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Roles of Calcium and Cadmium on Cd-Containing Intra- and Extracellular Formation of Ca Crystals in Tobacco

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Growth was severely inhibited when tobacco plants were exposed to toxic levels of cadmium (0.2 mM). However, when this treatment was combined with a high concentration of calcium (30 mM), the Cd-induced damage was strongly alleviated. Under these enhanced conditions, i.e., 30 mM Ca and 0.2 mM Cd, Ca crystals not only were heavily deposited in the leaves but were also actively excreted from the trichomes. The X-ray spectrum from our Energy Dispersive analysis revealed that both intra- and extracellular Ca crystals contained detectable amounts of Cd. Moreover, intracellular Ca deposition in the leaves was stimulated only by a high Ca concentration (30 mM); moderate levels of Ca (3 mM) or a toxic amount of Cd (0.2 mM) alone resulted in crystal deposition that was undetectable under a light microscope. In contrast, extracellular crystal formation on the trichomes was stimulated by toxic Cd treatment but not by high Ca concentrations alone. Finally, Inductively Coupled Plasma Spectroscopy revealed that a high level of Ca (30 mM) suppressed Cd accumulation while also increasing the endogeneous Ca concentration in the leaves. These observations imply that the amelioration of Ca against toxic Cd in tobacco plants is a result of not only the inhibition of Cd uptake, but also the extra- and intracellular sequestration of cadmium via Ca crystallization.

Keywords: cadmium, calcium, crystallization, heavy metal, tobacco

Heavy metals in soil and water can seriously affect the growth and development of all organisms. Plants possess various means for detoxification (Salt et al., 1998). A regulated network of metal transport, chelation, trafficking, and sequestration activities functions to provide for their uptake, distribution, and detoxification (Salt et al., 1998; Clemens, 2001). One well-known defense mechanism is the inactivation of metal ions by their complexation with heavy-metal-binding polypeptides, e.g., cysteine-rich metallothioneins and phytochelatins (Salt et al., 1998).

As part of the final detoxification process in plants, vacuolar accumulation of toxic ions is the definitive event for intracellular sequestration (Vögeli-Lange and Wagner, 1990). In addition, plant trichomes serve as critical compartmentation and sequestration sites. In Indian mustard leaves, cadmium is preferentially accumulated in the trichomes (Salt and Rauser, 1995). Likewise, in *Alyssum lesbiacum*, *Thlaspi goesingense*, and *Arabidopsis halleri*, such heavy metals as cadmium and nickel are preferentially accumulated in those trichomes (Krämer et al., 1997; Küpper et al., 2000; Zhao et al., 2000). In water lilies, epidermal glands on their leaves also accumulate treated heavy metals (Lavid et al., 2001).

In tobacco, trichomes actively exude cadmium by Ca-Cd crystallization under toxic stress (Choi et al., 2001). In general, calcium crystallization occurs within the intravacuolar membrane chambers of specialized cells called crystal idioblasts (Franceschi and Horner, 1980; Webb et al., 1995; Nakata, 2003). Various roles have been hypothesized for calcium oxalate formation, including Ca regulation, plant defense, and detoxification (Franceschi and Horner, 1980; Webb et al., 1995; Nakata, 2003). The primary function of these idioblasts is as a Ca sink for reducing the apoplastic toxic concentration of these molecules around the adjacent cells (Frank, 1972; Franceschi and Horner, 1980; Borchert, 1985). However, calcium oxalate crystals are deposited even when the Ca supply is limited (Frank, 1972). For example, in *Eichhornia crassipes*, treated heavy metals (e.g., cadmium, lead, and strontium) are incorporated into idioblast oxalate crystals (Mazen and El Maghraby, 1997), while in *Lemna minor*, strontium, but not Cd, manifests this type of incorporation (van Steveninck and Fernando, 1995). Finally, treated heavy metals are never incorporated into the Ca crystals of the water lily (Lavid et al., 2001).

Here, we examined the role of Ca and Cd in the formation of intra- and extracellular Ca crystals in tobacco plants, and investigated whether this crystal

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formation could ameliorate the effects of calcium on cadmium detoxification.

