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Cellular compartmentation of nickel in the hyperaccumulators *Alyssum lesbiacum*, *Alyssum bertolonii* and *Thlaspi goesingense*

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Abstract

Nickel uptake and cellular compartmentation were investigated in three Ni hyperaccumulators: *Alyssum bertolonii* (Desv), *Alyssum lesbiacum* (Candargy) and *Thlaspi goesingense* (Hálácsy). The three species showed similar hyperaccumulation of Ni, but *T. goesingense* was less tolerant to Ni than the two *Alyssum* species. An addition of 500 mg Ni kg^{-1} to a nutrient-rich growth medium significantly increased shoot biomass of all three species, suggesting that the Ni hyperaccumulators have a higher requirement for Ni than normal plants. Energy-dispersive X-ray microanalysis (EDXA) was performed on frozen-hydrated tissues of leaves (all species) and stems (*Alyssum* only). In all species analysed, Ni was distributed preferentially in the epidermal cells, most likely in the vacuoles, of the leaves and stems. In stems, there was a second peak of Ni in the boundary cells between the cortical parenchyma and the vascular cylinder. The non-glandular trichomes on the leaf surfaces of the two *Alyssum* species were highly enriched with Ca, but contained little Ni except in the base. In the leaves of *T. goesingense*, the large elongated epidermal cells contained more Ni than the cells of the stomatal complexes. The role of cellular compartmentation in Ni hyperaccumulation is discussed.

Key words: Nickel, hyperaccumulator, cellular compartmentation, Brassicaceae.

Introduction

In the late 1940s, it was reported that *Alyssum bertolonii* (Desv), growing in the Tuscany region of Italy, contained an extraordinarily high Ni content of about 10 000 μg g^{-1} in the shoot dry matter (Minguzzi and Vergnano, 1948). This was the discovery of the first Ni accumulator plant in the world, which was about 30 years before the term 'hyperaccumulator' was used to describe plants that accumulate more than 1000 μg Ni g^{-1} of dry weight in their shoots in the natural habitats (Brooks *et al*., 1977). Although there are hyperaccumulators for other heavy metals or metalloids, Ni hyperaccumulators are the most numerous, currently with a total number of 318 taxa distributed mainly in the tropical to warm temperature regions with Ni-rich ultramafic (serpentine) soils (Baker *et al*., 2000; Reeves and Baker, 2000).

Metal hyperaccumulators have received increased attention in recent years, due to the potential of using these plants for phytoremediation of metal-contaminated soils (McGrath, 1998; Salt *et al*., 1998) or phytomining (Brooks *et al*., 1998). Some of the Ni hyperaccumulators, e.g. *A. bertolonii* and *Berkheya coddii*, are particularly attractive for these purposes because of their relatively high biomass production (Robinson *et al*., 1997*a*, *b*). Progress has also been made in recent years in the understanding of the Ni hyperaccumulation phenomenon. It was found that exposing several Ni hyperaccumulator species of *Alyssum* to Ni elicited a large increase in the levels of histidine in the xylem sap (Krämer *et al*., 1996). The results suggest that complexation of Ni with histidine may be involved in the tolerance to Ni in

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Abbreviations: EDXA, energy dispersive X-ray analysis; ICP-AES, inductively coupled plasma atomic emission spectroscopy; SEM, scanning electron microscope.

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roots and the delivery of Ni from roots to shoots. The histidine response may not be a universal feature associated with all Ni hyperaccumulators. For example, Persans *et al*. did not observe any Ni-inducible responses in terms of histidine concentrations in the roots, shoots and xylem sap of the Ni hyperaccumulator *Thlaspi goesingense* (Hálácsy), nor did they find any regulation by Ni of three cDNAs encoding the enzymes involved in the histidine biosynthetic pathway (Persans *et al*., 1999). In leaves, Ni appears to be associated mainly with organic acids, such as citrate (Lee *et al*., 1978; Homer *et al*., 1991; Sagner *et al*., 1998; Krämer *et al*., 2000), malate and malonate (Pancaro *et al*., 1978; Brooks *et al*., 1981).

Apart from complexation with organic ligands, cellular compartmentation is likely to be involved in the detoxification of metals in hyperaccumulator plants. In the Zn/Cd hyperaccumulator *Thlaspi caerulescens*, it has been shown that Zn and Cd are sequestered mainly in the vacuoles, particularly in the epidermal cells (Vázquez *et al*., 1994; Küpper *et al*., 1999). In the Ni hyperaccumulator *T. goesingense*, vacuolar sequestration appeared to be important too, although substantial cell wall binding was also implicated (Krämer *et al*., 2000). Cellular distribution of Ni in hyperaccumulator plants has been reported in only a few papers (Mesjasz-Przybylowicz *et al*., 1994; Heath *et al*., 1997; Krämer *et al*., 2000; Psaras *et al*., 2000), and the results appear to be contradictory. Therefore, the aim of this study was to investigate the cellular compartmentation of Ni in three species of Ni hyperaccumulators within the Brassicaceae family: *Alyssum bertolonii*, *Alyssum lesbiacum* and *Thlaspi goesingense*.

Materials and methods

Plant culture

Alyssum bertolonii, *Alyssum lesbiacum* and *Thlaspi goesingense* were sown into vermiculite in trays. Sixteen days later, seedlings were transferred to plastic pots, each containing 500 g compost (John Innes No. 1, moisture content = 27%). Three seedlings were planted in each pot. Because of a poor germination of *A. lesbiacum* in vermiculite, seeds of this species were later sown directly on to the pots containing compost. Each species was treated with four different Ni concentrations in the compost: control (no addition), 500, 2000 and 4000 mg kg^{-1}. Nickel was applied as $NiSO_4$ and mixed thoroughly with the compost before transplanting. Each treatment was replicated three times. Plants were grown in a greenhouse with the following conditions: 20/16 °C day/night temperature, natural sunlight supplemented with 1 kW SON-T lamps to maintain a minimum light intensity of 250 μmol m^{-1} s^{-1}. *A. bertolonii* and *T. goesingense* were harvested 86 d after transplanting, and *A. lesbiacum* was harvested 64 d after emergence of seedlings.

