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RESEARCH PAPER

## Distribution of cadmium in leaves of *Thlaspi caerulescens*

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### Abstract

Knowledge of the intracellular distribution of Cd in leaves is necessary in order to understand the mechanisms of hyperaccumulation in *Thlaspi caerulescens*. Ganges and Prayon, two ecotypes accumulating Cd to different levels, were grown in nutrient medium containing varying concentrations (0, 5, 10, 50, and 100  $\mu\text{M}$ ) of Cd. Several different approaches were combined in this study to (i) validate the results obtained by a specific method and (ii) establish the link between observations and measurements performed at different scales. In both ecotypes, Cd, localized by autoradiography, was found mainly at the edges of the leaves, but also in points of higher concentration spread over the whole limb surface. This localization was clearly correlated with the necrotic spots observed on Prayon leaves. Scanning electron microscopy coupled with energy dispersive X-ray microanalysis (cryo-SEM-EDXMA) and tissue fractionation (apoplasm, cell walls, mesophyll protoplasts, and lower epidermis) showed that Cd had similar patterns of distribution in leaf cells of both ecotypes. Cadmium was found both inside the cells and in the cell walls, mainly in the large epidermal cells but also in small epidermal cells. All the methods used agreed well and the results indicated that metal storage in the plants studied involves more than one compartment and that Cd is stored principally in the less metabolically active parts of leaf cells.

Key words: Autoradiography, cadmium (Cd), cell walls, compartmentation, energy dispersive X-ray microanalysis, hyperaccumulation, metal storage, *Thlaspi caerulescens*.

### Introduction

In view of the risk posed by Cd as an environmental pollutant (Sanità di Toppi and Gabbriellini, 1999; Kabata-Pendias and Pendias, 2001), there has been interest in developing the use of hyperaccumulator plants to extract Cd from contaminated soils (Brown *et al.*, 1995b; Hammer and Keller, 2003). However, a lack of understanding of the mechanisms involved in heavy metal tolerance and hyperaccumulation prevents the optimization of the technique. It is therefore a research priority to gain basic information on sink capacities and transport mechanisms of Cd into the cells of hyperaccumulator species.

*Thlaspi caerulescens*, a hyperaccumulator of Zn, Cd, and Ni (depending on the population tested) has been recognized as an interesting model for studying hyperaccumulation (Assunção *et al.*, 2003). However, to date no data are available for Cd localization in leaves of *T. caerulescens*. The major efforts have been focused on the assessment of root uptake and Cd localization because this is the primary step leading to hyperaccumulation. For example, kinetic studies of Cd uptake in *T. caerulescens* were performed on roots only (Lombi *et al.*, 2001; Zhao *et al.*, 2002) and Cd was found in both the apoplast and the vacuole (Vázquez *et al.*, 1992; Boominathan and Doran, 2003). Knowledge of Cd intracellular localization in leaves, where concentrations are higher, is necessary to improve current understanding of *Thlaspi caerulescens* hyperaccumulation mechanisms.

Earlier studies have shown that the phytochemistry involved in metal transport and storage seems to vary considerably with plant species (Brooks *et al.*, 1998). In tobacco (*Nicotiana rustica*), Cd was localized predominantly in the vacuoles of leaf cells (Vögeli-Lange and Wagner, 1990). The cell walls were further identified as another site of Cd storage in *Zea mays* shoots (Khan *et al.*, 1984; Lozano-Rodriguez *et al.*, 1997). In *Brassica napus* leaves, Cd was

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found both in vacuoles and in cell walls (Carrier *et al.*, 2003). In *Brassica juncea* and *Silene vulgaris*, the leaf epidermis was a major site of Cd accumulation (Salt *et al.*, 1995b; Chardonnens *et al.*, 1998), whereas in *Arabidopsis halleri* and *B. napus* the largest concentration of Cd was found in the mesophyll (Küpper *et al.*, 2000; Carrier *et al.*, 2003).

However, due to low Cd concentrations found in plants and methodological difficulties, in a number of studies the cellular distribution of Cd has been analysed only in cell fractions separated by biochemical or physical methods (Vázquez *et al.*, 1992; Chardonnens *et al.*, 1998; Carrier *et al.*, 2003) with the attendant possibility of artefacts and/or Cd losses during preparation as mentioned by Frey *et al.* (2000b). Moreover, physical methods such as atomic absorption spectroscopy (AAS) after tissue disruption (Brown *et al.*, 1995a; Chardonnens *et al.*, 1998), secondary ion mass spectroscopy (SIMS) imaging (Lazof *et al.*, 1996), or short-term desorption with radiotracers (Lasat *et al.*, 1996; Blaudez *et al.*, 2000) are appropriate to spot compartments, but not to localize metals precisely. Energy dispersive X-ray microanalysis (EDXMA), electron spectroscopic imaging (ESI) and electron energy loss spectroscopy (EELS) have been used with some success to plot the subcellular distribution of metals (Nassiri *et al.*, 1997; Bringezu *et al.*, 1999; Küpper *et al.*, 1999; Frey *et al.*, 2000a; Zhao *et al.*, 2000), but lack of sensitivity and interference with other cations (e.g. K) are limiting for Cd detection.

To learn more about the general allocation of Cd in leaves of *T. caerulescens* and to overcome the methodological difficulties described above, in the present study different approaches were combined in order to (i) validate the results obtained by a specific method and (ii) make the link between observations and measurements performed at different scales. Two different populations of *T. caerulescens* were compared: Ganges a Cd-hyperaccumulating ecotype, and Prayon ecotype with a lower degree of accumulation (Lombi *et al.*, 2000; Zhao *et al.*, 2003). The concentrations tested were chosen according to the sensitivity of the method employed. Macro-autoradiography was conducted on plants grown in solution culture and treated with increasing concentrations of Cd. Autoradiographs of whole leaves were compared with visual symptoms, biomass production, and metal concentrations in shoots at various exposure times for the evaluation of the general process of Cd allocation. The distribution of Cd at the macroscopic level was completed by observation at the cellular and subcellular levels in leaves by EDXMA combined with cryo-scanning electron microscopy (SEM-EDXMA) of leaf cuttings. Finally, biochemical and physical separation of leaves was performed to measure in detail the involvement of different plant compartments (apoplasm, cell wall, mesophyll protoplasts, and epidermis) in Cd storage.

## Materials and methods

### Plant material and culture

The plants studied were *T. caerulescens* ecotype Ganges ('Les Avinières', St-Laurent-le Minier, southern France), a known Zn and Cd hyperaccumulator (Robinson *et al.*, 1998), and *T. caerulescens* ecotype Prayon (Belgium), known to hyperaccumulate Zn, but to accumulate Cd to a lesser degree (Lombi *et al.*, 2000; Zhao *et al.*, 2003).