MATERIALS AND METHODS

Cadmium and Calcium Treatments in Tobacco Plants

Plants were produced from the seed of tobacco (*Nicotiana tabacum* L. cv. Xanthi). When they reached about 5 cm in height, they were transferred to 300-ml glass culture bottles (five plants each) that contained 1/2 MS media (Murashige and Skoog, 1962), which was solidified with 0.6% agar and 1% sucrose, and supplemented with various levels of $CdCl_2 \cdot 2.5H_2O$ (0.00, 0.05, 0.10, 0.20, or 0.50 mM), and/or $CaCl_2 \cdot 2.5H_2O$ (0, 3, 10, 20, or 30 mM). Cultivation took place in a growth room (25°C, 16-h photoperiod, 35 μmol $m^{-2}s^{-1}$ from white fluorescent tubes). Fifteen plantlets were cultured in each experiment and each treatment was repeated three times. Heights and dry weights were measured after one month of growth.

To understand how calcium interacts with cadmium in the growth of young seedlings, 20 germinated seeds each were placed in Petri dishes containing 1/2 MS media that was supplemented with 0.6% agar, 1% sucrose, and 0.2 mM $CdCl_2 \cdot 2.5H_2O$, plus various levels of $CaCl_2 \cdot 2.5H_2O$ (0.3, 1.0, or 30.0 mM). Culture conditions were as described above, and seedling growth was monitored for one month.

Crystal Isolation from Tobacco Leaves

To isolate their accumulated intracellular Ca crystals, leaves were incubated for 10 h (25°C, gentle shaking at 50 rpm) in 40 ml of a protoplast-isolation medium containing 2% (w/v) cellulase RS (Yakult, Japan), 0.5% macerozyme (Sigma, USA), 0.7 M mannitol, 3% (w/v) KCl, and 0.5% (w/v) $CaCl_2$ (pH 5.6). The digested tissues were then vortexed for 10 s and allowed to settle for 5 min. Supernatants were removed and the crystal sediments were collected and prepared for analysis.

Extracellular Ca crystals that had been produced on the leaf surfaces through the trichomes also were isolated, using the method described by Choi et al. (2001).

Energy-Dispersive X -Ray (EDX) Analysis

We observed the crystals through a low-vacuum scanning electron microscope (LV-SEM, S-3500N, Hitachi Science Systems, Japan). Leaf segments that showed trichomes with crystals, as well as the isolated crystals, were mounted on aluminum stubs and placed on a chamber stage after cooling to −120°C. Their elemental composition was analyzed through the LV-SEM, which was fitted with an EDX analysis system (EMAX-7000, Horiba, Japan). Chamber pressure was 30 Pa and the accelerating voltage was 15 KV.

Ca and Cd Measurements by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS)

Tobacco plants were grown for one month on 1/2 MS media containing 0.6% agar, 1% sucrose, 0.2 mM $CdCl_2 \cdot 2.5H_2O$ (0.2 mM), and/or 3 mM or 30 mM $CaCl_2 \cdot 2.5H_2O$. After the whole plants were harvested and freeze-dried, the tissues were weighed and digested with a 10:4:1 (v:v:v) mixture of HNO_3:$HClO_4$:H_2SO_4 by heating (150°C to 200°C) under vacuum until dry. The digests were then dissolved in 1 M nitric acid, incubated for 1 h, and diluted with distilled water. To determine their Ca and Cd contents, each sample was analyzed by ICP-MS (JY 38 Plus, Jobin-Yvon, France). Three individual plants were measured in each experiment, and each treatment was repeated three times.

Histological Observations of Leaves

To histologically observe the accumulated Ca crystals, tobacco leaves were fixed for 4 h at 4°C in 1% glutaraldehyde (phosphate buffer, pH 6.8). They were then post-fixed for 2 h in 2% OsO_4. These samples were dehydrated with ethyl alcohol and embedded by the method of Spurr (1969). After semi-thin sections were made with an LKB-V ultramicrotome, they were stained with 1% toluidine blue O and observed by light and phase-contrast microscopy.

RESULTS

Amelioration Effect of Calcium Against Toxic Cadmium Damage in Tobacco

Tobacco seedlings did not survive in the presence of 0.2 mM Cd (Fig. 1A). However, when that level of treatment was combined with a high concentration of Ca (30 mM), cadmium-induced damage was markedly reduced (Fig. 1, 2A), although growth was still slightly retarded (Fig. 1A).

Ca and Cd Contents in Tobacco Plants

ICP-MS analysis revealed that a treatment with 30 mM Ca suppressed Cd uptake (Fig. 2B). Moreover, the Ca content in plants treated at that concentration was 3-fold higher than in those exposed to 3 mM Ca (Fig. 2B). Nevertheless, calcium levels did not differ significantly between plants treated with a toxic amount of cadmium (0.2 mM) versus those that received no Cd (Fig. 2B). However, the high Ca treatment did decrease the endogenous Cd concentration in plants.

