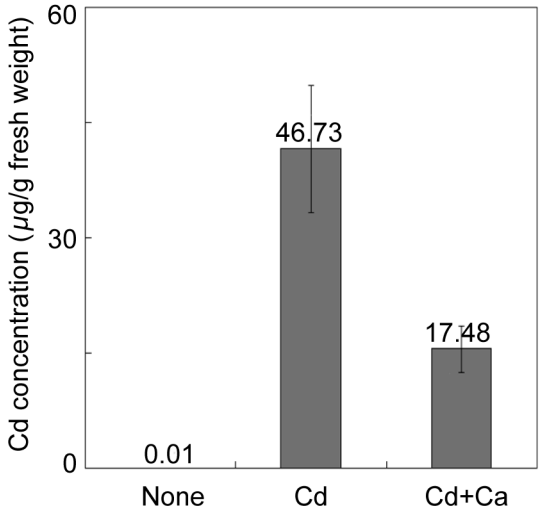

Figure 3. Measurement of Cd contents in plants by atomic absorption/film emission spectrophotometer. Endogenous Cd was extracted with Tris-HCl solution. Whole plants were treated for 24 h with Cd (500 μM) or Cd plus Ca (30 mM).

addition of Ca prevented the brown color from appearing and restored the fluorescence of fluorecein diacetate (Figure 4D, E).

Cell death and metabolic alteration

Sublethal concentration of Cd seriously inhibited root elongation without inducing cell death. Affected tissues contained enlarged and unusual form of cells, instead of the normal small and dense cytoplasmic cells that divide and expand in size (Figure 4F, G, H, I). Yellow or blue fluorescence derived from compounds was detected in Cd-damaged regions (Figure 4J, K, L, M), and in particular, areas with bright yellow fluorescence were stained red with safranin, indicating the presence of phenolics (Figure 4).

Discussion

In this study, the effects of Cd on root tissues and its relation with Ca were described. 100–200 μM Cd caused serious damage to the root elongation of Arabidopsis, and cell death in the same zone was observed. Ca caused a decrease of the Cd content in plant of up to 1/3 compared to plant treated with Cd alone and reduced Cd toxicity.

In higher plants, non-essential heavy metals such as Cd are likely to be transported across membranes via nutrient transporters or channels that are not completely selective (Clemens et al. 1998). Cd was assumed to enter cells via either the high affinity uptake system for iron or low affinity system for Ca or Zn uptake (Roosens et al.