Seeds were germinated in the dark on filter papers moistened with deionized water. Two-week-old seedlings were transferred to 1.0 l pots (four plants per pot) filled with modified quarter-strength Hoagland's nutrient solution (Sigma, St Louis, USA) supplemented with 20 µM Fe-N, N'-di (2-hydroxybenzyl) ethylenediamine-N, N'-diacetic acid monohydrochloride hydrate (HBED; Strem chemical, Newburyport, USA). Fe(III)-HBED was prepared as described by Chaney *et al.* (1998) in such a way that all HBED was saturated with Fe. Plants were allowed to grow for 2 weeks in solution culture before treatment with Cd was commenced.

Germination and plant culture were performed in a climate chamber (day/night period 16/8 h, day/night temperatures 20/16 °C, and a light intensity of 500 lx). The nutrient solution was renewed every week and aerated continuously.

The concentrations tested were chosen according to the sensitivity of the method employed. When necessary, and depending on the method chosen (see below) and the device used, labelled Cd (<sup>109</sup>Cd) was used alone or in addition to the different Cd treatments. Radiotracers are very convenient and sensitive for the determination of low concentrations in aqueous extracts, but they cannot be measured by β-counting in acidic samples or in intact leaves. When Cd had to be measured in such samples, unlabelled Cd was used in higher concentrations to allow detection by inductively coupled plasma-atomic emission spectroscopy (ICP-AES).

### Biomass production and accumulation of Cd in plants

The total concentration of Cd was measured in leaves of *T. caerulescens* Ganges and Prayon treated for 8 weeks with non-labelled Cd (0, 5, 10, 50, and 100 µM Cd). Four mature plants grown in hydroponics were harvested. Shoots were quickly rinsed with deionized water, separated from the roots, and dried at 80 °C for 1 week. Leaves were weighed and hot-digested in HNO<sub>3</sub> 65% suprapur (Fluka, Buchs, Switzerland) and HClO<sub>4</sub> 70% *pro analysis* (Fluka, Buchs, Switzerland). The concentration of Cd in digests was measured by ICP-AES (Plasma 2000; Perkin Elmer, Wellesley, USA).

### Autoradiography of Cd in plants

For the macro-autoradiographs, both ecotypes of *T. caerulescens* were tested with four different treatments (0, 5, 10, and 50 µM Cd) and three times of exposure to the metal (3, 15, and 30 d). An additional treatment, 100 µM Cd, was included but for only 15 d. All nutrient solutions were labelled from the beginning of the Cd treatments with 0.1 kBq ml<sup>-1</sup> (=2.2×10<sup>-3</sup> µM) of <sup>109</sup>CdCl<sub>2</sub> (NEN Life Science Products, Boston, USA). Shoots were collected, quickly rinsed with deionized water, and then blotted dry. The samples were arranged as flat as possible and wrapped in a single layer of thin polyethylene film. To obtain autoradiographs of adaxial and abaxial sides, samples were sandwiched between 2 X-OMAT AR-5 autoradiography films (Kodak, Rochester, USA) at room temperature. Time of exposure varied from 2 h to 10 d depending on the <sup>109</sup>Cd activity present in the samples. Autoradiographs were developed with an automatic film processor (SRX-101A, Konica, Tokyo, Japan) and subsequently enumerated on a Linotype-Hell Tango digitizer (Heidelberg, Kiel, Germany). Control plants grown without <sup>109</sup>Cd were processed at the same time to detect any possible artefacts on

the autoradiographs. Distribution of Cd within each leaf was determined by examining the exposed film. Leaves of plants grown in 50  $\mu\text{M}$  Cd were then individually dried at 80 °C, weighed, hot-digested for 1 h at 95 °C in  $\text{HNO}_3$  65% suprapur, and analysed for Cd concentration.

#### Energy dispersive X-ray microanalysis

The microanalysis of leaves was performed on both Ganges and Prayon populations grown for 8 weeks without Cd prior to growth for 4 weeks in nutrient solution supplemented with 100  $\mu\text{M}$  Cd. The aim was to reach high biomass production and high Cd concentrations in the leaves and to avoid the problem of Cd phytotoxicity. Indeed, in contrast to seedlings, older plants were able to withstand this extremely high Cd concentration without showing toxicity symptoms. Old leaves of plants grown in high Cd concentrations were used because preliminary analyses and autoradiographs had shown that Cd concentrations in the whole leaves were 10 times greater in old leaves. In fact, Cd could not be detected in young leaves or in plants grown in lower Cd concentrations. Fresh leaves were cut into small pieces (approximately 4×4 mm). The samples were mounted vertically on an SEM stub using a Tissue-Tek and rapidly frozen by plunging into liquid propane as previously described (Frey *et al.*, 2000a). Microanalysis of freeze-fractured leaves was performed in a Philips SEM 515 equipped with an SEM cryo unit (SCU 020, Bal-Tec, Balzers, Liechtenstein) and a Tracor Northern energy dispersive X-ray analysis system interfaced with a Noran System Six Version 1.3 software package. Electron-induced X-rays were detected by a Si(Li) spectrometer detector (Tracor Northern 30 mm<sup>2</sup> Microtrace) with an ultra-thin beryllium window. The microscope was operated at an acceleration voltage of 18 kV with a beam current of 80 nA and the stage-tilt was adjusted to obtain a take-off angle of 44°. Working distance was 12 mm. The temperature on the SEM cold stage was kept below -120 °C. After preliminary measurements, analyses were carried out in the spot mode at ×10 000 magnification (50 nm spot size). Selected spots at the edge and in the middle of the leaf ( $n=1-5$ ) and on mesophyll and epidermal cells as well as in the vein, the cuticle, the cell walls, and the intercellular space were analysed using a standard detection time of 100 live seconds. The depth resolution at 18 kV in frozen-hydrated samples was estimated to be 2.4  $\mu\text{m}$  (Van Steveninck and Van Steveninck, 1991). This may be satisfactory for determining the elemental contents in vacuolar and cell walls in the spot mode. Spectra were processed using the standardless analysis program of the Noran System Six software package for automatic background subtraction and calculations of net counts of elements of interest. The program uses a digital top hat filter to remove background and a multiple least squares fit to determine net peak counts. Any net intensity less than 50 counts per 100 s was designated 'not detected'. Net counts of Cd were obtained at Cd  $L_{\alpha}$  3.13 keV. Elemental maps were also produced to determine the distribution of S, Ca, K, P, C, and Cd at the edge of the leaf in the Ganges population, but did not give additional information. It was not possible to obtain a discriminative distribution of Cd within the leaf.

