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Increase of free cysteine and citric acid in plant cells exposed to cobalt ions

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Abstract

Cobalt complexation was investigated in a suspension cell culture of the cobalt hyperaccumulator *Crotalaria cobalticola*. *C. cobalticola* cells were more tolerant towards cobalt ions than the suspension cells of the non-accumulators *Rauvolfia serpentina* and *Silene cucubalus*. While the concentration of various compounds increased in cells of *C. cobalticola* challenged with cobalt ions, phytochelatin biosynthesis was not induced. Instead, the exposure to cobalt ions resulted in the increase of citrate and cysteine in cells. Size exclusion chromatography demonstrated the co-elution of cobalt and cysteine in *C. cobalticola* cell extracts. A significant increase in cysteine was observed also in cells of *R. serpentina* and *S. cucubalus* when they were exposed to cobalt ions. These results suggest that free cysteine is involved in cobalt ion complexation in plant cells. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *Crotalaria cobalticola* Duvign.; Fabaceae; Cobalt complexation; Metal hyperaccumulation; Cysteine; Citric acid

1. Introduction

A considerable number of plants are known to accumulate heavy metal ions such as nickel, zinc, copper, cobalt, and lead in unusually high concentrations in their shoots. These plants are termed metal hyperaccumulators (Baker et al., 1999). While the phenomenon of metal hyperaccumulation by plants has been known for many years, the biochemical and molecular fundamentals of metal detoxification and accumulation are only recently better understood.

The compounds involved in metal complexation in hyperaccumulators include organic acids and free histidine. The existence of various metal-organic acid complexes in plants was demonstrated (Lee et al., 1977; Mathys, 1977; Sagner et al., 1998) or predicted by computer modelling (Rauser, 1999). When the nickel hyperaccumulator *Alyssum lesbiacum* was exposed to nickel ions, a strong increase in histidine was observed and the complexation of nickel by histidine was also demonstrated (Krämer et al., 1996). However, histidine was clearly not involved in nickel accumulation in nickel hyperaccumulator *Thlaspi goesingense* (Persans et al., 1999), and it was suggested that the "histidine effect" is limited only to selected hyperaccumulators (Clemens, 2001). Phytochelatins (PCs), cysteine-rich peptides that are crucial for the detoxification of many metals in plants, were not reported to be involved in the hyperaccumulation of any heavy metal.

While it has been known for many years that cobalt is an essential element for humans, animals and prokaryotes, a biological function for this element in higher plants has not been identified. The cobalt-containing vitamin B_{12} does not occur in plants. Until now, there is only one study that indicates that cobalt might have a physiological function in algae. The enzyme that catalyses decarbonylation of a fatty aldehyde to a hydrocarbon and carbon monoxide in the green alga

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Botyrococcus braunii was suggested to contain a porphyrin moiety with cobalt in its centre (Dennis and Kolattukudy, 1992). No other functions of cobalt in plants have been reported until present. While normally plants contain Co^{2+} in concentrations as low as 1–50 μg g^{-1} dry weight (Peterson and Girling, 1981), 28 cobalt hyperaccumulators were reported to accumulate more than 1000 μg cobalt g^{-1} dry weight in their shoots (Baker et al., 1999).

Cobalt detoxification and accumulation mechanisms in plants have not been extensively studied until now. It has been reported previously that cobalt is one of those heavy metals that do not activate PC synthase (Zenk, 1996). It was therefore suggested that cobalt cannot be detoxified via the phytochelatin system in plant cells. In contrast, two other studies claimed that cobalt can activate *Arabidopsis* and rice PC synthases (Vatamaniuk et al., 2000; Yan et al., 2000). While the reports on PC synthase activation by cobalt have been recently refuted (Oven et al., 2002), other ligands which could be involved in the complexation of this metal in plants have not yet been identified.

We addressed the question of cobalt complexation in plants in the present study. We used a suspension cell culture of the cobalt hyperaccumulator *Crotalaria cobalticola* Duvign. as a model system. This plant grows endemically in the Shaba province of the Democratic Republic of Congo, and is reported to accumulate cobalt in its shoots in concentrations as high as 3000 μg Co^{2+} g^{-1} of dry matter (Brooks et al., 1981). Our goal was to clarify PC synthase activation by cobalt ions and to identify compounds that are involved in cobalt complexation in plant cells.