Freeze-fracturing and energy dispersive X-ray analysis (EDXA)

EDXA analysis was performed on both *Alyssum* species growing in the 4000 mg Ni kg^{-1} treatment. Because *T. goesingense* failed to survive in the 4000 mg Ni kg^{-1} treatment, EDXA was done using plants from the 2000 mg Ni kg^{-1} treatment. For quantification of elements in each type of tissue, samples were taken from two or three different plants, two or three samples were taken from each plant, and two or three analyses were done per sample. For leaf and stem samples, mature but not senescent tissues were taken. When leaf surfaces were analysed, these were mounted flat on the surface of carbon stub using conductive carbon tabs. Cross-sections of plant tissue were prepared in the following way: a 0.5 cm wide strip of leaf was taken by two cuts perpendicular to the central vein or a piece about 0.5 cm long was cut out of the mature part of the stem. These pieces of tissue were mounted in a sample holder made of carbon. Samples were then rapidly (within 1 min after excision) frozen in melting nitrogen slush and transferred to a fracturing chamber cooled to −170 °C. To obtain cross-sections of leaves and stems, a sharp blade was used to cut through the cells rather than breaking the tissue at the cell surfaces. Thus the leaves and stems were cut in half, the lower of which stayed fixed in the sample holder and was used for analysis. Subsequently, the specimens were evaporatively coated with carbon to produce an electrically conductive surface and thus prevent charging under the electron beam. Carbon was used instead of metal coating to avoid interference with the elements measured.

EDXA was performed in a scanning electron microscope (SEM, Philips XL 40) on a cryostage (−160 °C to −180 °C), using an acceleration voltage of 30 kV and a working distance of 10 mm. The low temperature during analysis is essential to avoid evaporation of water during exposure of the sample to the electron beam. Tests have shown that even after continuously scanning an area of 200 × 200 μm for 8 h, at this temperature no visible or measurable damage occurred. Electron penetration depth (R) is

$$R = \frac{4120}{\rho} E^{(1.265 - 0.0954 \ln E)}$$

where E is primary electron energy in MeV; ρ is specific gravity of the absorber in g cm^{-3}.

With the chosen acceleration voltage, the maximum penetration depth (i.e. in an aqueous compartment like a vacuole) of the electron beam is about 15 μm. In materials with a higher specific gravity, the electron penetration depth is smaller (inversely proportional to specific gravity), so if, for example, a dense cell wall has a specific gravity of 2.5 g cm^3, the maximum penetration depth would be decreased to about 6 μm. The horizontal resolution (determined mainly by the diameter of the electron beam) is less than 1 μm. Hence, differences in concentration, for example, between small organelles such as chloroplasts or mitochondria, and the cytoplasm, cannot be resolved. However, differences between the cell wall and the intercellular compartment can be measured by choosing cell walls that were approximately parallel to the electron beam. Nevertheless, cell wall data will always contain some counts from the neighbouring intracellular compartment, and if that compartment contains higher concentrations, this can lead to overestimation of element concentrations in the apoplast. Hence, measurements of intracellular concentrations are generally more accurate than those of cell walls.

The distribution of elements across a section of a sample was measured semi-quantitatively by displaying the count rate within a narrow spectrum window within its peak (0.6 × peak half width) along a transect line. A two-dimensional distribution pattern was also recorded by scanning an area of the specimen repeatedly for up to 2 h and integrating the counts for Ni, O, Ca, K, and S within their respective spectrum windows into dot-maps. In both line and area scans, the spectrum-analysis

software did not allow for the separation of the element-specific X-rays (net peak counts) from the background counts. Therefore, these scans were performed only on those elements with a peak/background ratio greater than 2. The latter could be determined by recording an average EDXA spectrum of the region; this was done automatically during the recording of a dot-map. A dot-map of oxygen distribution was recorded before any further analysis of the specimen. Because of its low energy, the X-rays emitted by oxygen are most strongly affected by shading (caused by the sample holder or neighbouring cells) inside the sample, so that an oxygen dot-map clearly shows these shaded parts of the sample. The regions of specimen that suffered from shading effects were not used for quantitative analysis.

Spectra from 0–20 keV were collected at increments of 10 eV per channel with the electron beam focused on a rectangular area in the centre of selected cells. The spectra were analysed using the program Superquant (EDAX, San Francisco, USA), which separates the background from the element-specific peaks and fully deconvolutes the spectra for the correction of interference between elements. Thus, the analysis with this program yielded the net peak count rate and the peak/background ratio for each element, which were used for quantification. Quantification of EDXA data was done as described in detail (Küpper *et al.*, 2000) using the oxygen Kα line as an internal standard. Since the EDXA analysis of bulk-frozen tissues does not allow a solution or a suspension of an element to be distinguished, the term 'concentration' always refers to the apparent concentration of the element, meaning that not all of this is necessarily in soluble form.

Determination of element concentrations in whole plant tissues

At harvest, plant shoots were washed with deionized water, blotted dry, and oven-dried at 80 °C for 24 h before dry weights were determined. Dried samples were ground to <0.5 mm, and a 0.2 g subsample was digested with a mixture of HNO_3 and $HClO_4$ (Zhao *et al.*, 1994). Concentrations of Ni and other elements in the digests were determined using inductively coupled plasma atomic emission spectrometry (ICP-AES; Fisons ARL Accuris).

Means and standard errors are presented. Where appropriate, analysis of variance or Student's *t*-test was performed.