Analyses of variance (ANOVA) were performed to test the effect of ecotype, position in leaf and type of cell on the Cd signal (ANOVA, SYSTAT 10.2, SYSTAT Software Inc.). The effect of a given factor was considered significant when the  $P$ -value was <0.05. In addition, Student's  $t$ -tests were performed to test whether the Cd count obtained for Ganges for a given type of cell differed from that of the Prayon ecotype. The difference between means was significant when the  $P$ -value was <0.05.

#### Leaf tissue fractionation

To measure the involvement of different plant compartments (apoplasm, cell walls, and protoplasts) in Cd storage, mesophyll protoplasts were prepared from leaves of eight different 12-week-old *T. caerulescens* plants grown with or without Cd (0, 5, 10, and 50  $\mu\text{M}$

Cd) spiked with 0.1 kBq ml<sup>-1</sup> ( $=2.2 \times 10^{-3}$   $\mu\text{M}$ ) of <sup>109</sup>Cd for better accuracy and because of the small volumes and low concentration involved.

The method used for extraction of protoplasts has been described in detail elsewhere (Cosio *et al.*, 2004). The abaxial side of leaves, consisting of the lower epidermis and the cuticle, was peeled using tweezers, weighed, and placed in a counting vial. Peeled leaves were weighed and placed in a cell-wall-digesting medium composed of sorbitol medium (500 mM sorbitol, 10 mM MES, 10  $\mu\text{M}$  CaCl<sub>2</sub>, pH 5.3), 0.75% (w/v) Cellulase Y-C (Kikkoman, Tokyo, Japan), and 0.075% (w/v) Pectolyase Y-23 (Kikkoman, Tokyo, Japan). The leaves were incubated for 2–4.5 h at 30 °C until digestion was judged satisfactory but had not reached the epidermal cell layer. The resulting suspension was centrifuged at 400  $g$  for 7 min on top of a 100% Percoll medium cushion comprising 500 mM sorbitol, 10  $\mu\text{M}$  CaCl<sub>2</sub>, 20 mM MES, pH 6 solubilized in Percoll (Sigma, St Louis, USA). The supernatant corresponding to the apoplasm/cell walls fraction was removed, filtered through 0.45  $\mu\text{m}$  to remove all the debris, and 10–20 aliquots were placed in counting vials. The layer of mesophyll protoplasts was resuspended in the residual liquid. Percoll medium (100%) was added to the protoplasts mix to obtain a final Percoll medium of 50% (1:1 v/v) sorbitol medium with 100% Percoll medium), which was overlaid with a Percoll medium of 40% (3:2 v/v) sorbitol medium with 100% Percoll medium) and finally with a layer of sorbitol medium. The gradient was centrifuged at 400  $g$  for 5 min. The protoplasts were collected from the upper interface. All centrifugation steps were performed at 4 °C. The number of protoplasts per ml obtained in the concentrated preparation was counted with a haemocytometer. Depending on the plant sample, 10–20 aliquots of 200  $\mu\text{l}$  concentrated protoplasts solution were placed in counting vials. For technical reasons (poor quality of the material) the upper epidermis was not recovered. Four ml of scintillation solution (Ultima Gold LSC-cocktail, Packard Bioscience, Meriden, USA) were added to the counting vials with all the aliquots (lower epidermis, apoplasm/cell walls and protoplasts). The <sup>109</sup>Cd was quantified by scintillation counting (Packard, Tri-Carb, liquid scintillation analyser, Meriden, USA).

The percentage of viable protoplasts was determined by staining with fluorescein diacetate (Flucka, Buchs, Switzerland) as described by Cosio *et al.* (2004) and inspected using a fluorescence microscope. Protoplasts showing bright fluorescence were counted as viable. Protoplast viability ranged between 80% and 95%. In addition, the size of protoplasts was measured to assess homogeneity between the different samples. Protoplasts were similarly distributed for all the plants tested and the different treatments.

#### Preparation of the apoplast wash fluid and cell walls

Two experiments were conducted to further differentiate Cd distribution between apoplasm and cell walls.

**Extraction of apoplast wash fluid:** As the apoplast wash fluid extraction resulted in very small volumes, the treatment giving the largest signal was used and plants were grown in the presence of the radiotracer only ( $=2.2 \times 10^{-3}$   $\mu\text{M}$  <sup>109</sup>Cd) to avoid dilution of the activity. Leaves of eight 12-week-old *T. caerulescens* Ganges and Prayon were cut at the base with a razor blade and weighed. Leaves were then infiltrated with deionized water under vacuum three times for 4 min to ensure complete infiltration. Fully infiltrated leaves sank and were darker in colour. Leaves were blotted dry, weighed, and packed tip down into a 5 ml syringe that was centrifuged at 4 °C for 5 min at 200, 500, or 4500  $g$ . The volume of the collected apoplastic solution was measured. The <sup>109</sup>Cd was quantified as described previously by scintillation counting. Two grams of infiltrated leaves were then recovered from the syringe and hot-digested for 1 h at 95 °C in  $\text{HNO}_3$  65% suprapur for the determination of Cd concentration.



All samples were also tested for cytoplasmic contamination. Since  $K^+$  occurs in the cytosol at concentrations at least one order of magnitude higher than in the apoplasmic solute (Lohaus *et al.*, 2001), the concentration of  $K^+$  in the apoplasmic wash fluid and in the digest was analysed by flame-AAS (AAAnalyst 300; Perkin Elmer, Wellesley, USA). Due to the large volumes necessary for flame-AAS analysis, samples were pooled before analysis. The values obtained for  $K^+$  are therefore a mean ( $n=4$ ) without SD.

The intercellular gas space was estimated by weighing the leaves before and after complete infiltration. It was calculated that 8%, 15%, and 30% of the total apoplast wash fluid was recovered at 200, 500, and 4500 g, respectively. In order to avoid underestimation, the Cd and  $K^+$  contents measured in the extracted apoplast wash fluid were therefore extrapolated to 100% by multiplying each result with the ratio of total intercellular gas space volume to the volume extracted experimentally.