2. Results

2.1. Cultured cells of C. cobalticola are tolerant towards cobalt

The comparison between cobalt tolerance of *C. cobalticola* cells and cells of two randomly chosen non-accumulating plants, *R. serpentina* and *S. cucubalus*, is presented in Fig. 1. While 50% growth inhibition of *R. serpentina* and *S. cucubalus* cells occurred at 2.8 mM $CoCl_2$ and 3.2 mM $CoCl_2$ in the medium, respectively, the same level of growth inhibition of *C. cobalticola* cells occurred when the Co^{2+} concentration in the medium was as high as 24 mM (Fig. 1). The analysis of cobalt distribution in the *C. cobalticola* system showed that 68% of the recovered Co^{2+} was found in the medium and 32% of the recovered Co^{2+} was associated with the filtered cells. When filtered cells were washed with 10 mM EDTA, another 10% of the recovered Co^{2+} were removed, indicating that this portion of metal ions was bound to the cell wall. We conclude that

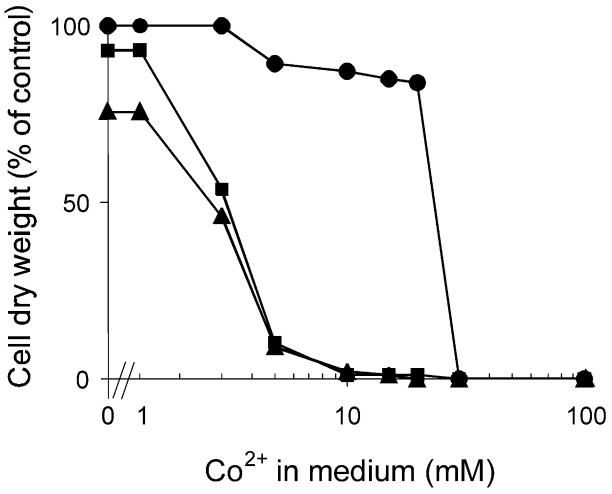

Fig. 1. Growth inhibition of plant suspension cells by cobalt ions. Cells of *Silene cucubalus* (▲), *Rauvolfia serpentina* (■), and *Crotalaria cobalticola* (●) were grown in LS medium containing $CoCl_2$ at various concentrations for 7 days.

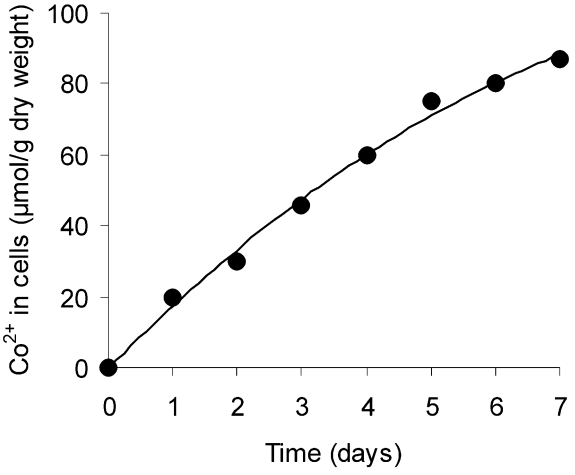

Fig. 2. Time-course of Co^{2+} uptake by *Crotalaria cobalticola* suspension cells. Cells were grown in LS medium supplemented with 10 mM $CoCl_2$ for 7 days, harvested at 24-h intervals and washed with 10 mM EDTA prior to AAS analysis.

the rest, i.e. 22% of recovered Co^{2+} (corresponding to 75 μmol Co^{2+} g^{-1} dry matter), was taken up by *C. cobalticola* cells. The kinetic studies showed that Co^{2+} was taken up by *C. cobalticola* suspension cells during the entire cultivation period (Fig. 2).