Results

Plant growth and Ni uptake

The compost used in this experiment is a nutrient-rich all-purpose growth medium. However, an addition of 500 mg Ni kg^{-1} to the growth medium increased plant growth of all three Ni hyperaccumulators significantly ($P<0.01$) compared to the control (Fig. 1a). *T. goesingense* was clearly less tolerant to Ni than the two *Alyssum* species. The 4000 mg Ni kg^{-1} treatment was lethal to *T. goesingense* within a few weeks of exposure. The plants grown on 2000 mg Ni kg^{-1} also exhibited symptoms of phytotoxicity, and the shoot dry weight was decreased to about 50% of that in the control. In comparison, plants of both *A. bertolonii* and *A. lesbiacum* survived the highest Ni treatment, showing no obvious symptoms of phytotoxicity, although the shoot dry weights decreased. Shoot dry weights of *A. lesbiacum* were smaller than those of *A. bertolonii*, mainly because the former species was grown for a shorter period.

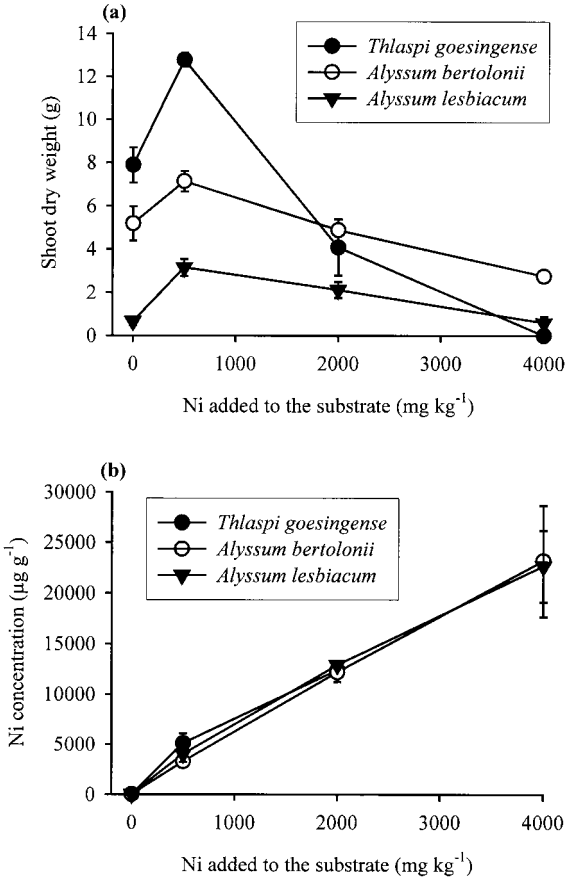

Fig. 1. Effects of Ni addition on shoot dry weight (a) and the concentration of Ni in the shoots (b). The concentration of Ni was determined with ICP-AES. The error bars represent the standard errors ($n=3$).

The concentrations of Ni in the shoots of all three species increased almost linearly with the increasing addition of Ni to the growth medium (Fig. 1b). The patterns of Ni accumulation were remarkably similar in all three species. The highest Ni concentration obtained in this experiment was about 23 000 mg kg^{-1} shoot dry weight in the two *Alyssum* species grown on the 4000 mg Ni kg^{-1} treatment, whereas for *T. goesingense* a maximum of 12 400 mg Ni kg^{-1} shoot dry weight was obtained in the 2000 mg Ni kg^{-1} treatment.

Cellular distribution of Ni and other elements in leaves

Leaves of all species analysed had a rather similar pattern of Ni distribution in different cell types (Fig. 2). In all three species the Ni concentration was significantly higher ($P<0.01$) in the epidermal cells than in the mesophyll cells or various types of cells inside the vascular bundles (Fig. 2). No significant difference was observed between

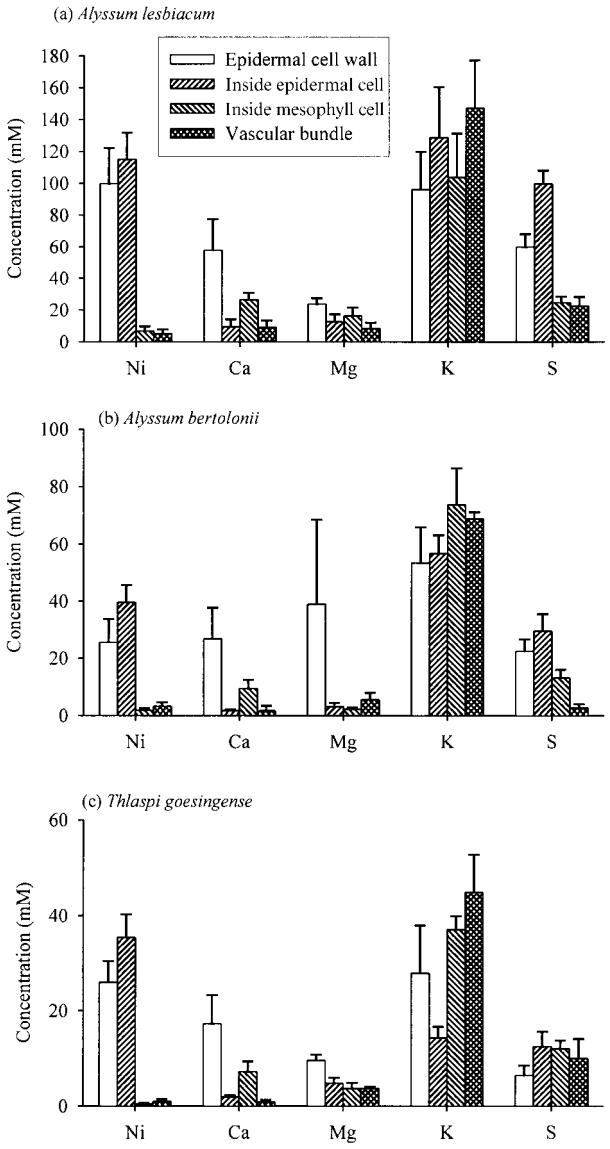

Fig. 2. Concentrations of Ni, Ca, Mg, K, and S in different leaf cells as determined by EDXA of cross-sections. (a) *A. lesbiacum*; (b) *A. bertolonii*; (c) *T. goesingense*; *A. lesbiacum* and *A. bertolonii* were from the 4000 mg Ni kg^{-1} treatment, and *T. goesingense* from the 2000 mg Ni kg^{-1} treatment. Error bars represents standard errors ($n = 3$–14). The concentrations in *A. bertolonii* are generally lower than in *A. lesbiacum* because samples from the latter species were taken later.