Intracellular Calcium Crystal Formation in Tobacco Plants

The combination of high Ca (30 mM) with toxic Cd (0.2 mM) resulted in heavy deposition of calcium crystals within the plants. After the leaves were bleached with 70% ethanol, those crystals appeared as numerous white spots (Fig. 3A, B) under a dissecting light

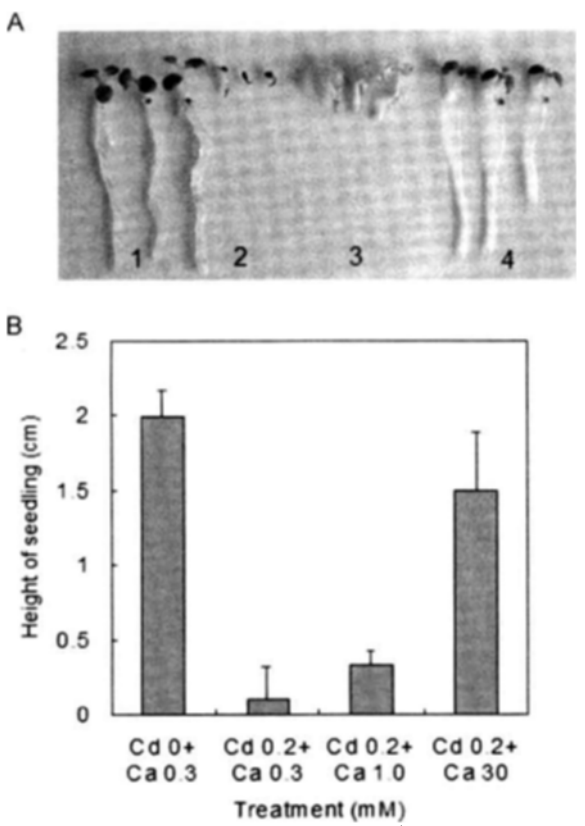

Figure 1. Growth of tobacco seedlings after one month of culturing on media with different concentrations of Cd and Ca. **A**, 1, Control; 2, 0.2 mM Cd + 0.3 mM Ca; 3, 0.2 mM Cd + 1.0 mM Ca; 4, 0.2 mM Cd + 30 mM Ca. **B**, Seedling heights.

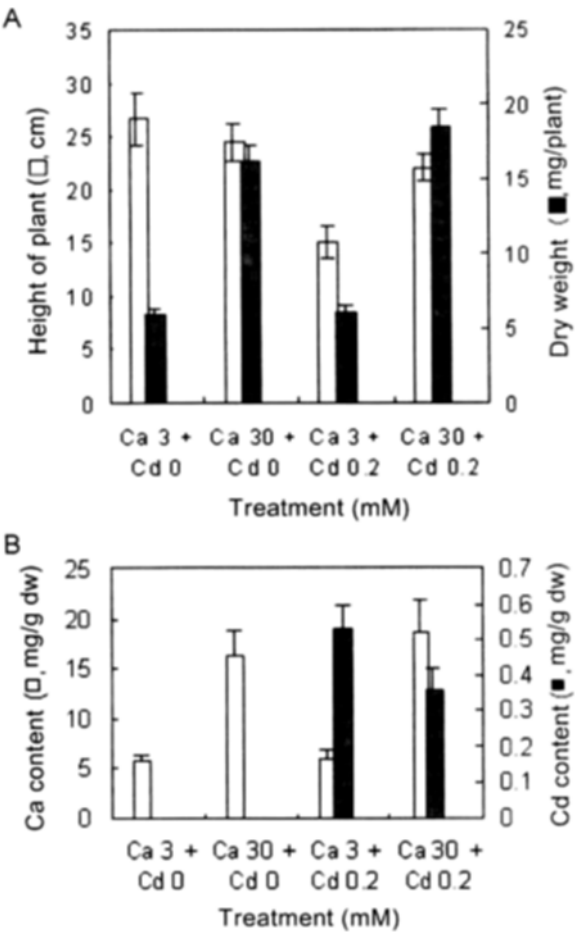

Figure 2. Growth of tobacco seedlings after one month of culturing on media with different concentrations of Cd and Ca. **A**, Heights (□) and dry weights (■). **B**, ICP analysis of Ca (□) and Cd (■) contents.

microscope, and as dark spots under a light microscope (Fig. 3C, arrows). Semi-thin sections of plastic-embedded leaf tissue revealed the accumulation of intracellular Ca crystals (Fig. 3D). These were evident under the phase-contrast microscope (Fig. 3E).