Studies of tolerance of *C. cobalticola* suspension cells towards other heavy metals such as Ni^{2+}, Cu^{2+}, and Cd^{2+} demonstrated that 50% growth inhibition occurred at 2.0 mM Ni^{2+}, 0.5 mM Cd^{2+}, and 0.5 mM Cu^{2+}. Similar levels of growth inhibition by these metal ions were observed also when suspension cells of non-accumulating plants were investigated (data not shown). *C. cobalticola* suspension cells can obviously tolerate especially high concentrations of cobalt, but not heavy metals in general.

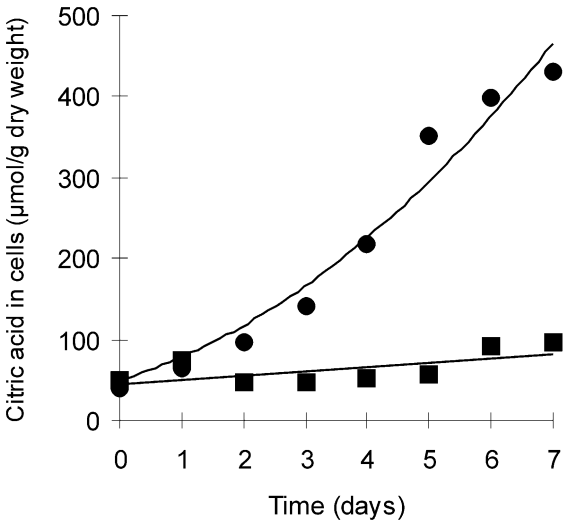

Fig. 3. Time-course of citric acid increase in *Crotalaria cobalticola* control cells (■) and in cells challenged with 10 mM $CoCl_2$ (●). *Crotalaria cobalticola* cells were harvested at 24-h intervals. Citric acid concentration in cell extracts was determined by the citrate lyase assay.

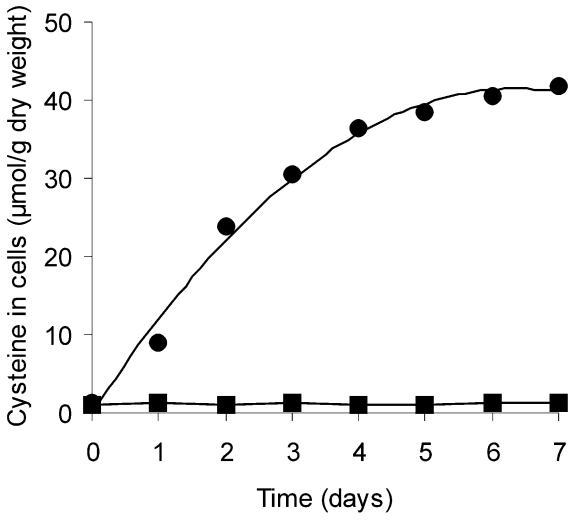

Fig. 4. Time-course of cysteine increase in *Crotalaria cobalticola* control cells (■) and in cells exposed to 10 mM $CoCl_2$ (●). Cell samples were collected every 24 h for 7 days. Cysteine concentration in cell extracts was determined by the cysteine assay.

2.2. Induction of low molecular weight compounds by cobalt ions

The occurrence of organic acids in extracts of *C. cobalticola* cells was investigated first by thin layer chromatography (TLC). The exposure of *C. cobalticola* cells to 10 mM $CoCl_2$ for 7 days resulted in the increase of one compound that was present in much lower concentrations in the control cells. This compound had the same R_f value as a citric acid standard. To verify that the induced compound was citric acid, the quantitative "citrate lyase" enzyme assay was applied. The results of this assay confirmed that exposure of *C. cobalticola* cells to 10 mM $CoCl_2$ for 7 days resulted in a 6.4-fold citric acid increase from 75 µmol g^{-1} dry weight (control cells) to 480 µmol g^{-1}. The kinetic studies showed that the citric acid concentration increased during the entire period of cell cultivation in cells exposed to cobalt ions, while no such change was observed in control cells (Fig. 3).