**Cell wall extraction:** The distribution of Cd between the cell walls and symplast of leaves and roots was further estimated after isolating the cell walls using the method developed by Hart *et al.* (1992). This treatment isolates the cell walls without destroying their morphology (Hart *et al.*, 1992). Plants were grown for 8 weeks without Cd prior to growth for 4 weeks in 100  $\mu\text{M}$  non-labelled Cd in order to reach high biomass production and concentrations above the detection limit of Cd by ICP-AES, and to avoid the problem of Cd phytotoxicity (see above). Shoots of four plants were harvested and quickly rinsed. Abaxial sides of leaves were peeled using tweezers to remove the cuticle. Peeled leaves and roots were weighed, soaked in 2:1 v/v methanol:chloroform for 3 d and then washed thoroughly in water. Tissues were processed as for the tolerance experiment and Cd was measured. The purity of the isolated cell walls was checked by analysis of the fatty acids composition of the extracts performed by the Lipid Analysis Unit of the Scottish Crop Research Institute in Dundee, Scotland. Briefly, lipids were converted to the methyl esters by reaction with 1% (v/v)  $\text{HSO}_4$  in methanol at 50 °C overnight (Christie, 2003). They were analysed by gas chromatography on a Hewlett Packard 5690 Series II gas chromatograph equipped with a CP-wax 52CB column (25 m $\times$ 0.25 mm  $\times$  0.2  $\mu\text{m}$ ; Chrompack, UK), temperature was programmed from 170 °C (held at this for 3 min) then increased at 4 °C  $\text{min}^{-1}$  to 220 °C (held for a further 3 min) with hydrogen (1 ml  $\text{min}^{-1}$ ) as the carrier gas. Fatty acid 21:0 was added as an internal standard. This confirmed that all membranes had been destroyed by the solvents and that cellular components were washed away.

The dry weight of the whole leaves was calculated from the fresh biomass weighed before the solvent extraction and based on previous observations (20% of fresh weight for shoots). The dry weight of the cell walls was determined after extraction.

## Results

### Biomass production and metal uptake

To assess the potential of the two *T. caerulescens* populations under this study's growth conditions, biomass produced and Cd concentrations in shoots were measured (Table 1). The Prayon ecotype grew best in the presence of Cd with no significant reduction in shoot biomass until 50  $\mu\text{M}$  Cd (43% decrease in biomass, Student *t*-test  $P < 0.05$ ). Ganges was more affected by low and medium Cd concentrations (36% decrease in shoot biomass at 10  $\mu\text{M}$  Cd,  $P < 0.05$ ) but showed a similar decrease in shoot biomass (51%,  $P < 0.05$ ) at 50  $\mu\text{M}$ . Prayon also produced more leaves

**Table 1.** Effect of Cd on biomass and accumulation: average dry weight (mg) and Cd concentration ( $\text{mg kg}^{-1}$ ) per plant of 12-week-old *T. caerulescens* Ganges and Prayon grown for 8 weeks in hydroponics with or without Cd ( $n=4$ , SD in parentheses)

Cd in nutrient solution ( $\mu\text{M}$ )	Dry weight (mg)	Cd ( $\text{mg kg}^{-1}$ )
<b>Ganges</b>		
0	205 (37)	3 (2)
5	138 (61)	751 (299)
10	131 (39)	1091 (160)
50	101 (37)	2960 (635)
100	22 (9)	8163 (1058)
<b>Prayon</b>		
0	235 (73)	4 (4)
5	242 (5)	170 (11)
10	287 (52)	264 (44)
50	135 (58)	2457 (519)
100	43 (31)	5670 (558)

than Ganges (see below). On the other hand, Prayon exhibited necrotic spots on the leaves at 50 and 100  $\mu\text{M}$  Cd (Fig. 1a) but Ganges appeared healthy. None of the plants showed chlorosis.

Cadmium concentrations were always higher in Ganges than in Prayon (Table 1). For example, at 10  $\mu\text{M}$  Cd in the nutrient solution Ganges accumulated 4-fold more Cd in the shoots ( $1091 \pm 160 \text{ mg Cd kg}^{-1}$ ) than did Prayon ( $264 \pm 44 \text{ mg Cd kg}^{-1}$ ). However, this factor diminished with increasing concentrations of Cd and both plants exhibited similar high Cd concentrations in their shoots at 50  $\mu\text{M}$  Cd, reaching  $2960 \pm 635 \text{ mg Cd kg}^{-1}$  in Ganges and  $2457 \pm 519 \text{ mg Cd kg}^{-1}$  in Prayon.

### Visualization of heavy metals in plants

Autoradiographs were prepared to assess whether the concentration of Cd in the nutrient solution or the duration of exposure to Cd had any influence on Cd distribution in the leaves. The darkness of the autoradiographs depended on the  $^{109}\text{Cd}$  only. The concentration of  $^{109}\text{Cd}$  remained constant ( $2.2 \times 10^{-3} \mu\text{M } ^{109}\text{Cd}$ ) in all solutions, whereas total Cd concentration increased from  $2.2 \times 10^{-3} \mu\text{M}$  to 100  $\mu\text{M}$ . Thus the  $^{109}\text{Cd}$  activity present in the samples decreased proportionally and the autoradiographs became lighter with increasing total Cd concentration (Fig. 2). Thus, the intensity of the darkening did not reflect the total concentration of Cd found in the leaves and the intensity of darkening could only be compared when plants were grown with the same  $^{109}\text{Cd}/\text{total Cd}$  ratio.

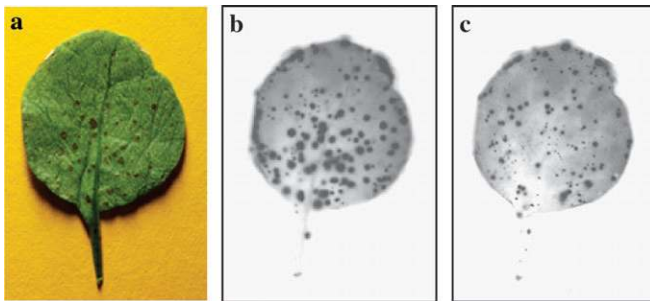
In Ganges leaves, a change in the Cd distribution pattern was observed with increasing Cd concentration (Fig. 1). At low Cd concentration, younger leaves exhibited higher Cd concentrations than older ones as shown by the darker print on the autoradiographs, but the reverse was true at high Cd concentrations. Cadmium was localized mainly at the edge of the leaves, but also in points of higher concentrations spread over the whole limb surface. The number of points

of higher concentrations increased with increasing Cd concentration. The autoradiographs of the adaxial and abaxial sides of the leaves were always different (data not shown).

A similar overall Cd distribution pattern was observed in Prayon (Fig. 2). Points of higher concentration and accumulation at the margin of the leaves were visible. Nevertheless, younger leaves exhibited lower Cd concentration than older ones, as shown by the lighter print on the autoradiographs observed for all the treatments. In addition, the

concentration in older leaves seemed to be more variable, some of the leaves appearing dark and others being very slightly coloured. In this ecotype, the localization of the spots observed on the autoradiographs at high Cd concentrations (50 and 100  $\mu\text{M}$ ) could be directly related to the necrotic spots also observed at these concentrations (Fig. 1). As for Ganges, the autoradiographs of the adaxial and abaxial sides of the leaves were different (Fig. 1).