To isolate and count the accumulated crystals, the leaves were incubated in a liquid medium containing cell wall degradation enzymes (0.5% cellulase and 0.2% macerozyme), which allowed for protoplast isolation of the crystals after vortexing (Fig. 4A). Their squashed powders comprised a group of tiny crystals (Fig. 4B). The EDX spectrum revealed calcium as the major component in the intracellular crystals from leaves treated with 30 mM Ca and 0.2 mM Cd (Fig. 5). Cadmium was also detected, indicating that it had been sequestered in the idioblasts under the high-Ca conditions. In contrast, our moderate-Ca and toxic-Cd treatments resulted in few crystals and undetect-

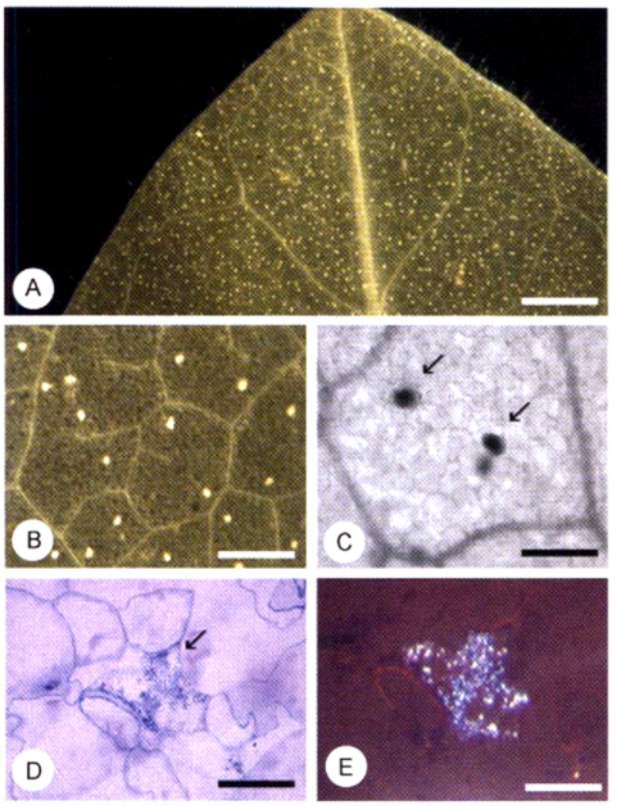

Figure 3. Intracellular Ca crystals accumulated in idioblasts of tobacco leaves. **A**, Numerous crystals, seen as white spots (bar, 600 μm). **B**, Close view of crystals (bar, 180 μm). **C**, Crystals (arrows) under light microscope (bar, 180 μm). **D**, Histological observation of crystal after semi-thin sectioning (bar, 120 μm). **E**, Phase-contrast light-microscopic observation of **D**.

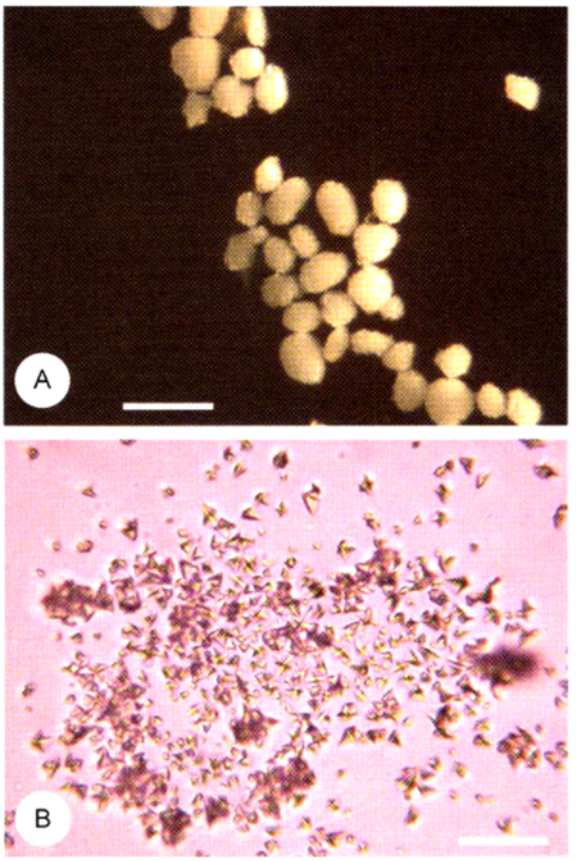

Figure 4. Purified Ca crystals from tobacco leaves by enzymatic digestion of cell wall. **A**, Isolated crystals (bar, 150 μm). **B**, Squashed crystals comprising tiny sands (bar, 15 μm).

able Cd levels of accumulation (data not shown).

