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Selection and characterization of nickel-tolerant tobacco cells

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Abstract

Tobacco (*Nicotiana tabacum* L. cv. BY-2) cell lines tolerant to 700 μM Ni in which unselected cells can not grow, were selected. The Ni-tolerant cells were also more tolerant to Co, but not to Cd than unselected cells. Ni concentrations in Ni-tolerant cells were always higher than those in medium. Since buthionine sulfoximine did not affect their Ni-tolerance, it is suggested that phytochelatins are not involved in Ni-tolerance of Ni-tolerant cells. On the other hand, histidine contents in Ni-tolerant and unselected cells, which were treated with Ni, were higher that those treated without Ni, and the degree of the elevation of histidine contents by Ni-treatment was higher in Ni-tolerant cells than in unselected cells. Additionally, exogenous histidine reduced the inhibitory effect of Ni on the growth of unselected cells. In addition, the cells that were tolerant to histidine-analogue, had higher contents of histidine and Ni-tolerance. These results suggest that histidine is involved in Ni-tolerance and the detoxification of Ni in symplast in Ni-tolerant cells.

Additional key words: Co-tolerance, histidine, *Nicotiana tabacum*, Ni-tolerance, triazole alanine.

Introduction

Exposure of plants and plant cells to high contents of Ni results in the reduction of their growth (Mishra *et al.* 1974). Generally, plant cells have various mechanisms against heavy metal stress: *1*) the cellular exclusion of heavy metals (*e.g.* Lolkema *et al.* 1986), *2*) the adsorption of those ions in cell walls (Turner *et al.* 1972), *3*) the synthesis of heavy metal-insensitive enzymes (Cox *et al.* 1976), *4*) the chelation of those ions by chelators such as phytochelatin (PC; Rauser 1995), amino acids (Krämer *et al.* 1996), organic acids (Lee *et al.* 1977), *etc*. PCs that have general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (Grill *et al.* 1987, Maitani *et al.* 1996), protect plant enzymes from Cd and Zn by forming a complex with those ions (Kneer and Zenk 1992). The involvement of PCs in tolerance to Cd is demonstrated by the findings that the inhibition of PCs' biosynthesis by treatment with buthionine sulfoximine (BSO), the inhibitor of γ-glutamylcysteine synthetase (Griffith and Meister 1979), increases the sensitivity to Cd (Mendum *et al.* 1990, Reese and Wagner 1987), and that Cd-sensitive mutants of *Arabidopsis* are deficient in PCs biosynthesis, and the mutants are also sensitive to Hg, Cu and Zn, but not to Mn (Howden *et al.* 1995a,b). It has not been fully elucidated whether PCs are involved in Ni-tolerance. Ni-hyperaccumulating plants *Alyssum lesbiacum* that survived on serpentine soils contained high amount of Ni in their shoots (above 1 - 3 % of dry mass) (Kramer *et al.* 1996). High concentration of free histidine and citrate in the xylem sap were detected. It is assumed that free histidine is involved in Ni-tolerance. However, it has not been elucidated whether free histidine is involved in the detoxification of Ni, because the cell sap prepared from intact plants must be contaminated by apoplast solutions such as xylem sap.

Cultured cells are a suitable model system for the investigation of the physiological mechanism of tolerance to environmental stress. Therefore, in this study, we selected and characterized the tobacco cell lines tolerant to $NiCl_2$. Then, the intracellular mechanism of Ni-tolerance was discussed from the data shown in this study.

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Abbreviations: BSO - L-buthionine-S,R-sulfoxime; GSH - glutathione; NIS - unselected, Ni-sensitive cell lines; NIT - Ni-tolerant cell lines; PC - phytochelatin; TRA - β-(1,2,4-triazole-3-yl)-DL-alanine
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Materials and methods

Plant cell cultures: Suspension cultures of tobacco (*Nicotiana tabacum* cv. BY-2) cells were cultured as described previously (Nakazawa *et al.* 2001). $NiCl_2$, $CdCl_2$, $CoCl_2$, histidine-HCl and citrate-Na_2 were added to medium. BSO, glutathione (GSH), and β-(1,2,4-triazole-3-yl)-DL-alanine (TRA) were added as filter-sterilized solutions. The cultures were grown on a reciprocal shaker (100 rpm) at 26 °C in the dark, and maintained by regular inoculation of 2 cm^3 of a 7-d-old culture into 30 cm^3 of the fresh medium. The 7-d-old cultures were collected by vacuum-filtration and washed once in water, then stored at -85 °C.