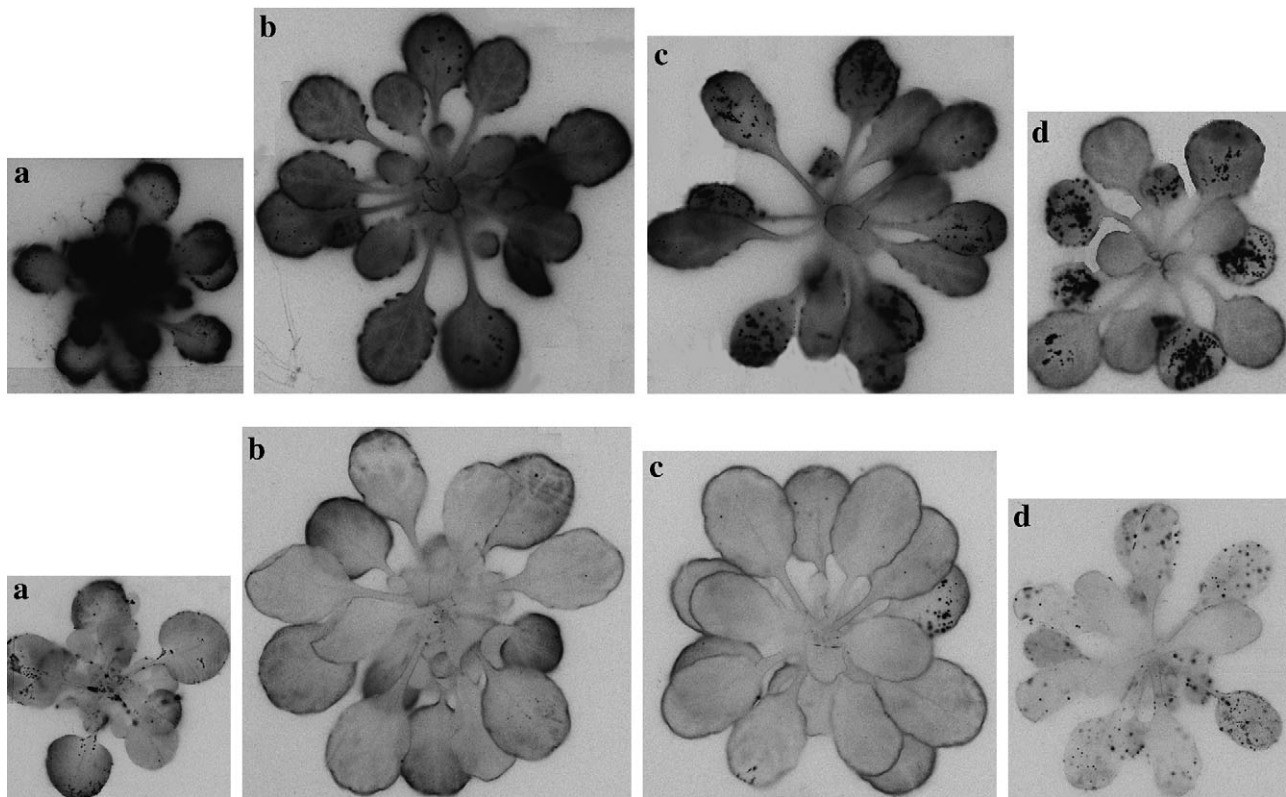
In both ecotypes the duration of exposure to the metal had no influence on the Cd distribution pattern. After 3, 15, or 30 d the distribution of Cd remained similar in the leaves, although Cd concentration increased in leaves as indicated by the increasing darkness of the autoradiographs (Fig. 3).



**Fig. 1.** Leaf of *T. caerulescens* Prayon grown for 15 d in hydroponics with 100  $\mu\text{M}$  Cd spiked with 0.1  $\text{kBq ml}^{-1}$  ( $=2.2 \times 10^{-3}$   $\mu\text{M}$ )  $^{109}\text{Cd}$ . (a) Photograph of the adaxial side, (b) autoradiograph of the adaxial side, and (c) autoradiograph of the abaxial side.

#### *Cadmium concentration in individual leaves of T. caerulescens*

To confirm the autoradiography results, individual leaves of Prayon and Ganges grown in 50  $\mu\text{M}$  Cd were weighed and the Cd concentration was measured (data not shown). Darker leaves on the autoradiographs of a plant corresponded to leaves with higher Cd concentrations. In Prayon, Cd concentrations found in leaves were proportional to leaf dry weight with a correlation coefficient of  $r=0.648$  (1 plant,  $n=80$ ; significant  $P < 0.001$ ). Cadmium concentration was higher in larger leaves. By contrast, the same comparison



**Fig. 2.** Autoradiographs of *T. caerulescens* leaves of the Ganges (top) and Prayon (bottom) ecotypes grown for 30 d in hydroponics with 0.1  $\text{kBq ml}^{-1}$  ( $=2.2 \times 10^{-3}$   $\mu\text{M}$ )  $^{109}\text{Cd}$  and (a) 0  $\mu\text{M}$  Cd, (b) 5  $\mu\text{M}$  Cd, (c) 10  $\mu\text{M}$  Cd, and (d) 50  $\mu\text{M}$  Cd.

for Ganges resulted in  $r=0.118$  (3 plants,  $n=37$ ; not significant). When the three Ganges plants tested were studied independently the results were  $r=0.694$  ( $n=8$ ; significant  $P < 0.05$ ),  $0.254$  ( $n=15$ ; not significant) and  $-0.750$  ( $n=14$ ; significant  $P < 0.001$ ). Surprisingly, the average concentrations in leaves of the three plants were similar with  $1142 \pm 180$  mg Cd kg<sup>-1</sup> for the first,  $1153 \pm 171$  mg Cd kg<sup>-1</sup> for the second, and  $1257 \pm 30$  mg Cd kg<sup>-1</sup> for the third. In Ganges, there was no obvious relationship between Cd concentration and the leaf weight.