the upper and the lower epidermis (data not shown). The preferential distribution of Ni in the epidermal cells can be seen clearly in a dot-map of Ni recorded from a cross-section of a mature leaf of *A. lesbiacum* (Fig. 3a, b). Using a higher magnification, it can be seen that the Ni concentration appeared to be higher inside the epidermal cells than in the epidermal cell walls (Fig. 2c, d). In all three species, the concentrations of Ni were higher in the intracellular compartment than in the cell walls of the epidermal cells (Fig. 3). Due to the large variations between individual tissue samples, the differences in concentrations between cell walls and intracellular compartments were statistically significant in a *t*-test ($P < 0.05$) only in *A. lesbiacum*. Therefore, line scans were performed to give a more direct comparison with the maximum possible spatial resolution, and these have consistently shown valleys of Ni counts in the cell walls and peaks in the intracellular compartments (Fig. 3c, d).

In contrast to Ni, the concentrations of Ca and Mg were higher in the epidermal cell walls than in the intracellular compartments (Fig. 2). In both *T. goesingense* and *A. lesbiacum*, there was a significant and positive correlation between Ni and Mg in the mesophyll cells ($n = 13$ and 12, $r = 0.72$ and 0.96, respectively, $P < 0.01$, data from all mesophyll cells analysed). The ratio of Mg to Ni was 4.0 ± 1.2 and 4.5 ± 1.3 for *T. goesingense* and *A. lesbiacum*, respectively. Potassium was abundant in most cell types, particularly in the vascular bundles. In the two *Alyssum* species, the S concentration was higher in the epidermal cells than in the mesophyll and vascular bundle cells, and there was a significant correlation between S and Ni concentrations in the intracellular compartments of different cells (for *A. lesbiacum*: $n = 32$, $r = 0.72$, $P < 0.001$; for *A. bertolonii*: $n = 26$, $r = 0.79$, $P < 0.001$; data from all cells analysed). In *T. goesingense*, the difference in S concentration between cell types was smaller, and the correlation between S and Ni was not significant. In the epidermal cells, cell walls appeared to have high S concentrations, which were comparable to the concentrations of Ca (Fig. 2).

Both *Alyssum* species have many trichomes on the leaf surfaces which are non-glandular and stellate in shape (Fig. 4a for *A. lesbiacum*). EDXA dot-map scans of leaf surfaces revealed that the trichomes contained little Ni compared to the other epidermal cells, whereas Ca was highly enriched in the trichomes (Fig. 4b, c). A further test was performed by immersing leaves in a solution of dimethylglyoxime (1% w/v in ethanol), which forms a red coloured precipitate with soluble Ni. Three hours later, leaves were dissected and observed by light microscopy. Some of the trichomes showed a pink colour in their basal part, possibly vacuoles (Fig. 4d), suggesting the presence of Ni.

There are no trichomes on the leaf surfaces of *T. goesingense*, but the size of epidermal cells varies widely (Fig. 5a). Ni was preferentially accumulated in the large elongated epidermal cells, whereas the small cells around the stomatal complex and the guard cells contained lower Ni concentrations (Fig. 5b).

Cellular distribution of Ni and other elements in stems

Distributions of Ni and other elements in different cells in the stem were determined in the two *Alyssum* species, and both showed similar patterns (Fig. 6). Similar to the

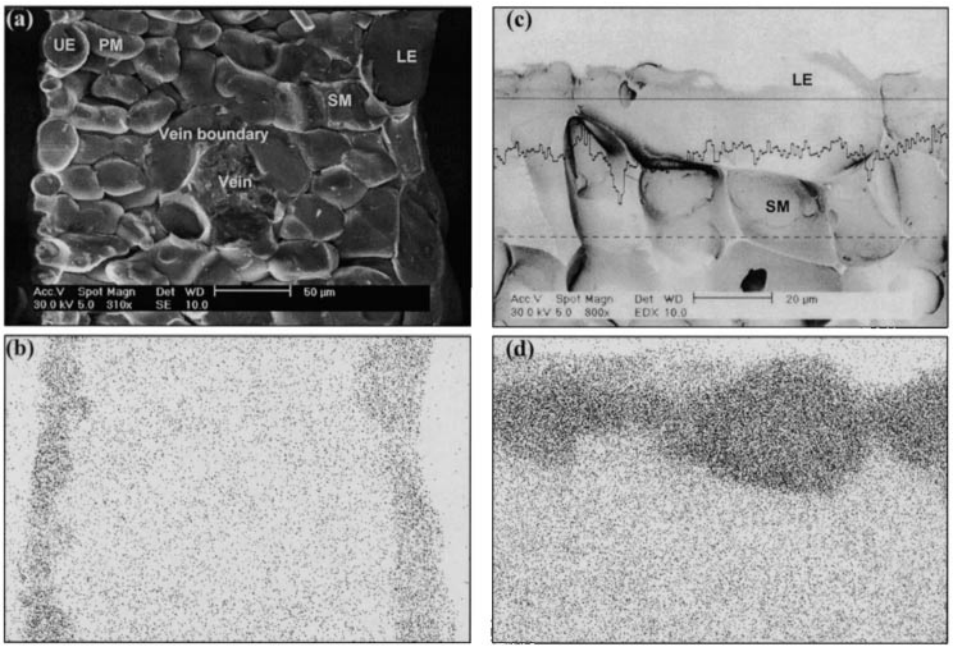

Fig. 3. Distribution of Ni across different leaf cells of *A. lesbiacum* as revealed by EDXA analysis of frozen-hydrated tissues. UE, upper (adaxial) epidermis; PM, palisade mesophyll; SM, spongy mesophyll; LE, lower (abaxial) epidermis. (a, b) Cross-section of a leaf and the corresponding dot-map for Ni; (c) EDXA line scan (Ni Kα line 0.6 × half width, analysis along the solid line, dashed line represents zero Ni counts) across the lower epidermis, also showing the adjacent mesophyll cells and (d) the corresponding dot-map of the Ni Kα (0.6 × half width). Plants were grown on 4000 μg Ni g^{-1}.