Effects of Ca and Cd on Crystal Formation

Previously we reported that Cd induces extracellular Ca crystal formation on tobacco leaves (Choi et al., 2001, 2004). Here, we compared the effect of Ca and Cd feeding on extra- and intracellular Ca crystal formation after tobacco plants were cultured on media supplemented with various concentrations of Ca (0, 3, 10, and 30 mM) and/or Cd (0.00, 0.05, 0.10, 0.20, and 0.50 mM). Extracellular crystals formed on the head-cell surfaces of the trichomes (Fig. 6), and were especially numerous when plants were exposed to 0.2 mM Cd plus 30 mM Ca (Fig. 6). Therefore, we believe that this extracellular crystal formation was slightly enhanced by the higher level of Ca (Fig. 7A).

Intracellular Ca deposition within the leaves also was increased markedly as the Ca concentration rose

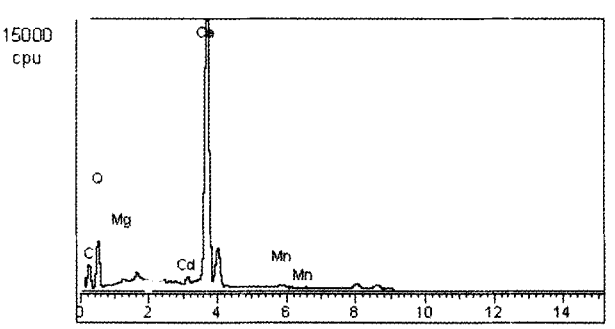

Figure 5. EDX spectrum of crystal from leaf of plant cultured for one month on medium with 30 mM Ca and 0.2 mM Cd.

(Fig. 7A). However, no crystals were observed at moderate calcium levels, regardless of the amount of Cd applied (Fig. 7B). Furthermore, extracellular crystal formation on the trichomes was greatly stimulated when cadmium concentrations were elevated to toxic amounts (0.2 mM), but was decreased under the severely toxic concentration of 0.5 mM Cd (Fig. 7B).

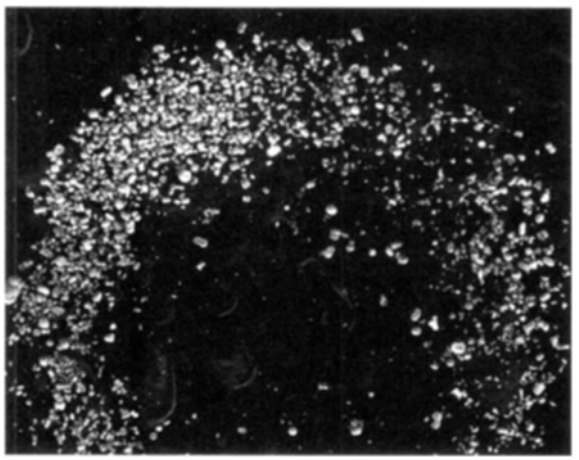

Figure 6. Extracellular Ca crystals formed on head cells of tobacco trichomes in the presence of toxic Cd (0.2 mM) and high Ca (30 mM). Crystals were isolated from three plants and observed by SEM.