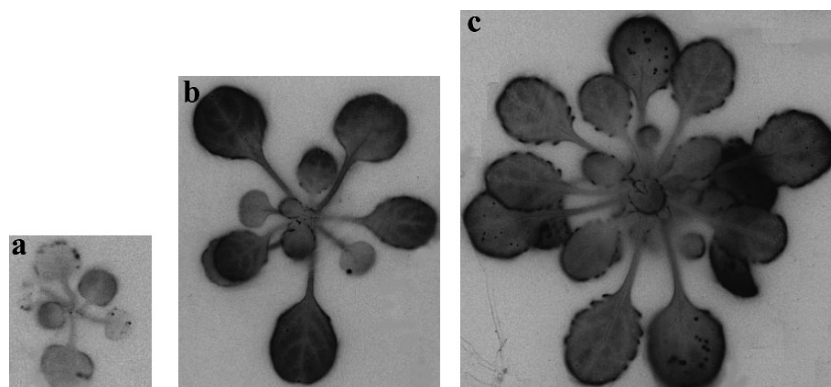
#### Localization of Cd in leaves by SEM-EDXMA

The localization of Cd at the edges of old leaves was studied further with SEM-EDXMA. Examples of scanning electron micrographs and the spots analysed are presented in Fig. 4. The pattern of Cd distribution was similar for both Ganges and Prayon (Fig. 5) and no difference between the two ecotypes was observed in the Cd counts when all cells were taken into account (ANOVA  $F=3.32$ , not significant). However, the location of the cells (edge versus middle of the leaf) significantly affected the Cd signal (ANOVA,  $F=15.49$ ,  $P < 0.001$ ). At the edge of the leaves, the Cd

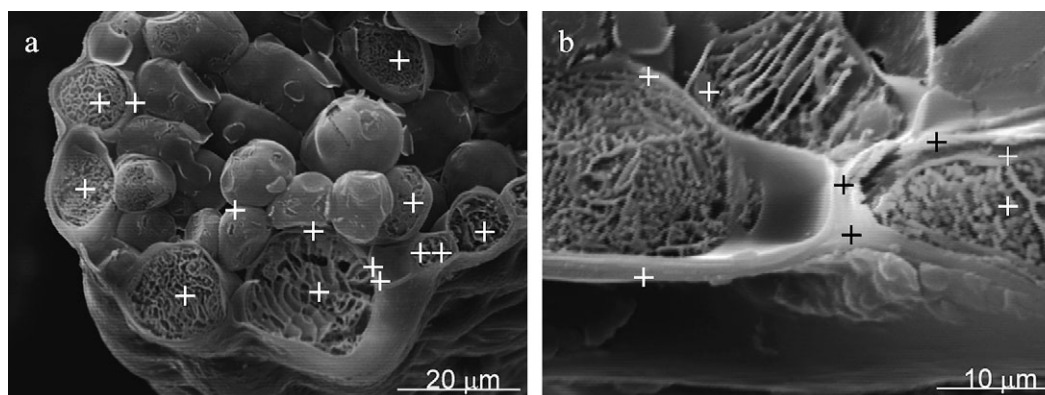
counts were significantly different between the ecotypes (ANOVA  $F=20.17$ ,  $P < 0.001$ ), but also depended on the location of the analysis (epidermal or mesophyll cells, intercellular space; ANOVA  $F=16.11$ ,  $P < 0.001$ ). Cadmium was found mainly in the large epidermal cells at the edge of the leaves and in a larger amount in Ganges than in Prayon. In fact, the only significant difference between the two ecotypes was the size of the signals measured in the epidermal cells (Student  $t$ -test  $P < 0.05$  for both the large and the small epidermal cells), probably resulting from the 1.5-fold Cd concentration difference found between leaves of both populations when grown at 100  $\mu$ M Cd (Table 1). However, Cd was also detected in the mesophyll cells at the edge of the leaf and in epidermal and mesophyll cells in the middle of the leaves (Fig. 5), but in significantly lower amounts than at the edges and no significant difference was found between the two ecotypes. Cadmium was virtually absent from the lateral veins. No central vein was analysed.

#### Subcellular distribution of Cd in *T. caerulescens*

The overall Cd storage of different leaf compartments was quantified using a protoplast extraction procedure.

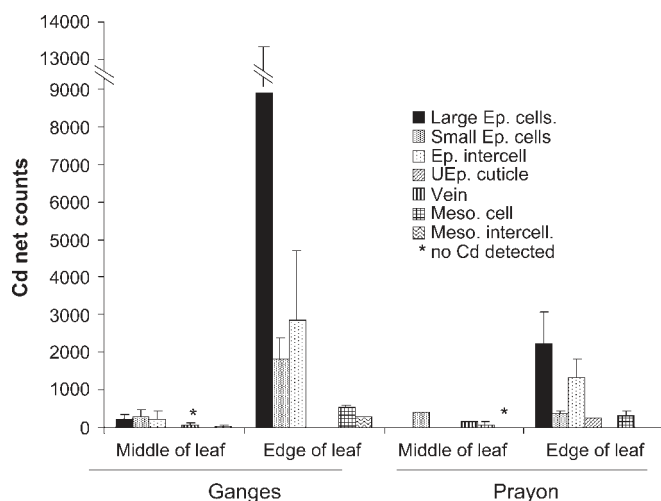


**Fig. 3.** Autoradiographs of leaves of *T. caerulescens* Ganges grown in hydroponics with 5  $\mu$ M Cd spiked with 0.1 kBq ml<sup>-1</sup> ( $\approx 2.2 \times 10^{-3}$   $\mu$ M) <sup>109</sup>Cd at (a) 3 d, (b) 15 d, and (c) 30 d.



**Fig. 4.** Scanning electron micrographs of (a) the leaf edge of Ganges and (b) epidermal cells of the leaf edge of Prayon. The crosses represent the single spot analyses performed on the freeze-fractured samples.



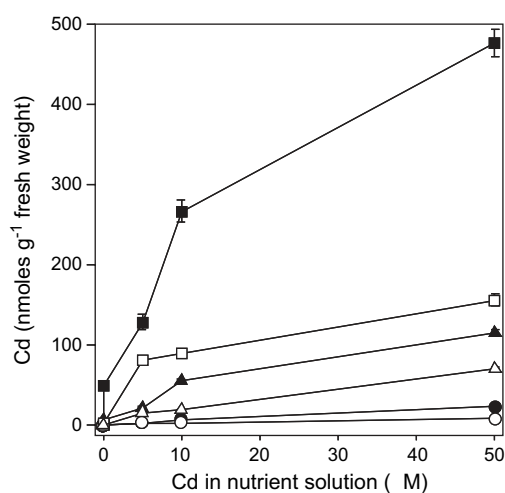


**Fig. 5.** Cadmium net counts in different leaf compartments of Ganges and Prayon grown with 100  $\mu\text{M}$  Cd determined by SEM-EDXMA (Ep., epidermis; UEp., upper epidermis). Analyses of variance (ANOVA) were performed to test the position in leaf (significant), effect of ecotype and type of cell (both significant when the edge of leaf was considered) on the Cd signal. The difference between Prayon and Ganges was significant when large and small epidermal cells from the edge of the leaves were compared (Student *t*-test,  $P < 0.05$ ).

Cadmium distribution between apoplasm/cell walls, lower epidermis, and mesophyll protoplasts was similar for the different concentrations tested (5, 10, and 50  $\mu\text{M}$  Cd) and for both ecotypes (Fig. 6), although the Cd content of the different subcellular fractions increased with increasing Cd treatments. The ranking with respect to Cd concentration was as follows: apoplasm/cell walls > lower epidermis > mesophyll protoplasts. In Ganges 5.4-fold more Cd was measured in the apoplasm/cell walls than in the lower epidermis (4-fold in Prayon). The concentration of Cd measured in the mesophyll protoplasts was negligible.