Selection of Ni-tolerant tobacco cells: Ni-tolerant tobacco cells were selected by progressively increasing the concentration of $NiCl_2$ in the medium. First, the parent cell lines (NIS) were inoculated into the medium containing 500 μM $NiCl_2$, then cultured for 11 d to produce a fresh mass of about 0.2 g cm^{-3}. Two cm^3 of this culture was inoculated and cultured into the medium containing 600 μM $NiCl_2$. When the cell fresh mass attained about 0.2 g cm^{-3}, 2 cm^3 of this culture was transferred again into the medium containing an equal content of $NiCl_2$. The same procedure was repeated 40 times. Then, 2 cm^3 of this culture was transferred into the medium containing 700 μM $NiCl_2$, then cultured and maintained for at least 57 generations. This cell line will be referred to as NIT700, and has maintained in the medium containing 700 μM $NiCl_2$. A portion of the Ni-tolerant cell cultures was transferred and maintained in the medium without Ni, and this cell line will be referred to as NIT 700-0. This cell line was grown in Ni-free medium for at least 10 generations before being the test of Ni-tolerance.

Selection of TRA-tolerant tobacco cells: TRA-tolerant tobacco cells were selected by progressively increasing the concentration of TRA in the medium. First, the cells were inoculated into the medium containing 20 μM TRA, then cultured to produce a fresh mass of about 0.2 g cm^{-3}. Two cm^3 of this culture was transferred again into the medium containing an equal amount of TRA. The same procedure was repeated for a year. Then, 2 cm^3 of this culture was transferred into the medium containing 30 μM TRA, then cultured and maintained.

Measurement of Ni contents in cells: The frozen cells (5 g f.m.) were homogenized in an equal volume of water, and homogenates were centrifuged at 15 000 *g* for 15 min. The precipitates were washed twice with 5 cm^3 of water. These washings and supernatants were combined (water-soluble fraction). The removed precipitates (water-insoluble fraction) were air-dried at 65 °C for 48 h, then digested with HNO_3-$HClO_4$. Ni concentrations in both fractions were measured by atomic absorption spectrophotometry (*Shimadzu AA-670*, Kyoto, Japan).

Measurement of cellular histidine contents in cells: The frozen cells were milled in liquid N_2. Ethanol [80 % (v/v)] was added to 0.5 g of the milled cell materials. The mixtures were incubated at 60 °C for 10 min, then centrifuged at 10 000 *g* for 10 min. The precipitates were washed twice with 5 cm^3 of 80 % (v/v) ethanol. The washings and supernatants were combined, and then ethanol and water evaporated under vacuum at 50 °C, and resolved in 2 cm^3 of 70 mM citrate-NaOH buffer (pH 2.2) containing 0.01 % (v/v) *n*-caprylic acid. Histidine concentration in the solution was measured by HPLC (*Shimadzu*, amino acid analyzer Na-type).

Results

Ni-tolerance of NIS, NIT700 and NIT700-0: We selected Ni-tolerant tobacco cells that could grow in the medium containing 700 μM $NiCl_2$ (NIT700 and NIT700-0). First, to test the Ni-tolerance of those cell lines, we compared the growth in the medium containing several concentrations of Ni for 7 d among the three cell lines (Fig. 1). The significant difference in the growth without $NiCl_2$ was not observed among those cell lines. The fresh mass of unselected cells treated with 700 μM $NiCl_2$ was 12 % of that without $NiCl_2$, on the contrary, the fresh mass of NIT700 and NIT700-0 cells treated with 700 μM $NiCl_2$ was not significantly different to the fresh mass of those cells grown without $NiCl_2$. From these results, it was confirmed the Ni-tolerance of NIT700 and NIT700-0 cells, and it was shown that the Ni-tolerance of NIT700 cells was maintained in the absence of

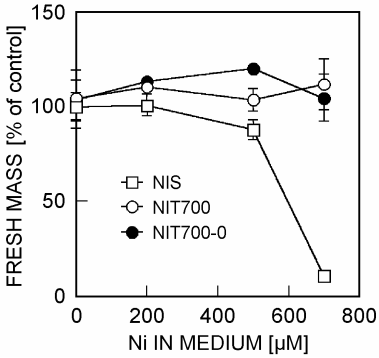

Fig. 1. Fresh mass of unselected (NIS) and selected (NIT700 and NIT700-0) cells grown in the medium containing different Ni concentrations for 7 d. The fresh mass of NIS treated without Ni was expressed as 100 %. Means ± SE (n = 9).

continuous selection pressure, since the Ni-tolerance of NIT700-0 cells were not significantly different to that of NIT700-0 cells.