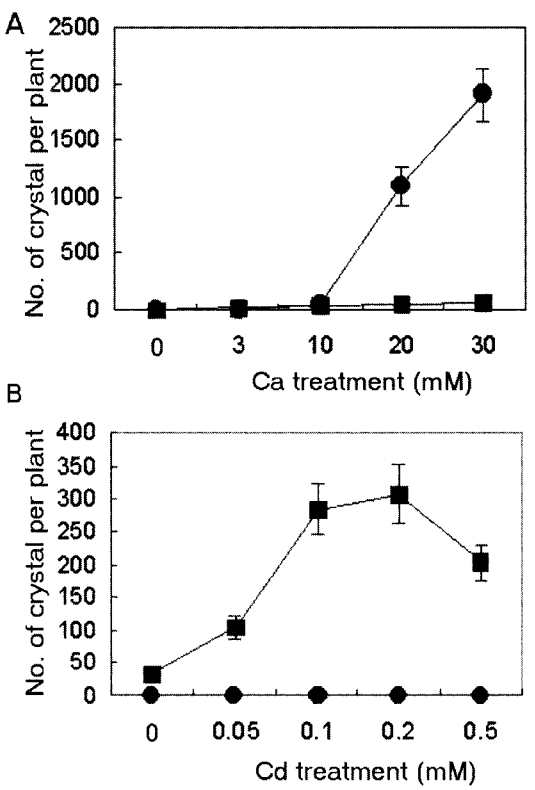

Figure 7. Ca crystals formed after one month of culturing. **A**, Number of extra- (■) and intracellular (●) crystals per plant on media with different Ca concentrations. **B**, Number of extra- (■) and intracellular (●) Ca crystals per plant on media with different Cd concentrations. Extracellular crystals were isolated by the method of Choi et al. (2001). Intracellular crystals were isolated and purified from protoplasts after enzymatic digestion of cell walls.

DISCUSSION

Amelioration Effect of High Calcium Against Toxic Cadmium

Cd-induced damage is markedly reduced under the high concentration of Ca (30 mM), indicating a strong amelioration effect by the latter. In general, this corresponds with the inhibition of Cd uptake because Ca competes for the common membrane channel in plant roots (Schroeder and Thuleau, 1991; Zhao et al., 2002). Several soil amendments, e.g., lime, are effective in reducing the translocation of metals from roots to shoots (Lehoczky et al., 2000; Tyler and Olsson, 2001). We also demonstrated here (via ICP analysis) that Cd uptake was lowered in the presence of high Ca (30 mM) compared with its activity at a more moderate Ca concentration (3 mM). Nevertheless, despite the fact that competition with Ca probably led to lower Cd uptake, this inhibition by calcium was not complete.

Intracellular Ca Crystal Formation in Tobacco Leaves

Numerous white calcium crystals accumulate in plants grown in media treated with high Ca and toxic Cd. Histological observations revealed that these might have been Ca oxalate crystals in the idioblasts. The squashed crystal sands were composed of tiny, tetrahedral-shaped crystals (Fig. 4B). The greater dry weights measured in plants grown on high Ca may have been caused by this heavy crystal accumulation (Fig. 3A). Bouropoulos et al. (2001) have also extracted Ca oxalate monohydrate (whewellite) crystals, with pseudotetrahedral shapes, from tobacco leaves.

After the cell wall digestion, EDX microanalysis detected Cd in our isolated Ca crystals, which implies that such depositions did sequester the Cd by incorporating it into those intracellular crystals. Choi et al. (2001) have previously suggested a Cd detoxification mechanism via this extracellular crystal exudation through the trichomes. Our current results further demonstrate that both intra- and extracellular crystallization in tobacco can sequester cadmium. In general, calcium idioblast crystals do indeed harbor some heavy metals (van Balen et al., 1980; Franceschi and Schueren, 1986; Webb et al., 1995; Mazen and El Maghraby, 1997). One exception to this is found with the lack of accumulation by the idioblast Ca crystals in the water lily (Lavid et al., 2001).

Role of Ca and Cd in Both Intra- and Extracellular Calcium Crystal Formation

We compared the role of Ca and Cd in both intra- and extracellular Ca crystal formation and found that the latter was stimulated on the trichomes by toxic Cd treatment but not by high Ca alone. In contrast, intracellular Ca accumulations in the leaves were markedly enhanced by increased Ca but not by toxic Cd. These results indicate that intra- and extracellular formations are separate physiological events. In previous research with NaCl, we had shown that both Na and Cl were preferentially accumulated in the insides of trichomes, but were not exuded (Choi et al., 2004). Therefore, intracellular formation is less active in sequestering Cd compared with the rate of extracellular crystallization on the trichomes. Nevertheless, cadmium incorporation in the idioblast crystals might also play some role in its toxic accumulation.

In conclusion, we have demonstrated here that the strong amelioration of Cd-induced damage by high Ca coincides with both intra- and extracellular Ca crystal formation in tobacco plants. This phenomenon can be explained by the inhibition of cadmium uptake in the roots due to ionic competition with calcium. Likewise, cadmium may be sequestered in extracellular and intracellular calcium crystals either on the trichomes or within the idioblast cells of the leaves.

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