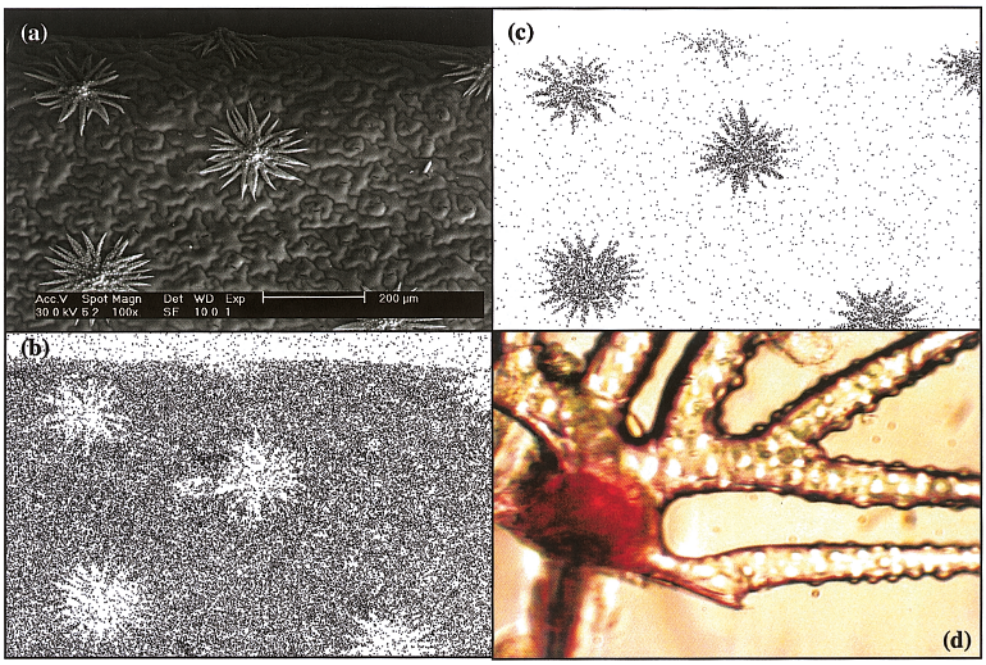

Fig. 4. Upper leaf surface and trichomes of *A. lesbiacum* (a), EDXA dot-map of Ni (b), EDXA dot-map of Ca (c), and (d) trichome stained with dimethyglyoxime. Plants were grown on 4000 μg Ni g^{-1}.

leaves, Ni concentrations were highest in the epidermis (Fig. 7). The concentrations of Ni in the intracellular compartment of epidermal cells were again higher than those measured in the epidermal cell walls ($P < 0.05$ and $P < 0.01$ for *A. lesbiacum* and *A. bertolonni*, respectively, in *t*-tests) (Fig. 6). An EDXA line scan along stem epidermal cells of *A. bertolonii* clearly shows that Ni was more enriched inside the cells than in the cell wall (Fig. 8). Ni concentrations in the stem epidermal cells (intracellular compartment) were around 120 mM in

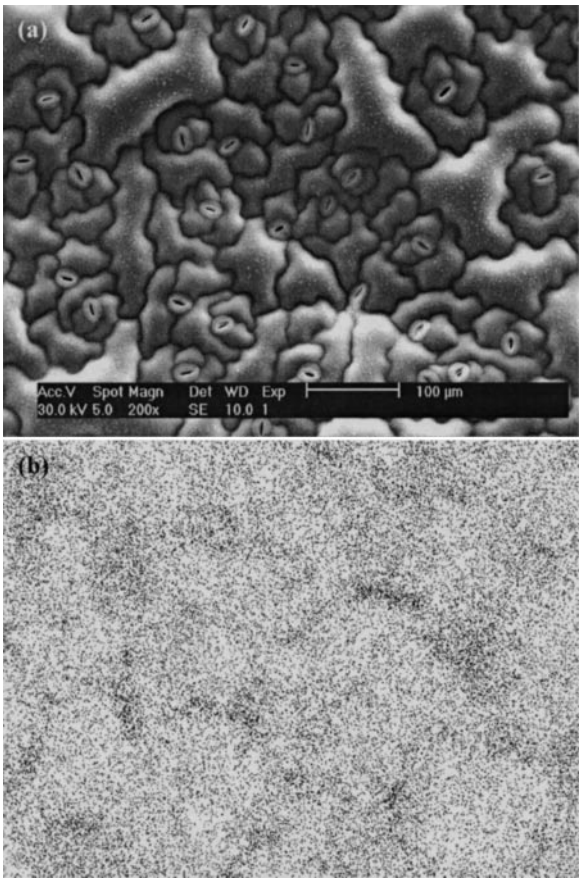

Fig. 5. Upper leaf surface of *T. goesingense* (a), and the corresponding dot-map of Ni (b). Plants were grown on 2000 µg Ni g^{-1}.