Tolerance to Cd and Co of NIT700-0 cells: To determine the tolerance to Cd and Co of NIT700-0 cells, the fresh mass of unselected and NIT700-0 cells treated with or without those ions for 7 d were compared (Fig. 2). The fresh mass of NIT700-0 cells treated with $CdCl_2$ was lower than that of control cells while fresh mass of NIT700-0 cells treated with excess $CoCl_2$ was higher than that of control cells. These results indicated that NIT700-0 cells were more tolerant to Co, but not to Cd than unselected cells. The tolerance to those ions of NIT700 cells was not examined, because the interactions between those ions and Ni which was contained in medium as selection pressure might affect the cell growth.

Ni contents in unselected, NIT700 and NIT700-0 cells: The total-Ni, water-soluble and water-insoluble Ni contents were not significantly different among cell lines (Fig. 3). Total-Ni concentrations in NIT700 and NIT700-0 cells were always higher than those in medium, and approximately 90 % of cellular Ni was in water-soluble fraction. From the results of the water-soluble Ni contents of NIT700 and NIT700-0 cells and their relative water contents (*ca*. 95.2 %), their Ni concentration in symplast was estimated as about 1.6 mM.

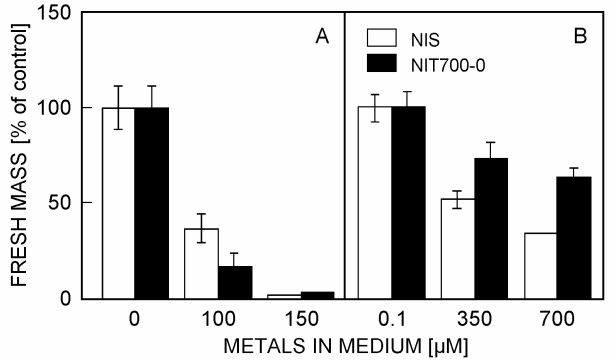

Fig. 2. Fresh mass of NIS and NIT700-0 grown in the medium containing different concentrations of $CdCl_2$ (*A*) or $CoCl_2$ (*B*) for 7 d. The fresh mass of NIS treated without Ni was expressed as 100 %. 0.1 μM $CoCl_2$ was contained in a standard medium. Means ± SE (n = 9).

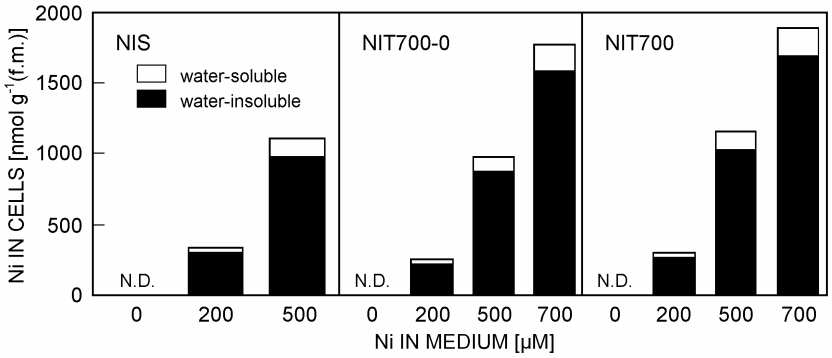

Fig. 3. Ni contents and distributions in NIS, NIT700 and NIT700-0 cells treated with $NiCl_2$. The measurements of Ni contents in NIS cells treated with 700 μM $NiCl_2$ were not done for the small amount of cell materials. Means ± SE (n = 9).