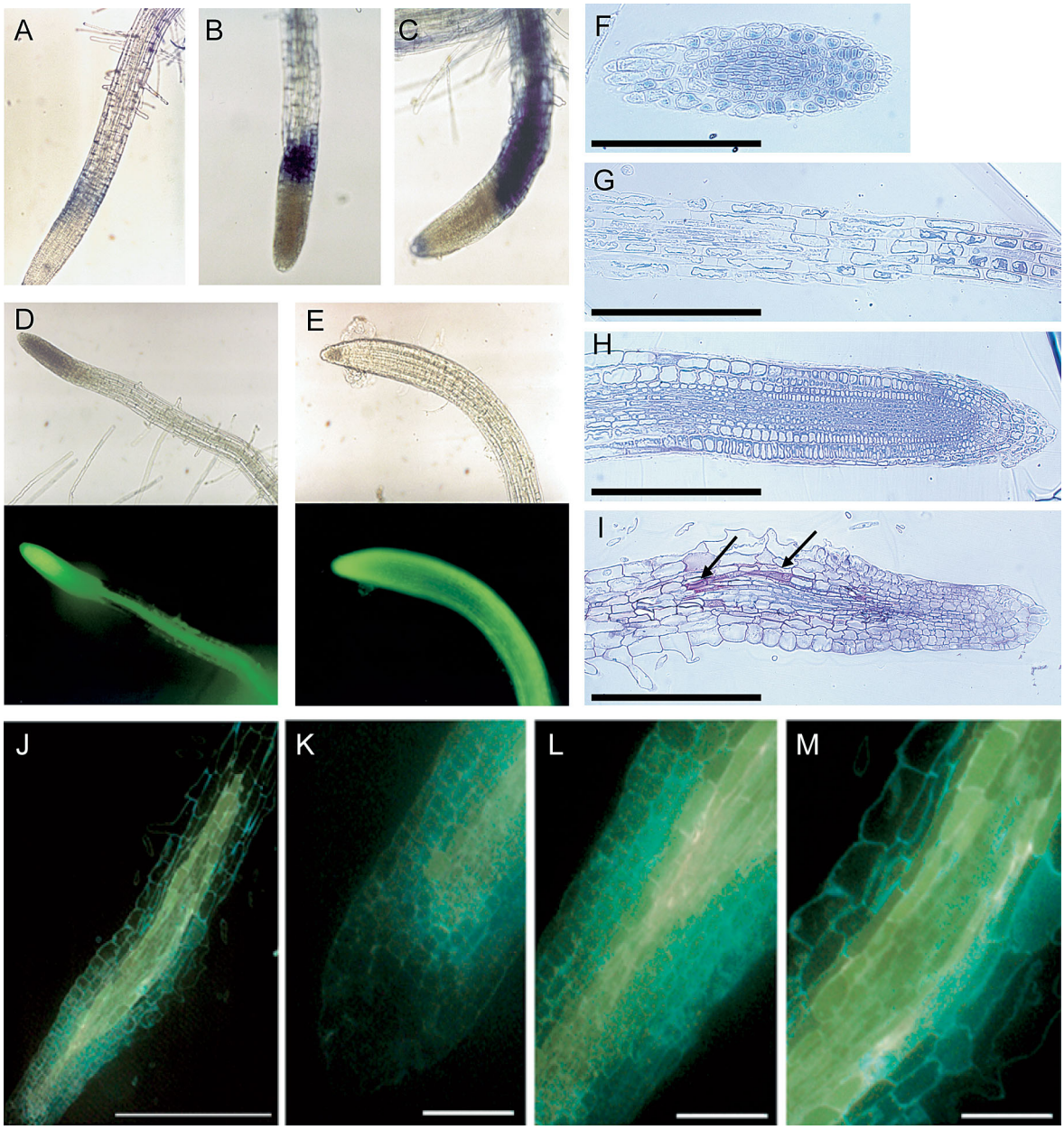

Figure 4. Effects of Cd on root cells. A through E show cell death in root tissue. 1-week-old seedlings of *Arabidopsis* were treated with 500 μM Cd (A, 0 h; B, 24 h; C and D, 48 h) or 500 μM Cd plus 30 mM Ca (E, 48 h). Samples were stained with evans blue (A, B, C) or fluorecein diacetate (D, E) and cell death was monitored. The excitation wavelength for fluorescence images (D, E) was from 470 to 490 nm (UV and IB-excitation filters). F trough I show morphological changes of root tissue. After treatment with a sublethal concentration of Cd (200 μM; F and G, control; H, 48 h; I, 2 weeks), roots were sectioned and stained with 0.2% safranin for 5 min and 0.5% fast green for 25 min. Black arrows indicate the localization of phenolics. J through M show accumulation of fluorescent chemicals in root upon Cd treatment. Plant was incubated for 2 weeks in medium with a sublethal concentration of Cd (200 μM) and fluorescence were captured by a CCD camera. The excitation wavelength is from 330 to 385 nm (UV and U-excitation filters). [Bar=250 μM (F–J) and 50 μM (K–M)].

2003; Lombi et al. 2002; Zhao et al. 2002; Pence et al. 2000). Multiple pathways were pointed out since the molecular mechanism responsible for metal uptake is very complex. It is known that aluminum (Al) also induces inhibition of root growth due to Al interactions within the root apex (Ryan and Kochian 1993). We also observed that cell death first appeared in root meristem or adjacent to meristem, probably forming in the root elongation zone, where protoxylem exists and cells are active, suggesting that influx of Cd causes cell death.

In addition, there is a report that indicates Cd influx at the positions of 1 to 1.5 mm from the root apex was significantly higher than that of positions further back from the apex by using microelectrode applied for Cd ion measurement (Pineros et al. 1998). The position is almost the same as that of dead cells that we observed.

However the reason why Cd influx is so high at this position is unknown, since Cd at high concentration confers serious damages to cells, high influx rate of Cd may also cause cell death at the root elongation zone.