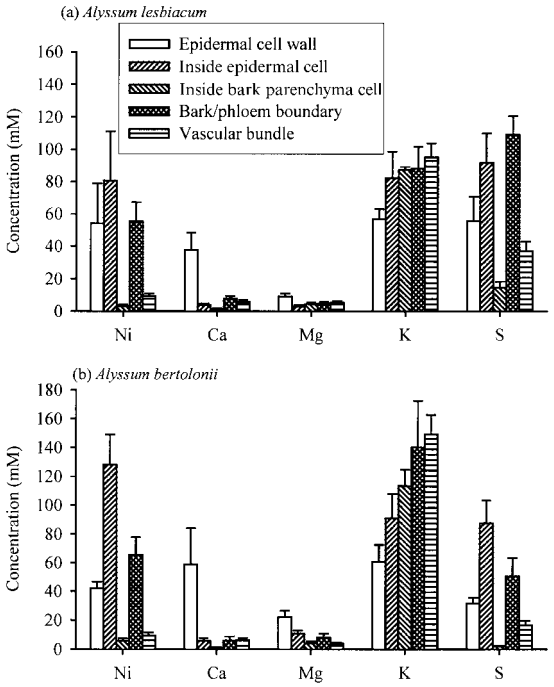

Fig. 6. Concentrations of Ni, Ca, Mg, K, and S in different stem cells as determined by EDXA. (a) *A. lesbiacum*; (b) *A. bertolonii*. Plants were from the 4000 mg Ni kg^{-1} treatment. Error bars represents standard errors ($n = 4$–14). The concentrations in *A. bertolonii* were generally lower than in *A. lesbiacum* because samples from the latter species were taken later.

A. bertolonii and about 80 mM in *A. lesbiacum*, whereas the concentrations in the epidermal cell wall were 40–50 mM in both species (Fig. 6). In stems there was a second peak of Ni accumulation in the boundary cells between the vascular cylinder and the cortical parenchyma cells (Fig. 7). The concentrations of Ni in these boundary cells (intracellular compartment) were around 55–65 mM, compared to <10 mM in the bark parenchyma and vascular bundle cells (Fig. 6).

The concentrations of Ca and Mg were markedly higher in the epidermal cell walls than in the intracellular compartment of the epidermis, and were low in all other cell types in the stems (Fig. 6). Potassium was again abundant in all cell types and tended to increase from epidermal cells to the vascular bundle cells. As in leaves, the patterns of S distribution in both *Alyssum* species were similar to those of Ni, with the intracellular compartment of epidermis and the boundary cells between bark parenchyma and vascular bundle containing more S than other cell types. In these cell types, there was also a significant ($P < 0.001$) and positive correlation between Ni and S. Also, cell walls apparently contained high concentrations of S.

Discussion

Growth of the Ni hyperaccumulators *A. bertolonii*, *A. lesbiacum*, and *T. goesingense* was enhanced by the addition of 500 mg Ni kg^{-1} as $NiSO_4$ to the growth medium, an all-purpose nutrient-rich compost. It is unlikely that the positive effect was due to the addition of S, because of the abundance of available S in the compost. Although Ni is an essential nutrient, the requirement for Ni by normal plant species (non-hyperaccumulators) is so low that Ni deficiency has never been reported in field-grown crops (Marschner, 1995). Even the small amount of Ni contained in seed is usually sufficient to sustain normal plant growth and development. To demonstrate that Ni is essential to higher plants, Brown *et al*. had to lower the content of Ni in the seeds of barley by growing plants for several generations with nutrient solution containing very high purity reagents that were further purified using an ion exchange column (Brown *et al*., 1987). The present study's results indicate that, compared to normal plant species, all three Ni hyperaccumulators tested have a much higher requirement for Ni. This is analogous to the higher requirement for Zn by the Zn hyperaccumulator *Thlaspi caerulescens* (Shen *et al*., 1997; Küpper *et al*., 1999). One beneficial effect of a highly elevated Ni concentration in the tissues of hyperaccumulator plants may be protection