Effect of BSO on the Ni-tolerance of NIT700-0: BSO was reported as the inhibitor of PC biosynthesis (Reese and Wagner 1987). Therefore, we next examined the effect of BSO on the growth of NIT700-0 cells treated with Ni (Fig. 4), to determine the relationship between PC and Ni-tolerance in the cells. The fresh mass of NIT700-0 cells treated with 50 μM BSO alone or 700 μM $NiCl_2$ alone was not significantly different to that of untreated NIT700-0 cells. The growth of the NIT700-0 cells treated with 50 μM $CdCl_2$ was 63.9 % of that of untreated NIT700-0 cells. However, the fresh mass of cells treated with both $CdCl_2$ and BSO was 0.6 % of that of untreated NIT700-0 cells. On the contrary, the fresh mass of the NIT700-0 cells treated with both $NiCl_2$ and BSO was not significantly different to that of untreated NIT700-0 cells.

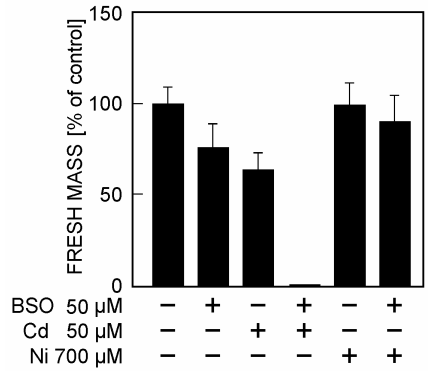

Fig. 4. Effect of the administration of BSO on the growth of NIT700-0 cells treated with $CdCl_2$ or $NiCl_2$. The fresh mass of NIS treated without this chemical was expressed as 100 %. Means ± SE (n = 9).

The effects of exogenous histidine, citrate and GSH on the growth inhibition of unselected cells by Ni: The effects of 700 μM of histidine, citrate and GSH on the growth of unselected cells treated with and without 700 μM $NiCl_2$ were examined (Fig. 5). The additions of those chelators alone did not significantly affect the growth of cells treated without $NiCl_2$. The cell growth in the presence of 700 μM $NiCl_2$ alone was approximately 10 % of that in the absence of $NiCl_2$, on the contrary, the growth of the cells simultaneously treated with $NiCl_2$ and histidine was not significantly different to that of control cells. The cell growth treated with both $NiCl_2$ and citrate, and $NiCl_2$ and GSH was 72 and 30 % of that of control cells, respectively. Thus, the cell growth inhibition by $NiCl_2$ was reduced by addition of histidine most effectively, followed by citrate and GSH. On the other hand, Ni concentration in unselected cells simultaneously treated with $NiCl_2$ and histidine, and that with $NiCl_2$ and citrate were 1.22 and 1.40 times higher than that treated with the equal amount of $NiCl_2$, respectively (data not shown).

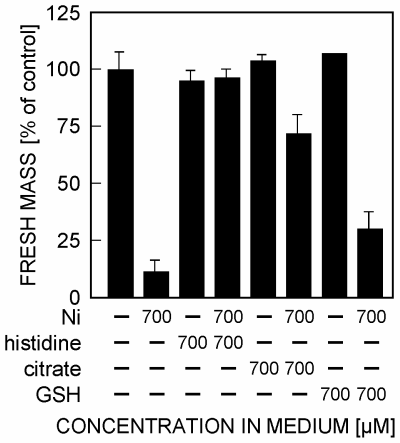

Fig. 5. Effects of the administrations of exogenous histidine, citrate and GSH on the growth of NIS cells treated with $NiCl_2$. The fresh mass of NIS treated without those chemicals was expressed as 100 %. Means ± SE (n = 9).

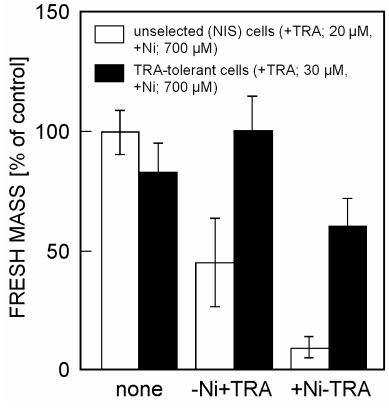

Fig. 6. Fresh mass of unselected (NIS), and TRA-tolerant cells grown in the medium containing TRA or Ni for 7 d. The fresh mass of unselected cells treated without TRA or $NiCl_2$ was expressed as 100 %. Means ± SE (n = 9).