The effect of cobalt on the free amino acid content in *C. cobalticola* cells was initially investigated by TLC as well. When compared to the extracts of control cells, TLC revealed that the concentration of one compound increased in cells exposed to 10 mM Co^{2+} for 7 days. This compound was identified as cysteine after co-chromatography with 20 proteinogenic amino acids. The identification of cysteine as a cobalt-induced compound was verified by RP–HPLC analysis with DTNB post-column derivatization and by a cysteine assay. The challenge of *C. cobalticola* cells with 10 mM Co^{2+} for 7 days resulted in a 31-fold increase of free cysteine from 1.1 µmol g^{-1} dry weight (control cells) to 35 µmol g^{-1} dry weight. Kinetic studies demonstrated a high cysteine

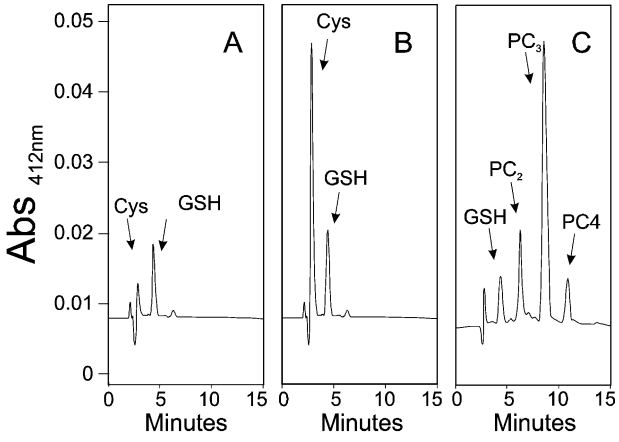

Fig. 5. HPLC analysis of thiol-containing compounds in *Crotalaria cobalticola* cells. Cells were grown (A) in LS-medium, (B) in LS-medium supplemented with 5 mM $CoCl_2$, or (C) in LS-medium supplemented with 100 µM $Cd(NO_3)_2$ for 7 days.

increase during the first 4 days after cells were challenged with cobalt ions (Fig. 4).

Our previous experiments demonstrated that cobalt does not induce phytochelatin biosynthesis in plants (Zenk, 1996). In contrast, recently it has been claimed that cobalt can activate recombinant *Arabidopsis* PC synthase (Vatamaniuk et al., 2000) and purified rice PC synthase (Yan et al., 2000). Fig. 5 B shows that PC synthesis in *C. cobalticola* cannot be induced by 5 mM $CoCl_2$ in the medium. Hence, low amounts of PC_2 could be detected also in cells exposed to cobalt ions, but since the same amount of PC_2 could also be observed in the control cells (Fig. 5A), this synthesis obviously resulted from Zn^{2+} and Cu^{2+} ions that were present in the medium (Grill et al., 1988). *C. cobalticola* PC synthase

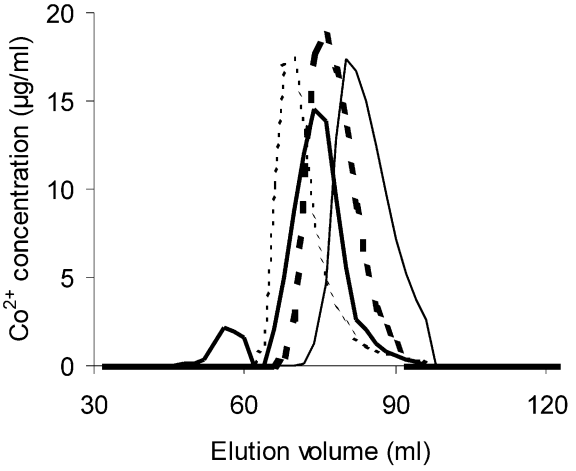

Fig. 6. Size exclusion chromatography of various cobalt complexes. Extracts of *Crotalaria cobalticola* cells grown in medium containing 10 mM $CoCl_2$ for 7 days (—), in vitro synthesized cobalt–citrate complex (- - -), in vitro synthesized cobalt–cysteine complex (- - -), and 1 mM $CoCl_2$ (—) were individually applied on a Sephadex G-25 column. Two millilitre fractions were collected and investigated for Co^{2+} by AAS.

