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BRIEF COMMUNICATION

Interactions between cadmium and nickel in phytochelatin biosynthesis and the detoxification of the two metals in suspension-cultured tobacco cells

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Abstract

We examined the effects of simultaneous treatments with Cd and Ni on the phytochelatin (PC) biosynthesis in suspension-cultured tobacco (*Nicotiana tabacum* cv. Bright Yellow-2) cells. The induction of PC biosynthesis in response to Cd was not affected by the coexistence of Ni. Cd and Ni formed complexes with different compounds in cells.

Additional key words: heavy metals, *in vitro* culture, *Nicotiana tabacum*.

When plant cells are exposed to heavy metals, the biosynthesis of heavy metal-binding peptides called phytochelatins (PCs) (Grill *et al.* 1987) with general structure (γ-Glu-Cys)$_n$-Gly was induced. PCs protect plant enzymes from Cd^{2+} by chelation with SH-groups (Kneer and Zenk 1992). PC synthesis uses glutathione as a substrate and is catalyzed by PC synthase (Grill *et al.* 1989). The enzyme is continuously present in plant cells, and the catalytic activity depends on presence of heavy metals (Loeffler *et al.* 1989). The involvement of PCs in tolerance to several heavy metals has been documented (Reese and Wagner 1987, Mendum *et al.* 1990, Howden *et al.* 1995a,b, Nakazawa *et al.* 2000a). However, since investigation on the induction of PC biosynthesis by simultaneous treatments with several heavy metals is limited, we have been studying the effects of simultaneous treatments with two metals on the induction of PC biosynthesis (Nakazawa *et al.* 2000a). In addition, it has been suggested that the biosynthesis of PCs does not contribute to Ni-tolerance of tobacco cells, and that Ni is chelated by histidine rather than PCs in tobacco cells (Kameda *et al.* 1998, Nakazawa *et al.* 2000c). These findings suggest that intracellular Cd is chelated by PCs, in contrast that intracellular Ni is chelated by the other chelators such as histidine. However, it has not been clarified the performance of Cd, Ni and PCs in plant cells simultaneously treated with the two metals. In this paper, we report the effects of simultaneous treatments with Cd and Ni on Cd absorption and PC biosynthesis of suspension cultured tobacco cells, and investigated the chemical forms of Cd and Ni in suspension cultured tobacco cells simultaneously treated with Cd and Ni.

Suspension cultures of tobacco (*Nicotiana tabacum* cv. Bright Yellow-2) cells were grown as described previously (Nakazawa *et al.* 2000a). As the medium, we employed a modified Linsmaier and Skoog (1965) medium, in which concentrations of KH_2PO_4 and thiamine-HCl were increased to 37 and 0.1 mg dm^{-3}, respectively, supplemented with 3 g dm^{-3} sucrose and 1 μM 2,4-dichlorophenoxyacetic acid. Tobacco cells were cultured on a reciprocal shaker (100 rpm) at 26 °C in the dark. $CdCl_2$ and $NiCl_2$ were added as autoclaved solutions into culture medium.

The cells were homogenized in an equal volume (v/m) of 10 % (m/v) 5-sulfosalicylic acid (SSA), and the homogenates were centrifuged at 15 000 *g* for 5 min at 4 °C. PCs in the supernatants were analyzed using C_{18}-HPLC (*Shimadzu Shim-Pack CLC-ODS*, Kyoto, Japan) as described by Gupta and Goldsbrough (1991). PC cellular contents were calculated as the amount of

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Abbreviations: Cys - cysteine; Glu - glutamate; Gly - glycine; PC - phytochelatins.
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SH-groups. The Cd contents in cells were calculated from the Cd concentration in SSA extracts measured by atomic absorption spectrophotometry (*Shimadzu AA-670*, Kyoto, Japan).

The cells were also milled in liquid-N_2, and then homogenized in an equal volume (v/m) of 20 mM Hepes-NaOH buffer (pH 7.0) containing 10 mM L-ascorbic acid. The supernatants were applied into the column of *Toyopearl HW-40C* (⌀ 2.6 × 40 cm) equilibrated with 0.1 M ammonium acetate (pH 7.0). The samples were eluted with 0.1 M ammonium acetate (pH 7.0) at 4 °C and at a flow rate of 0.8 cm^3 min^{-1}. Fractions of 5 cm^3 were collected in test tubes, and their concentrations of Cd and Ni were measured by atomic adsorption spectrophotometer.