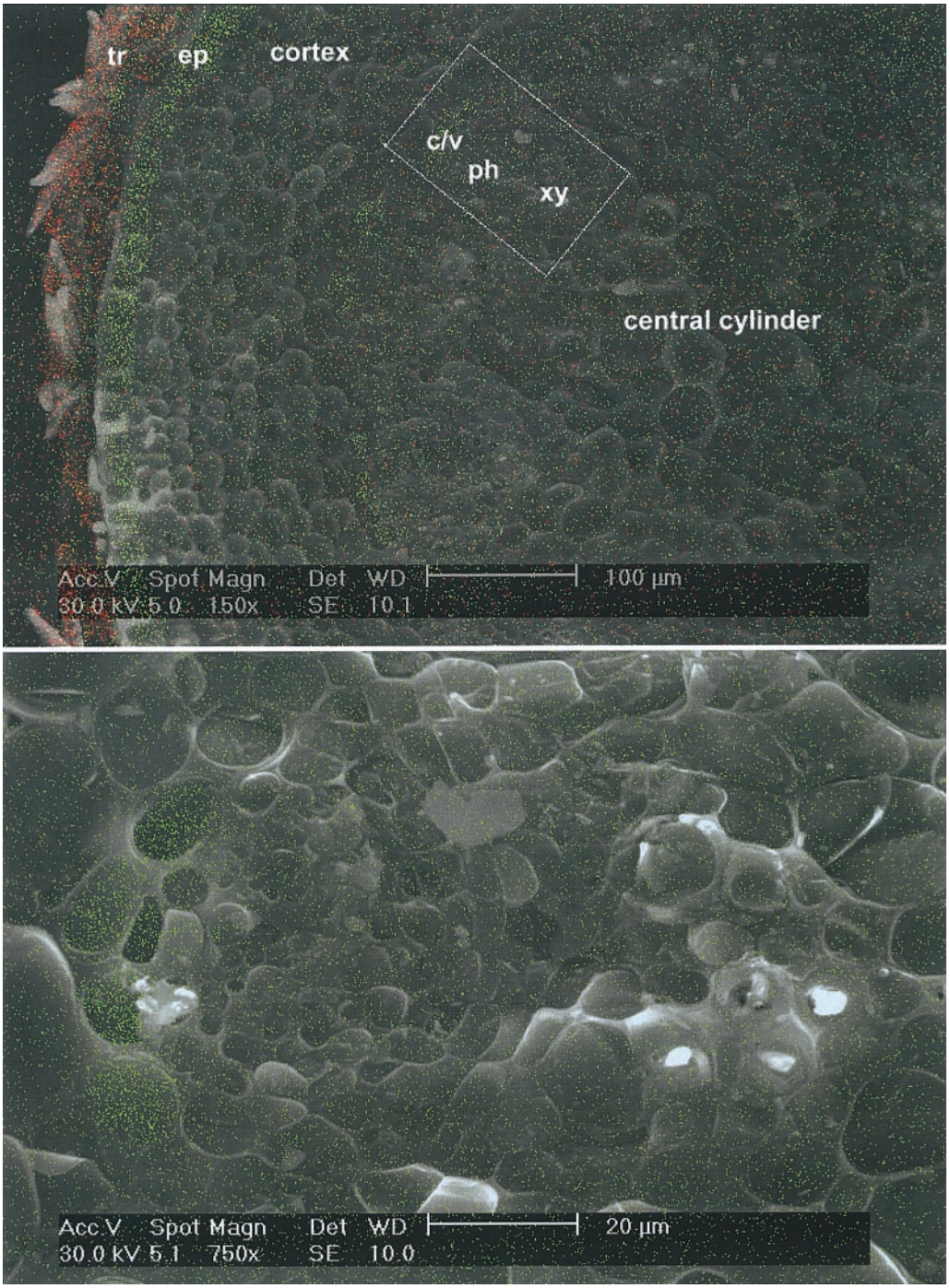

Fig. 7. Distribution of Ni and Ca across a section of a mature stem (side branch) of *A. bertolonii* as revealed by EDXA analysis of frozen-hydrated tissues. Tr, trichome(s); ep, epidermis; ph, phloem; xy, xylem; c/v, cortex/vein (phloem) boundary. The rectangle in the upper picture gives the approximate position of the enlarged view shown in the lower picture. Plants were grown on 4000 µg Ni g^{-1}. The dot-maps show the distribution of Ni (green) and Ca (red) as measured by the Kα lines (0.6 × half width) of their X-ray emission.

against pathogens and herbivores (Boyd, 1998). In other experiments (not reported here), it was also observed that fungal infections on the leaf surfaces of *T. goesingense* and the two *Alyssum* species were greatly reduced in the plants grown on +Ni treatments.

The three Ni hyperaccumulators studied all showed preferential accumulation in the epidermal cells. Mesophyll cells and the cells inside the vascular bundles in the leaves contained much less Ni than the epidermal cells. In the stems of *A. lesbiacum* and *A. bertolonii*, Ni

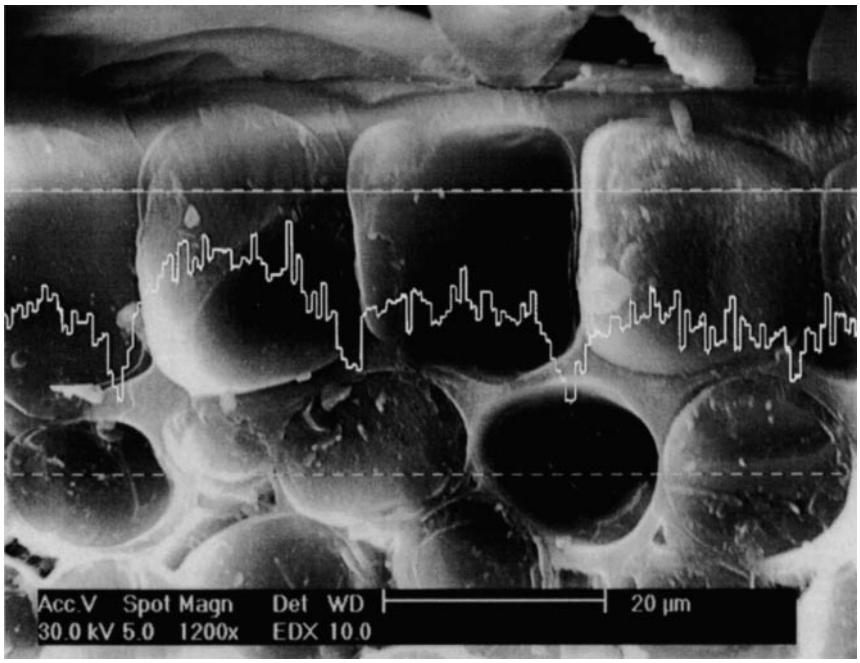

Fig. 8. Compartmentation of Ni in the stem epidermal cells of *A. bertolonii* (grown on 4000 µg Ni g^{-1}). The line in the electronoptic picture shows the intensity of the Ni Kα line across the epidermis; the measurement took place at the solid straight line, the dotted line shows the baseline (zero Ni counts). Plants were grown on 4000 µg Ni g^{-1}. The minima of the Ni counts in this line scan are slightly shifted to the left in comparison to the cell walls because of a slight movement of the sample between the recording of the electronoptic image and the line scan.

was also preferentially accumulated in the epidermal cells, although there was a second peak of Ni accumulation in the boundary cells between the vascular cylinder and the cortical parenchyma cells. The function of these cells is not known. The Ni accumulation in leaf epidermal cells was not uniform. In *T. gosingense*, cells in the stomatal complexes, which are small in size, had less Ni than the other large and elongated epidermal cells. This differs from a report (Heath *et al*., 1997) that Ni was accumulated in the subsidiary cells within the stomatal complexes in the leaves of the Ni hyperaccumulator *Thlaspi montanum*.