was, however, clearly activated when cells were cultivated in medium containing 100 μM $Cd(NO_3)_2$ (Fig. 5C). Although the cobalt concentration was 50-fold higher than that of cadmium ions, cobalt could not activate PC synthase. Instead of PCs, a strong increase in cysteine could clearly be detected in cells challenged with cobalt (Fig. 5B), but neither in the control cells nor in cells exposed to cadmium (Fig. 5A,C). In addition, it should be noted here that exposure of *C. cobalticola* cells to 5 mM $CoCl_2$ for 7 days did not effect GSH concentration in plant cells (Fig. 5A,B).

A cysteine increase was also detected when cells of non-accumulating plants were challenged with cobalt (Table I). A 12 and 9-fold increase in cysteine was observed in *R. serpentina* and *S. cucubalus* cells, respectively, after these cells were challenged with 1 mM $CoCl_2$ for 7 days.

2.3. Studies on cobalt complexation

The involvement of citric acid and cysteine in cobalt complexation in plant cells was investigated by size exclusion chromatography on a Sephadex G-25 column. This analysis revealed that cobalt occurs in two complexes in *C. cobalticola* cells (Fig. 6). While 0.24 μmol or 15% cobalt was found in a high molecular weight (HMW) complex in cells, 1.6 μmol or 85% cobalt occurs in a complex of lower molecular weight (LMW).

When size exclusion chromatography fractions were investigated for cysteine, this amino acid was found only in the cobalt-containing fractions of the LMW complex. The elution of an in vitro synthesized cobalt–cysteine complex correlated best with the endogenous cobalt-containing LMW complex of *C. cobalticola* cells. These observations suggest the occurrence of a cobalt–cysteine complex in cells, although the involvement of citrate in cobalt complexation cannot be fully excluded (Fig. 6). The cysteine content in fractions containing the LMW complex was 1.15 μmol, and the cobalt to cysteine ratio in the LMW complex was 1.4:1. The cobalt ligand in the HMW complex remains yet unidentified.

3. Discussion

We investigated cobalt complexation in cells of the cobalt hyperaccumulator *Crotalaria cobalticola*. Recently, it was suggested that cobalt ions activate PC synthase (Vatamaniuk et al., 2000; Yan et al., 2000). Our previous investigations performed with purified *S. cucubalus* PC synthase (Zenk, 1996), with recombinant *Arabidopsis* and *Glycine max* PC synthases (Oven et al., 2002) as well as the results presented in this study do not confirm that finding. We state here that phytochelatins are clearly not involved in cobalt complexation and detoxification in plants.

Although phytochelatins do not participate in cobalt complexation, their pivotal role in the detoxification and homeostasis of many other metals should not be doubted. Numerous studies have demonstrated that phytochelatins are essential for metal detoxification in plants (Howden et al., 1995; Zenk, 1996). This needs to be clearly stated here due to a recent report that suggests that phytochelatins have "a secondary role in Cd tolerance" in the Zn and Cd hyperaccumulator *Thlaspi caerulescens* (Ebbs et al., 2002). Claims have also been made that cadmium detoxification occurs via a "novel phytochelatin-independent mechanism" in azuki beans (Inouhe et al., 2000), which finding was later refuted (Oven et al., 2001). Cadmium tolerance is known to be a complex mechanism consisting of several components (i.e. metal uptake and transport over the membrane, complexation by phytochelatins, sequestration of metal–phytochelatin complexes in the vacuole and incorporation of labile sulphur into metal–phytochelatin complexes). The relative importance of anyone of these mechanisms cannot yet be assigned.

Since cobalt does not activate PC-synthase, plants must have developed a different detoxification mechanism in order to prevent the toxic effect of cobalt ions on cellular proteins. We observed a high increase in both citric acid (6.4-fold increase) and cysteine (31-fold increase) concentrations in *C. cobalticola* cells that were challenged with cobalt ions. Citric acid increases in metal accumulators were observed previously (Sagner et al., 1998; Rauser, 1999). The effect of heavy metal ions on the cysteine content of plant cells has not been reported until now. The observed cysteine increase was not limited to suspension cells of the cobalt accumulator *C. cobalticola*, but was significant also in cells of the

non-accumulating plants *S. cucubalus* and *R. serpentina* when they were cultivated in a cobalt-containing medium. These results suggest that a cysteine increase is a general response of plant cells that have been challenged with cobalt ions.