Table 1. Effects of 50 μM Cd and 350 μM Ni on phytochelatin (PC) and Cd contents [nmol g^{-1}(f.m.)] in tobacco cells. Means ± SE, $n = 3$, nd - not detected.

Treatment	PC_2	PC_3	PC_4	Total PC	Cd
Control	22.8 ± 4.9	12.0 ± 8.0	nd	34.8 ± 13.5	nd
Cd	59.9 ± 13.6	361.2 ± 35.5	69.7 ± 15.5	490.8 ± 39.0	134.7 ± 4.0
Ni	50.5 ± 5.5	195.0 ± 20.6	19.2 ± 6.4	286.1 ± 6.1	nd
Cd + Ni	50.0 ± 17.2	374.3 ± 22.2	81.0 ± 5.4	505.3 ± 44.7	112.7 ± 10.1

Effects of simultaneous treatments with Cd and Ni on growth of tobacco cells were examined. When the cells were cultured in medium containing 50 μM Cd and 350 μM Ni alone for 7 d, the fresh mass of the cells was not significantly different that of control cells, respectively. It had been confirmed that 50 μM Cd and 350 μM Ni were the maximum concentration which was non-toxic to tobacco cells, respectively (Nakazawa *et al.* 2000b). On the other hand, the fresh mass of the cells cultured in the medium containing both 50 μM Cd and 350 μM Ni was not significantly different from that of control cells. Thus, interaction between Cd and Ni in toxicity to tobacco cells, were neither synergistic nor additive. Previously, we reported that simultaneous treatments with Cd and As led to the synergistic growth inhibition of tobacco cells, and it was suggested that the growth inhibition was caused by the inhibition of PC biosynthesis in Cd-As-treated cells (Nakazawa *et al.* 2000a). In this study, it was indicated that interaction between Cd and Ni in toxicity to tobacco cells was not synergistic. From these findings, we assumed that the induction of PC biosynthesis in response to Cd-stress was not affected by the coexistence of Ni. Therefore, we next examined the effects of simultaneous treatments with Cd and Ni for 24 h on the induction of PC biosynthesis and Cd-contents in tobacco cells were examined (Table 1).

The fresh mass of the cells treated with Cd and/or Ni for 24 h was not significantly different from that of control cells (data not shown). The treatment with Cd and/or Ni alone led to the induction of (γ-Glu-Cys)$_2$-Gly (PC_2), (γ-Glu-Cys)$_3$-Gly (PC_3), and (γ-Glu-Cys)$_4$-Gly (PC_4). Total-PC content in the cells simultaneously treated Cd and Ni (Cd-Ni-treated cells) was not significantly different from that in the cells treated with

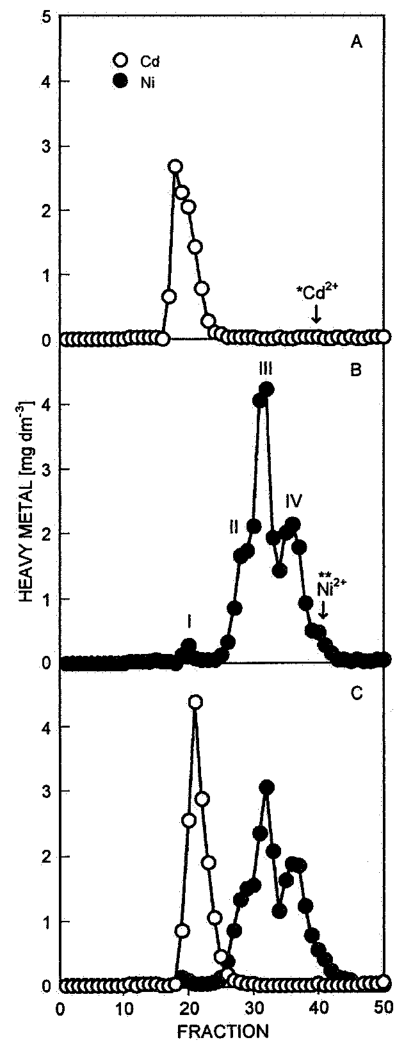

Fig. 1. Chromatography on *TOYOPEAL HW-40C* of the extracts from the tobacco cells treated with 50 μM $CdCl_2$ (*A*) and/or 350 μM $NiCl_2$ (*B, C*). *Closed symbols* - Cd concentration in fractions, *open symbols* - Ni concentration in fractions. * - elution fraction of $CdCl_2$ solution; ** - elution fraction of $NiCl_2$.