Histidine contents in NIT700-0 cells: The histidine contents in unselected and NIT700-0 cells treated with $NiCl_2$ were measured (Fig. 7). The significant difference in histidine cellular contents between unselected and NIT700-0 untreated cells was not observed. However, histidine contents in NIT700-0 cells increased due to treatment with 700 μM $NiCl_2$ than in unselected cells. The histidine contents of unselected cells treated with 700 μM $NiCl_2$ were not able to be measured, since the cells were dead by Ni-treatment.

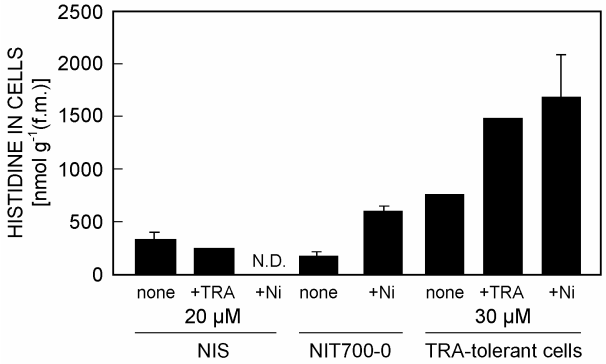

Fig. 7. Histidine content in unselected (NIS), NIT700-0 and TRA-tolerant cells treated with or without Ni. The measurements of histidine contents in unselected cells treated with 700 μM $NiCl_2$ were not done for the small amount of cell materials. Means ± SE (n = 9).

Selection of TRA-tolerant cells and those histidine contents and Ni-tolerance: Next, we selected suspension cultured tobacco cells which were tolerant to histidine analogue, TRA. First, to test the TRA-tolerance, we compared the growth in the medium containing 30 μM TRA for 7 d between unselected and TRA-tolerant cell lines (Fig. 6). The significant difference in the growth between cell lines, were not observed. On the other hand, the growth of unselected cells was fully inhibited by treatment with 30 μM TRA, on the contrary, the growth of TRA-tolerant cells treated with 30 μM TRA was not significantly different to the growth of untreated cells.

Next, to determine the Ni-tolerance of TRA-tolerant cells, the growth of unselected and TRA-tolerant cells treated with or without Ni for 7 d were compared (Fig. 6). The relative growth of TRA-tolerant cells treated with Ni was higher than that of control cells. These results indicated that TRA-tolerant cells were more tolerant to Ni than unselected cells.

The histidine contents in unselected and TRA-tolerant cells treated with and without TRA or Ni were measured (Fig. 7). The significant difference in histidine contents between unselected cells treated without TRA and those

with TRA was not observed. However, histidine contents in TRA-tolerant cells treated with or without TRA were higher than unselected cells without TRA. Additionally, histidine contents in TRA-tolerant cells were elevated by TRA-treatment or Ni-treatment.

Discussion

In this study, we selected and characterized Ni-tolerant tobacco cells (NIT700 and NIT700-0) to analyze Ni-tolerant mechanism. Ni-tolerant cells selected in this study can grow in the medium containing 700 μM $NiCl_2$, on the contrary, the growth of unselected cells is inhibited markedly (Fig. 1). NIT700-0 cells were also tolerant to excess $CoCl_2$ (Fig. 2). This result indicates that the physiological mechanisms of Ni-tolerance are similar to those of Co-tolerance in these cell lines. Probably, this is due to the similarities in chemical and biochemical properties between the two metals. For example, Ni is the co-factor of the enzyme urease (Dixon *et al.* 1975), and Co also activate this enzyme (Watanabe *et al.* 1994). In the xylem sap of *Alyssum lesbiacum*, Ni-hyper-accumulating plants, free histidine was accumulated by not only Ni-stress but also Co-stress (Kramer *et al.* 1996).

Next, we compared the Ni cellular contents and distributions in NIS and NIT cells (Fig. 3), to determine whether Ni-tolerance is due to Ni cellular exclusion from cytoplasm and the adsorption in cell wall. In this study, it is postulated that water-soluble Ni fraction corresponds to Ni in symplast, and water-insoluble fraction corresponds to Ni in cell wall. The difference in Ni contents among NIT700, NIT700-0 and NIS cells were not observed, in addition, the amount of water-soluble Ni was larger than that of water insoluble Ni. These results indicate the possibility that the Ni-tolerance may be connected with the synthesis of Ni-insensitive enzymes and/or the accumulation of chelators such as PC, histidine and organic acids.