To characterize the distribution of Cd between the apoplasm and the cell walls further, the apoplast wash fluid was extracted by centrifugation and separated from the cell walls (Table 2). In Ganges, K concentration was 1.3-fold higher at 500 g and 1.5-fold higher at 4500 g than at 200 g. In Prayon, cytoplasmic contamination was greater with a 15-fold increase in K concentration at 500 g and 81-fold at 4500 g compared with 200 g. However, even at 4500 g, Cd amounts measured in the apoplasm fluid were  $10^8$ -fold for Ganges and  $10^9$ -fold for Prayon smaller than the Cd amount in the whole leaf. It was concluded that cytoplasmic contamination was negligible compared with K concentrations measured in the whole leaf and that the Cd concentration found in the apoplasm wash fluid was very limited.

The cell wall extraction allowed further investigation and comparison of the Cd binding capacity of cell walls of both ecotypes (Table 3). Concentrations measured in cell walls after extraction with methanol:chloroform were similar to concentrations in whole shoots. Cell wall biomass accounted for 26% of shoot biomass in Ganges and 35% in Prayon.



**Fig. 6.** Cadmium concentrations measured in mesophyll protoplasts (circles), apoplasm and cell walls (squares) and lower epidermis (triangles) in Ganges (closed symbols) and Prayon (open symbols) leaves. Error bars=means ( $n=4$ )  $\pm$  SD. Error bars do not extend outside some data symbols.

Overall for both ecotypes, between 33% and 35% of the total Cd content of shoots could be allocated to the cell walls.

## Discussion

### Growth and metal uptake

The Prayon ecotype produced the larger biomass and number of leaves in these experimental conditions. Cadmium concentrations found in the leaves of Prayon were always smaller than in the leaves of Ganges, but they were above the 100  $\text{mg kg}^{-1}$  Cd threshold that defines Cd hyperaccumulation in the natural environment (Brown *et al.*, 1994). Although Ganges is a Cd hyperaccumulating ecotype, Prayon does not usually hyperaccumulate Cd in the field (Lombi *et al.*, 2000; Zhao *et al.*, 2003). It is, however, known that plants in solution culture accumulate more Cd than those in soil (Grant *et al.*, 1998). Roosens *et al.* (2003) similarly studied several populations of *T. caerulescens* in solution culture including one Prayon and two Ganges populations. They reported Cd concentrations in shoots of Prayon of the same order as those reported here (over 100  $\text{mg kg}^{-1}$  even at low Cd concentration in the growth medium). However, they also reported slightly higher Cd concentrations in shoots of Ganges ecotypes than the ones determined in this work. Different populations originate from the Ganges area and this may account for this discrepancy. Indeed, seeds were used from 'Les Avinières' whereas those used by Roosens *et al.* (2003) originated from 'St Félix-de-Pallières' and 'Les Malines'.

Overall, the differences in accumulation between both ecotypes tested in this work were smaller than those observed in the field. However, Prayon tolerated large amounts of Cd in its leaves. It was therefore interesting to compare the Cd



**Table 2.** Concentration of Cd and K measured in the apoplasm wash fluid and in the leaf extract in mg per kg of infiltrated leaves of 12-week-old *T. caerulescens* grown in hydroponics with 0.1 kBq ml<sup>-1</sup> (=2.2×10<sup>-3</sup> μM) of <sup>109</sup>Cd (n=4, SD in parentheses)

Centrifugation (g)	Apoplast wash (mg kg <sup>-1</sup> )		Leaf extract (mg kg <sup>-1</sup> )	
	Cd	K <sup>a</sup>	Cd	K <sup>a</sup>
Ganges				
200	4.2×10 <sup>-3</sup> (2.9×10 <sup>-5</sup> )	135	4432 (968)	4883
500	7.0×10 <sup>-3</sup> (1.7×10 <sup>-4</sup> )	179	4011 (247)	5460
4500	24×10 <sup>-3</sup> (3.1×10 <sup>-4</sup> )	205	3518 (821)	4512
Prayon				
200	0.4×10 <sup>-4</sup> (7.1×10 <sup>-6</sup> )	3	1035 (156)	5330
500	6.0×10 <sup>-4</sup> (1.2×10 <sup>-5</sup> )	39	1370 (68)	5910
4500	17.0×10 <sup>-4</sup> (2.9×10 <sup>-5</sup> )	204	9743 (4109)	6045

<sup>a</sup> Samples were pooled before analysis. The values obtained are therefore a mean without SD.

**Table 3.** Concentration of Cd of whole shoots and cell walls of 12-week-old *T. caerulescens* grown for 4 weeks in hydroponics with 100 μM of Cd (n=4, SD in parentheses)

	Cd (mg kg <sup>-1</sup> )	
	Shoots	Cell walls
Ganges	1122 (156)	1436 (278)
Prayon	920 (125)	938 (104)

distribution in its leaves with that in Ganges leaves obtained under the same growth conditions.

#### Distribution of Cd in leaves

To learn more about the general Cd allocation in leaves of *T. caerulescens*, macro-autoradiographs were performed on plants grown in solution culture and treated with increasing Cd concentrations. It was observed that the duration of exposure to the metal had no influence on the Cd distribution pattern within the plant. After 3, 15, or 30 d the allocation of Cd in the leaves remained similar, as also reported by Weigel and Jäger (1980) for Cd in bean plants. By contrast, Cd localization in leaves of Ganges changed according to the Cd concentration in the nutrient solution. At very low Cd concentration, the metal was preferentially located in the younger leaves, whereas at higher Cd concentration Cd was located mainly in the older leaves. Perronnet *et al.* (2003) measured 600 mg Cd kg<sup>-1</sup> in shoots of the Vivier population of *T. caerulescens* grown on contaminated soil, close to the concentrations that this study measured in shoots of Ganges grown at 5 μM Cd, and they similarly reported that younger leaves exhibited higher Cd concentrations. No such difference was found in Prayon between low and high Cd treatments and the highest Cd concentrations were always found in the larger, more mature leaves.