The identification of cysteine as a cobalt-induced compound prompted us to investigate cobalt complexation by cysteine. Several results suggest the involvement of cysteine in cobalt complexation in plants. First, when cobalt and cysteine solutions are mixed at pH 7.0, a yellow-coloured complex is instantly formed. Previous studies suggested the occurrence of a strong cobalt–cysteine complex at this pH (pK value 2.5×10^{15}; Dawson et al., 1993). This complex is significantly stronger when compared to, for example, a cobalt-citrate complex (pK value 10^5 at pH 7.0; Dawson et al., 1993). Second, size exclusion chromatography of *C. cobalticola* cell extracts demonstrates the co-elution of cobalt and cysteine in a low molecular weight cobalt-containing complex. Realizing the tendency for the formation of a cobalt–cysteine complex as discussed earlier, the occurrence of such a complex in fractions containing cobalt and cysteine is very likely. And third, the elution of the in vitro synthesized cobalt–cysteine complex correlated well with the elution of cobalt and cysteine in the LMW complex from *C. cobalticola* cells.

The electronic status of cobalt, which belongs to the ninth group of the periodic system, might be the reason why cobalt fails to activate PC synthase. Recently we reported that only 15 metals belonging to the 11th to 15th group in the fourth, fifth and sixth period of the periodic system can activate PC synthase (Oven et al., 2002). Furthermore, while the occurrence of metal–thiolate complexes was recognized to be essential in solutions containing soft metal ions and thiol compounds (Lippard and Berg, 1995; Falbe and Regitz, 1999), we also observed that the characteristics of individual metals and individual thiols are crucial for PC synthase activation. For example, while a cadmium–cysteine complex could strongly activate *Arabidopsis* PC synthase, the level of enzyme activation by a zinc-cysteine complex was 74-fold lower (Oven et al., 2002). The characteristics of a cobalt-cysteine complex might be a reason for cobalt failing to activate PC synthase in vivo.

The observed cobalt to cysteine ratio in the low molecular weight complex was found to be approximately 1.4:1. This ratio suggests that cysteine is not the only compound involved in cobalt complexation in *C. cobalticola* cells. If cysteine would be the only cobalt ligand in this complex, then the occurrence of at least two cysteinyl −SH groups per cobalt ion should be expected. When metal coordination is considered, even more cysteinyl −SH groups should be anticipated in the complex. Since citric acid is known to be involved in complexation of various metals in plants (Sagner et al., 1998; Rauser, 1999), this compound cannot be ruled out as a possible cobalt ligand in *C. cobalticola* cells. Citric acid concentration was shown to significantly increase in cobalt-exposed *C. cobalticola* cells (Fig. 3), and size exclusion chromatography results do not exclude the involvement of citric acid in cobalt complexation (Fig. 6). We have observed that the citric acid to cobalt ratio in *C. cobalticola* cells was approximately 5.6:1 and that the concentration of citric acid alone would be sufficient to complex cobalt ions in these cells. Although the involvement of citrate in cobalt complexation is indeed possible, the structure of cobalt complex(es) in *C. cobalticola* have not yet been elucidated. It is possible that cysteine is a cobalt ligand in the cytosol, while citrate could complex cobalt ions in the vacuole. A similar model was proposed recently for nickel complexation in the Ni-hyperaccumulator *Thlaspi goesingense* (Krämer et al., 2000). These authors suggested that free histidine shuttles nickel ions through the cytoplasm and organic acids are nickel ligands in the vacuole (Krämer et al., 2000). Alternatively, both cysteine and citrate might be ligands in individual cobalt complexes in *C. cobalticola*. Further studies addressing cobalt, cysteine and citrate compartmentation are necessary to better understand cobalt complexation in plant cells.