Plant cells treated with excess heavy metals such as Cd induce the biosynthesis of PC (Grill *et al.* 1987, Maitani *et al.* 1996). As described in introduction, it is considered that PC is involved in Cd-tolerance, whereas, it is unclear whether PC is involved in Ni-tolerance. Therefore, we next examined the effects of BSO on the growth of NIT700-0 cells treated with Ni. BSO was reported as the specific inhibitor of γ-glutamylcysteine synthetase which was the first step enzyme of GSH biosynthesis pathway (Griffith and Meister 1979), and the treatment with BSO to plant cells inhibited the biosynthesis of GSH and PC, and simultaneous treatment with Cd and BSO caused the increase of their Cd-sensitivities and growth inhibition (Mendum *et al.* 1990, Reese and Wagner 1987). In this study, the similar effect of Cd and BSO was observed in NIT700-0 cells (Fig. 4), which confirmed that PC biosynthesis play an important role in the Cd-tolerance of this cell line. On the contrary, BSO-treatment to the NIT700-0 cells did not affect their Ni-tolerance (Fig. 4). On the other hand, the addtion of GSH slightly reduced the inhibitory effect of Ni on the growth of unselected cells (Fig. 5). In previous paper, we reported that GSH inducted of PC biosynthesis in response to Cd exposure and the Cd-tolerance in tobacco cells (Nakazawa and Takenaga 1997). Furthermore, the Cd-tolerance of NIT700-0 cells was not stronger than that of NIS (Fig. 2). These findings suggest that the physiological mechanism of Ni-tolerance of NIT700-0 cells is dissimilar to Cd-tolerance mechanism, and the biosynthesis of PC dose not play an important role in the mechanism of Ni-tolerance in NIT700-0 cells. Therefore, we next analyzed the relationship between the Ni-tolerance of NIT700-0 cells and several chelators that were reported as heavy metal-scavengers in plant tissue.

In xylem sap and cell sap from several Ni-hyper-accumulating plants, high contents of free histidine and citrate were detected (Kramer *et al.* 1996, Lee *et al.* 1977), and the concentration of histidine was elevated under Ni-stress (Kramer *et al.* 1996). Hence, we examined the effects of exogenous histidine and citrate on Ni-toxicity to NIS (Fig. 5). The results were that the inhibitory effect of Ni (700 μM) on the cell growth was fully neutralized by 700 μM histidine. The addition of the same concentration of citrate also partially reduced the cell growth inhibition by Ni. These results suggest that histidine and citrate participate in the mechanism of Ni-tolerance of tobacco cells. Moreover, Ni-contents in NIT700-0 cells treated with 700 μM Ni and those chelators were higher than that in NIT700-0 cells treated Ni alone. Thus, the reduction of Ni-toxicity was not due to the prevention of Ni-absorption by chelating Ni in medium.

Next, we compared histidine contents in NIS and NIT700-0 cells untreated and treated with Ni (Fig. 7). It was suggested that the biosynthesis of histidine was induced by Ni-stress, and that the biosynthesis abilities of NIT700-0 cells were higher than NIS. However, histidine contents in NIT700-0 cells treated with Ni were lower than their Ni contents (Fig. 3, Fig. 7). These results indicate that the other chelators such as citrate may also participate in Ni-tolerance, or that a part of Ni may be distributed in vacuole as free ion in NIT700-0 cells.

For the confirmation of involvement of histidine in Ni-tolerance, we analyzed plants with altered histidine contents. We selected suspension cultured tobacco cells tolerant to histidine analogue TRA, and analyzed their Ni-tolerance. Histidine biosynthesis is regulated by negative feedback inhibition of ATP phosphoribosyl

transferase (EC 2.4.2.17, first enzyme of histidine biosynthesis pathway) by histidine. TRA inhibit the enzyme as well as histidine, consequently, TRA-treatment results histidine-deficiency and the inhibition of growth. TRA-tolerant cells had higher contents of histidine and Ni-tolernce than unselected cells (Figs. 6, 7).

In conclusion, from the results shown in this paper and the finding that histidine forms a complex with Ni ion *in vitro* (Kramer *et al.* 1996), it is strongly suggested that free histidine participates in the cellular detoxification of excess Ni.

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