The distribution of Cd was highly uneven in both ecotypes. Although having spots spread over the leaf surface and along the edges, the autoradiographs of adaxial and abaxial sides of the same leaf were not similar, indicating highly localized Cd accumulation. In Prayon, the spots of high Cd accumulation observed on the autoradiographs were directly correlated with the necrotic spots observed on the leaves. It is believed that this indicates that the necrosis resulted directly from Cd accumulation, which decreased cellular viability and finally resulted in cell death. Salt *et al.* (1995a) analysed Cd in leaves of Indian mustard by autoradiography. They found that Cd toxicity induced chlorosis in young leaves and that Cd preferentially accumulated within these young leaves, explaining the localization of the chlorosis. However, contrary to these results with *T. caerulescens*, they found an even distribution on the leaf surface that they correlated with trichomes localization. Sequestration of metals in trichomes has been reported in a number of other annual hyperaccumulators and non-hyperaccumulators (Küpper *et al.*, 1999, 2000; Zhao *et al.*, 2000; Choi *et al.*, 2001; Ager *et al.*, 2002). However, *T. caerulescens* does not possess trichomes and mechanisms must, therefore, be based on a different compartment or cell type.

#### Localization of Cd at the subcellular level

To learn more about the cellular and subcellular Cd allocation in leaves of *T. caerulescens* several different approaches were tested to examine the importance of different leaf parts in Cd storage. Indeed, as mentioned by Frey *et al.* (2000b) artefacts and/or Cd losses may occur during the preparation of cell fractions separated by biochemical and physical methods (epidermal strips, protoplast extraction, apoplast wash fluid, and cell wall extraction). Cryo-SEM-EDXMA is more reliable in this respect, but lack of sensitivity and interference with other cations (e.g. K) impose the use of high concentrations of Cd that do not always represent a realistic environment. Consequently, the test concentrations were selected according to the sensitivity of the method employed. The different approaches were combined in this study in order to (i) validate the results obtained by each specific method and (ii) make the link between observations and measurements performed at different scales.

Overall, the results of the different experiments were consistent with one another. Furthermore, it was demonstrated with the protoplast extraction procedure that Cd distribution between compartments remained similar for the different concentrations tested, although the Cd content of the different subcellular fractions increased with increasing Cd treatments. The SEM-EDXMA analysis confirmed that an important site of Cd allocation was at the edges of the leaves in both Ganges and Prayon ecotypes.

Mizuno *et al.* (2003) pointed out the importance of Ni allocation at the leaf edges of *T. japonicum*. They suggested

that Ni was transported along with the transpiration stream and that Ni in excess was excreted together with guttation. It was earlier reported that the guttation fluid commonly functions to exclude various substances including K, Mg, and Ca in sunflower, and recombinant protein in tobacco (Komarnytsky *et al.*, 2000; Tanner and Beevers, 2001). Very low Cd concentrations were obtained from the apoplastic wash fluid. It is therefore questionable whether this would be an acceptable hypothesis for Cd in *T. caerulescens*. However, this experiment did not take into account the process kinetics and must be regarded as a snap shot performed at one particular time. A high rate of Cd loading/unloading as well as a possible time-dependent mechanism could not be assessed and thus cannot be excluded.

Cellular Cd is known to accumulate preferentially within vacuoles in the leaves of a number of different plant species including tobacco and *Silene vulgaris* (Vögeli-Lange and Wagner, 1990; Chardonens *et al.*, 1998, 1999), and this has often been proposed as a possible mechanism of Cd tolerance in plants. Chardonens *et al.* (1998, 1999) also showed that, in *S. vulgaris*, Cd accumulated mainly in the lower leaf epidermis. In this study Cd was mostly found in the epidermal cells as observed previously for Zn in *T. caerulescens* (Vázquez *et al.*, 1992, 1994; Küpper *et al.*, 1999; Frey *et al.*, 2000a), but no consistent differences were observed between the upper and lower epidermis. The variation in relative Cd concentration in the epidermal cells appeared to be mainly associated with the cell size as shown for Zn (Küpper *et al.*, 1999; Frey *et al.*, 2000a). This relationship suggests that vacuolation may be an important driving force for Cd sequestration in epidermal cells of *T. caerulescens* leaves.

However, Cd was found both inside the vacuoles and in the cell walls. This latter result was obtained by both the SEM-EDXMA observations and the compartmental studies and in both ecotypes, indicating an additional storage mechanism. Indeed, it was shown that up to 35% of Cd could be bound to cell walls (epidermal or mesophyll) in contrast to Salt *et al.* (1999) who reported 12% Zn in the cell walls of *T. caerulescens*. Accumulation of Cd in the cell walls may therefore be an important tolerance mechanism in *T. caerulescens* as in other organisms or plants (Galli *et al.*, 1994; Turnau *et al.*, 1994; Blaudez *et al.*, 2000). However, the role of cell walls in Cd binding and storage in plants remains controversial. For example, Vögeli-Lange and Wagner (1990) and Vázquez *et al.* (1992) failed to show substantial Cd binding in cell walls of tobacco and *T. caerulescens* roots, but the importance of Cd binding to cell walls and the limitation of its subsequent translocation to shoots has been demonstrated for root cells of non-hyperaccumulating plants (Wagner, 1993; Grant *et al.*, 1998) and was recently described for *T. caerulescens* hairy roots (Boominathan and Doran, 2003). In *Zea mays*, cell walls were assumed to play the most important role in Cd accumulation (Khan *et al.*, 1984), but Carrier *et al.*

(2003) reported that Cd was distributed both in vacuoles and in cell walls of *B. napus* leaves. Cell walls can easily interact with metal ions because of the presence of many enzymes and negative charge sites on their surfaces (Wang and Evangelou, 1995), thereby potentially lowering toxic Cd ions in the cytoplasm. The low Cd concentrations measured in the apoplasm fluid could indeed be due to the role of the cell walls in continuously reducing metal-ion activity in the intercellular spaces through tight metal binding (Bringezu *et al.*, 1999). However, in the literature, the emphasis is currently given to vacuolar metal compartmentation and sequestration by phytochelatin. So far, less attention has been devoted to other tolerance mechanisms, although different tolerance mechanisms could operate in combination as proposed by Baker (1987).

To date, two mechanisms have been proposed to explain partly the high uptake of metals by hyperaccumulators: (i) enhanced absorption of metal into the roots (Lasat *et al.*, 1996) coupled with high rates of translocation of metal from roots to shoots (Shen *et al.*, 1997; Lasat *et al.*, 1998), and (ii) foraging for metal by the roots, involving preferential allocation of root biomass into regions of metal enrichment (Schwartz *et al.*, 1999; Whiting *et al.*, 2000) and a large root system compared with shoot dry matter that might further favour soil prospecting as well as heavy metal uptake (Keller *et al.*, 2003). Kochian *et al.* (2002) hypothesized that this increased transport could include a stimulated metal influx across the leaf cell plasma membrane and an enhanced storage in the leaf vacuole. According to these results, this may be possible because the only differences found between the two ecotypes in this study were in the Cd concentrations found in the storage sinks, vacuoles, and cell walls.

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