The identification of cysteine as a compound involved in cobalt complexation might be useful for designing cobalt-tolerant or cobalt-accumulating plants in the future. Recently it was shown that the cysteine biosynthetic pathway can be genetically manipulated in plants (Dominguez-Solis et al., 2001). Furthermore, plants with increased cysteine synthesis tolerated cadmium significantly better than non-transformed plants, presumably because of the cysteine involvement in phytochelatin synthesis (Dominguez-Solis et al., 2001). However, the manipulation of cysteine biosynthesis alone might prove to be insufficient for the design of cobalt tolerant and hyperaccumulating plants. Cobalt ions induced a cysteine increase not only in *C. cobalticola* cells, but also in cells of non-tolerant plants (Table 1), suggesting that there are other cellular mechanisms that enable cobalt tolerance and hyperaccumulation. A similar effect has been recently observed when nickel complexation was investigated

Table 1
Cysteine content in *Silene cucubalus* and *Rauvolfia serpentina* control cells and in cells challenged with 1 mM $CoCl_2$ for 7 days (in μmol cysteine per g cell dry weight). Cysteine concentration was determined by HPLC analysis

	Control cells	Cells exposed to $CoCl_2$	Cysteine increase (-fold)
R. serpentina	0.41	5.0	12
S. cucubalus	0.73	6.3	9

(Krämer et al., 2000). In that study, free histidine was shown to be involved in nickel complexation in both the Ni-hyperaccumulator *T. goesingense* and in its non-accumulating relative *Thlaspi arvense*, but in contrast to *T. goesingense*, *T. arvense* could not tolerate high Ni-concentrations in the media (Krämer et al., 2000). These authors proposed that an efficient vacuolar compartmentation rather than cytosolic complexation of nickel by histidine is decisive for nickel tolerance and accumulation by *T. goesingense* (Krämer et al., 2000). If this model proves to be correct, then the manipulation of cysteine biosynthesis alone might prove to be beneficial, but insufficient where the design of commercially useful cobalt tolerant and cobalt-accumulating plants would be concerned.

4. Experimental

All experiments were performed in triplicate.

4.1. Plant material

Suspension cells of *C. cobalticola., R. serpentina* and *S. cucubalus* originated from the plant cell culture collection of the Institut für Pharmazeutische Biologie, Universität München and had each been maintained in culture for more than 10 years. Cells were cultivated in 1-l Erlenmeyer flasks containing 400 ml of Linsmaier-Skoog medium (Linsmaier and Skoog, 1965) at 23 °C in diffuse light (650 lux) on a gyratory shaker (100 rpm). Suspension cells were transferred into fresh medium every seven days.

4.2. Chemicals

All chemicals were of p.A. grade and originated from Fluka (Neu-Ulm), Merck (Darmstadt), Roth (Karlsruhe) and Sigma Aldrich (Deisenhofen).

4.3. Extraction of plant cells

Plant suspension cells were vacuum filtered, washed and flash frozen with liquid nitrogen. For thin layer chromatography and for the cysteine assay, frozen cells were collected and extracted in 20 mM Tris–HCl pH 8.0 (1 ml buffer per 1 g cells). Cell extracts were sonicated (pulse sonication, 10 s), centrifuged (10 min, 10 000×*g*, 4 °C) and used in subsequent analysis.

For HPLC analysis, the extraction proceeded by combining 400 mg frozen cells and 600 μl 1 N NaOH that contained $NaBH_4$ (1 mg ml^{-1}). Following sonication (Branson Sonifier B-12; pulse sonication, 1×10 s) and centrifugation (5 min, 10 000×*g*, room temperature), 500 μl supernatant were transferred to a new tube and acidified by the addition of 100 μl 3.6 N HCl. Samples were incubated for 5 min on ice, once more centrifuged (5 min, 10 000×*g*, room temperature) and injected onto an HPLC.