Free Cd and other heavy metal ions often reduce cellular activities for a variety of reasons, for example, by generation of oxidative stresses and inhibition of enzyme reactions. Plants have developed a variety of mechanisms to tolerate such heavy metals. One of the major defense mechanisms is to inactivate metal ions by complexing them with phytochelatins and CXXC motif proteins such as metallothionein, Cys rich proteins (di Toppi and Gabbrielli 1999; Suzuki et al 2002; Song et al. 2004). Phytochelatin is a cystein rich peptide that is synthesized from glutathione and chelates heavy metals, including Cd, Cu, Zn and Ag on its thiol moieties (Zenk 1996). Usually, chelated Cd ions are stabilized by the acid-labile sulfide and sequestered in cells (di Toppi and Gabbrielli 1999). When radish seeds were exposed to 300 μM Cd for 24 h, the level of acid-soluble thiols in the embryo axes decreased by approximately 75% (Rivetta et al. 1997). Cells of the root elongation zone may have also died due to the depletion of thiols.

The inhibition of root growth which was observed may have been caused by the death of cells in the root elongation zone. When plants were incubated for 2 weeks in a sublethal level of Cd (200 μM), root cells survived but with irregular thickening of cell walls, and enlarged and unusually formed cells. Accumulation of chemical compound which have yellow or blue fluorescence in the endodermis, pericycle, or cambium cells was observed (Figure 4). Although it is not clear which mechanism caused the morphological change to occur, Cd may have altered some metabolic pathways. For example, GSH contains thiol moiety, and its deficiency was shown to lead to cell division block in plant roots (Vernoux et al. 2000). Cd was shown to affect GSH metabolism (Xiang and Oliver 1998).

It is notable that the accumulation of soluble chemicals, yellow or blue fluorescence was detected in Cd-damaged regions, and in particular, areas with bright yellow fluorescence were stained red with safranin, indicating the presence of phenolics. Scots pine was reported to accumulate soluble phenolics under Cd stress (Schutzendubel et al. 2001). Phenolics are believed to contribute to H_2O_2 destruction in the phenol-coupled APX reaction together with ascorbate, resulting in protection of tissues from oxidative stress (Schutzendubel et al. 2001). In cultured tobacco cells, phenolics were found to protect cells from aluminum toxicity (Yamamoto et al. 1998). Cd would generate oxidative stresses (Stohs and Bagchi 1995; Arduini et al. 1996; Arisi et al. 2000; Schutzendubel et al. 2001) and due to these results, such chemicals may be accumulated. Despite these observations, a direct protective function of phenolics against Cd is yet to be determined.

In contrast, the exposure of plants to Cd in the presenece of Ca alleviated root growth inhibition when compared to the presence of Cd alone. Cell death was not observed when seedlings were cultured in the presence of Ca. Cd content in the plants decreased up to 1/3 compared to plants treated with Cd alone. In the presence of Al^{3+}, H^+, or Na^+, supplementation of the medium with higher levels of Ca alleviates growth inhibition (Kinraide and Parker 1987; Yan et al. 1992; Yermiyahu et al. 1997). Several reasons for Ca alleviation of mineral toxicity have been considered. A proposed mechanism is the displacement of cell-surface toxic cations by Ca. Since plasma membrane surface are usually negatively charged, high level Ca^{2+} would reduce cell-surface negativity and alleviate the harmfulness of cationic toxicants (Kinraide et al. 1998). The other proposed mechanism is the uptake of Cd through calcium channels to mimic Ca (Perfus-Barbeoch et al. 2002). There is a report that the uptake of Cd is inhibited by the Ca channel blockers, diltiazem, verapmil, nifedipine and nitrendipine (Blazka and Shaikh 1991). Due to high concentrations of Ca around Ca channels, Cd uptake was possibly decreased by competition for metal ion influx. However, since a large number of carrier proteins are involved in the transport of Cd (Maser et al. 2001) in Arabidopsis, the precise roles of Ca in decreased Cd influx in plants have yet to be elucidated.

In this report, we showed that Ca reduced the uptake and toxic effects of Cd in Arabidopsis. In tobacco, Cd tolerance was increased in the presence of Ca, probably due to the active exclusion of toxic Cd by the formation and excretion of Cd/Ca containing crystals through the head cells of trichomes (Choi et al. 2001). It was also previously reported that the accumulation of Cd on seed germination of radish is reduced by high concentration of Ca (Rivetta et al. 1997).

In recent years, phytoremediation, which is the use of plants to clean up contaminated area, has been proposed as an environmentally friendly, inexpensive way to remove toxic elements because plants would provide an efficient system for heavy metal removal from soils (Cunningham and Ow 1996; Salt et al. 1995). Studies on the effect of heavy metal ions of plant would be useful for our understanding of the physiology of heavy metal uptake and accumulation in plants.

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