Cd alone (Cd-treated cells). Additionally, significant difference in individual PCs (PC_2, PC_3, and PC_4) contents between Cd-Ni-treated cells and Cd-treated cells was not observed. On the contrary, total-PC contents in the cells treated with Ni alone (Ni-treated cells) was 58 % of those in Cd-treated cells, and the proportion of individual PCs to total-PC in Cd-treated-, Cd-Ni-treated-, Ni-treated cells was $PC_3 > PC_4 > PC_2$, $PC_3 > PC_4 > PC_2$, and $PC_3 > PC_2 > PC_3$, respectively. Thus, the induction of the biosynthesis of total-PC and individual PCs of Cd-Ni-treated cells was similar to those of Cd-treated cells. From these results, it is suggested that the induction of PC biosynthesis in response to Cd is not affected by the coexistence of Ni, in contrast to the coexistence of As (Nakazawa *et al.* 2000a). In previous paper, it was suggested that PC biosynthesis contribute to Cd-tolerance and not to Ni-tolerance in tobacco cells (Nakazawa *et al.* 2000c). This finding imply that PC in Cd-Ni-treated cells contribute to Cd-tolerance and not to Ni-tolerance. Therefore, it is suggested that Cd-toxicity to tobacco cells is not influenced by the coexistence of Ni. Additionally, we have previously reported that Cd affected the activation of the catalytic activity of PC synthase predominantly to Ni (Nakazawa and Takenaga 1998). Since the response of PC biosynthssis to Cd-Ni-stress is similar to the activation of PC synthase by the coexistence of Cd and Ni, the activation of PC synthase by Cd and Ni *in vitro* may affect the biosynthesis of PC in response to Cd-Ni stress. On the other hand, Cd-contents in Cd-Ni treated cells were lower than those in Cd-treated cells (Table 1). Generally, it is considered that heavy metals are distributed to cell wall, cytoplasm, and vacuole, and that PC synthase is mainly activated by cytoplasmic heavy metals. Unfortunately, the effects of simultaneous treatments with Cd and Ni on the distribution of the two metals in cells have not been determined in this study.

Next, to investigated the chemical forms of Cd and Ni in Cd-Ni-treated cells, the gel filtration of the extracts from the tobacco cells simultaneously treated with Cd and Ni was examined. In tobacco cells treated with 50 μM Cd alone for 7 d (Cd-treated cells), a Cd-peak was obtained (M_r = 10 kDa) (Fig. 1*A*). On the contrary, in cells treated with Ni alone for 7 d (Ni-treated cells), two major Ni-peaks and two minor Ni-peaks were observed. Those peaks were named I, II, III, and IV, respectively (Fig. 1*B*). The M_r of peak I was 10 kDa. The M_rs of peak II, III, IV were below 1 kDa, and were higher than that of $NiCl_2$. In the cells simultaneously treated with Cd and Ni for 7 d (Cd-Ni-treated cells), a Cd-peak and four Ni-peaks were detected (Fig. 1*C*). The M_rs of the peaks corresponded to those of the peaks detected in Cd-treated and Ni-treated cells, respectively. Additionally, the M_r of Ni-peak I corresponded to M_r of Cd-peak. From these results, it is suggested that Ni mainly formed complexes with the several low-molecular-compounds which are different from Cd-binding compounds. In previous papers, we reported that the catalytic activity of PC synthase was activated by treatment with Cd and/or Ni in tobacco cells, and that PC biosynthesis contributed to Cd-tolerance, but not to Ni-tolerance in tobacco cells (Nakazawa and Takenaga 1998, Nakazawa *et al.* 2000c). Additionally, it was reported that Ni was chelated with histidine in tobacco cells (Kameda *et al.* 1998). These findings indicate that the two metals may be selectively detoxified by different compounds.

Thus, a part of the performance of Cd and Ni in Cd-Ni-treated cells, was elucidated in this paper. Further investigations on biochemical interactions between the two metals will be required.

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