Because vacuoles generally occupy the majority of cell space, particularly in epidermal cells, the Ni signal recorded in the central intracellular compartment of a cut cell essentially reflects the concentration of Ni in the vacuole. The EDXA data obtained from this study show that the majority of Ni in leaves and stems of the three hyperaccumulator species was located in the vacuoles, rather than in the cell wall. The pattern of cellular distribution of Ni seen in this study is in contrast to that of Al distribution in the mature leaves of tea (*Camellia sinensis*; Matsumoto *et al*., 1976; Memon *et al*., 1981), where Al was found to be located predominantly in the cell wall. The exclusive localization of Al in the cell wall of tea leaves was confirmed in the laboratory using the same EDXA technique (unpublished results). The results of this study disagree with the conclusion of Krämer *et al*., who reported that 67–73% of the Ni in the leaves of *T. goesingense* was associated with the cell wall (Krämer *et al*., 2000). There are two possible explanations for the difference. Firstly, Krämer *et al*. used young plants that were exposed to relatively low concentrations of Ni for short periods. The Ni concentrations in the leaves were far below 1000 mg kg^{-1}, the concentration commonly used to define Ni hyperaccumulation. In contrast, the leaf samples used for the EDAX analysis in this study contained $>10\,000$ mg Ni kg^{-1} (dry weight), which is more typical of hyperaccumulator plants. Secondly, Krämer *et al*. used the techniques of cell fractionation and X-ray absorption spectroscopy to quantify cellular distribution of Ni. The accuracy of both techniques for quantifying *in situ* cellular distribution of elements, particularly in the cell wall, is not without doubt. Other studies with the Zn hyperaccumulator *T. caerulescens* indicate that Zn was located mainly in the vacuoles rather than in the cell wall (Vázquez *et al*., 1994; Küpper *et al*., 1999; Frey *et al*., 2000).

Previous studies showed inconsistent evidence regarding the localization of Ni in the trichomes in *Alyssum* species. Using a proton microprobe to examine dehydrated and fixed leaf specimens, Krämer *et al*. showed that Ni was sequestered to a considerable degree in the trichomes of *A. lesbiacum* (Krämer *et al*., 1997). In contrast, Psaras *et al*. found exclusion of Ni in the trichomes in dry leaves of eight Ni hyperaccumulators, including *A. lesbiacum*, which were obtained from plant materials kept in a herbarium (Psaras *et al*., 2000). In the

present study, EDXA surface scans indicated exclusion of Ni from trichomes of *A. bertolonii* and *A. lesbiacum*. However, staining with dimethylglyoxime appeared to indicate the presence of Ni in the basal part of the trichomes, most likely in the vacuoles. The discrepancy may be caused by the different methods of sample preparation used. Dehydration and fixation prior to elemental mapping (Krämer *et al*., 1997) may lead to the redistribution of mobile ions. In the present study, EDXA was performed on hydrated tissues immediately after the specimens were frozen in melting nitrogen, which involved minimum sample preparation and was most likely to provide information of the *in situ* distribution of elements. One possible limitation of EDXA surface scans is that the electron beam may not penetrate the thick cell walls of trichomes, which may also be fortified with the deposition of calcite (Lanning, 1960). In the Zn hyperaccumulator *Arabidopsis halleri*, Zn was found to accumulate in the basal compartment of trichomes, most likely in the vacuoles (Küpper *et al*., 2000; Zhao *et al*., 2000).

Preferential accumulation of Ni in the leaf epidermis has previously been observed in the Ni hyperaccumulator *Senecio coronatus* (Mesjasz-Przybylowicz *et al*., 1994). Also, Zn was found to be sequestered preferentially in the vacuoles of leaf epidermal cells in the Zn hyperaccumulator *Thlaspi caerulescens* (Vazquez *et al*., 1996; Küpper *et al*., 1999), except the guard cells and subsidiary cells within the stomatal complexes (Frey *et al*., 2000). This pattern of metal accumulation may be seen as protecting both photosynthesis in the mesophyll cells and the function of stomata against metal toxicity. However, preferential accumulation of metals in the epidermis is apparently not a universal feature associated with all metal hyperaccumulators. For example, in the Zn/Cd hyperaccumulator *Arabidopsis halleri*, Zn and Cd were found to be stored mainly in the mesophyll vacuoles and in the basal compartment of leaf trichomes, but not in the other epidermal cells which tend to be very small in size (Küpper *et al*., 2000; Zhao *et al*., 2000). It is possible that the size of vacuoles is an important factor influencing metal accumulation. This hypothesis is supported by the observation that the concentration of Zn correlated positively with the size of epidermal cells in *T. caerulescens* (Küpper *et al*., 1999).

The reason for the apparent high concentrations of S in the epidermal cell walls is not clear. Because the cell wall is thinner than the intracellular compartment, it is more difficult to quantify element concentrations in the cell wall than inside the cell. It is possible that the counts measured in the cell wall may also include small amounts of the signal from intracellular contents. This will cause an overestimation of the cell wall concentrations if the intracellular concentrations are higher than in the neighbouring cell wall, as is the case for S and Ni (Figs 2, 6). If so, it follows that the Ni concentration in the epidermal cell walls could be overestimated. This actually reinforces the conclusion that Ni sequestration in the vacuoles is more dominant than binding in the cell walls.

The concentrations of Mg and Ni in the mesophyll cells of *A. lesbiacum* and *T. goesingense* were found to correlate positively. A similar relationship was observed between Mg and Cd in the mesophyll cells of *A. halleri* (Küpper *et al*., 2000). It is possible that increased Mg may be a defence response of the plants to resist the substitution of Mg in chlorophyll by Ni or Cd (Küpper *et al*., 1996). In the case of S, a cellular distribution pattern similar to that of Ni, and a positive correlation between S and Ni at the single cell level suggests two possibilities. First, sulphate may be an important counter ion for Ni^{2+} in the vacuoles. Indeed, the treatments in these experiments were created by supplying different concentrations of $NiSO_4$. Second, S-containing ligands may be involved in complexing Ni. The second possibility is thought to be less likely, because organic ligands that have been identified to play a role in Ni complexation in Ni hyperaccumulator plants include organic acids (Lee *et al*., 1978; Brooks *et al*., 1981; Homer *et al*., 1991; Sagner *et al*., 1998; Krämer *et al*., 2000) and histidine (Krämer *et al*., 1996), but not S-containing ligands.

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