For size exclusion chromatography, 10 ml extraction buffer (10 mM Tris–HCl pH 8.0 with 10 mM β-mercaptoethanol) were added to 10 g frozen cells. Thawed cell extracts were sonicated (Branson Sonifier B-12; pulse sonication, 3×30 s) and centrifuged (10 min, 10 000×*g*, 4 °C).

4.4. TLC

For the investigation of organic acids, 20 μl of cell extract was applied to a cellulose plate (20×20 cm; Merck). The plate was placed in a glass chamber containing the mobile phase formic acid/ethanol/water 48.8:48.8:2.4, v/v/v. Following resolution, the plate was thoroughly dried for 1 day at room temperature and sprayed with a dichlorophenol–indophenol reagent (100 mg 2,6-dichlorophenol-indophenol in 100 ml ethanol). Blue spots on the plate indicated the occurrence of organic acids.

TLC for amino acid analysis was performed by resolving 20 μl of supernatant on a silica gel plate (20×20 cm; Kieselgel 60; Merck; mobile phase chloroform/methanol/25% ammonium hydroxide/water 40:40:15:15, v/v/v/v). Following separation, the plate was air dried and sprayed with a ninhydrin reagent (200 mg ninhydrin in 100 ml ethanol). Red spots indicated the occurrence of individual amino acids.

4.5. RP-HPLC analysis

Cell extracts were prepared as given earlier. The acidified supernatant (100 μl) was injected onto an RP–HPLC (Pharmacia HPLC System; solvent A: 99.9% water, 0.1% trifluoroacetic acid; solvent B: 79.9% water, 20% acetonitrile, 0.1% trifluoroacetic acid; flow rate: 2 ml min^{-1}; column CC250/4 Nucleosil 100–10 C18 with a precolumn CC8/4 Nucleosil 100–10 C18, both Macherey-Nagel). The resolved samples were detected at 412 nm after an online post-column derivatization by a DTNB reagent (75 μM DTNB in 50 mM potassium phosphate buffer pH 8.0 with 10% acetonitrile, v/v), and thiol compounds were detected at 412 nm as described previously (Kneer and Zenk, 1997).

4.6. Cysteine assay

A quantitative determination of cysteine in plant cells was conducted using a photometric method (Gaitonde, 1967). Sixty microlitres of 50 mM DTT were added to 60 μl of cell extracts. The sample was incubated at room temperature for 15 min, after which 120 μl acetic acid and 120 μl acid–ninhydrin reagent (250 mg ninhydrin with 6 ml acetic acid and 4 ml HCl) were added to the

sample. The assay mixture was incubated at 95 °C for 10 min, after which cold ethanol (840 μl) was added. The mixture was then chilled on ice and the cysteine content measured spectrophotometrically at 560 nm.

4.7. Citrate-lyase assay

The citric acid content in plant cell extracts was determined by a citrate lyase enzymatic assay according to the manufacturer's instructions (''citric acid'' kit, Boehringer Mannheim).

4.8. Determination of cobalt

Cobalt concentration was determined by atomic absorption spectroscopy (AAS) using a Perkin-Elmer 1100B Atomic Absorption Spectrometer (flame-mode, hollow cathode lamp, wavelength 240.7 nm; Bachofer).

4.9. In vitro synthesis of cobalt–cysteine and cobalt–citrate complexes

The cobalt–cysteine complex was assembled in vitro by reacting 1 mM cysteine and 1 mM $CoCl_2$ in 20 mM Tris–HCl pH 8.0 at room temperature. The in vitro formation of a cobalt–citrate complex resulted from combining 1 mM citric acid and 1 mM $CoCl_2$ in 20 mM Tris–HCl pH 8.0 at room temperature.

4.10. Size exclusion chromatography

Size exclusion chromatography was performed on a Bio-Logic Workstation (BioRad; flow-rate 2 ml min^{-1}). Cell extracts were prepared as described earlier. Supernatant (5 ml) was applied onto a Sephadex G-25 column (640×16 mm; Pharmacia) that was pre-equilibrated with running buffer (10 mM Tris–HCl pH 8.0 containing 100 mM NaCl). Fractions of 2 ml were collected and analyzed for cobalt (AAS) or thiol (RP–HPLC) content.

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