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Hyperaccumulation of Cadmium and Zinc in *Thlaspi caerulescens* and *Arabidopsis halleri* at the Leaf Cellular Level

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Vacuolar compartmentalization or cell wall binding in leaves could play a major role in hyperaccumulation of heavy metals. However, little is known about the physiology of intracellular cadmium (Cd) sequestration in plants. We investigated the role of the leaf cells in allocating metal in hyperaccumulating plants by measuring short-term 109Cd and 65Zn uptake in mesophyll protoplasts of *Thlaspi caerulescens* “Ganges” and *Arabidopsis halleri*, both hyperaccumulators of zinc (Zn) and Cd, and *T. caerulescens* “Prayon,” accumulating Cd at a lower degree. The effects of low temperature, several divalent cations, and pre-exposure of the plants to metals were investigated. There was no significant difference between the Michaelis-Menten kinetic constants of the three plants. It indicates that differences in metal uptake cannot be explained by different constitutive transport capacities at the leaf protoplast level and that plasma and vacuole membranes of mesophyll cells are not responsible for the differences observed in heavy metal allocation. This suggests the existence of regulation mechanisms before the plasma membrane of leaf mesophyll protoplasts. However, pre-exposure of the plants to Cd induced an increase in Cd accumulation in protoplasts of “Ganges,” whereas it decreased Cd accumulation in *A. halleri* protoplasts, indicating that Cd-permeable transport proteins are differentially regulated. The experiment with competitors has shown that probably more than one single transport system is carrying Cd in parallel into the cell and that in *T. caerulescens* “Prayon,” Cd could be transported by a Zn and Ca pathway, whereas in “Ganges,” Cd could be transported mainly by other pathways.

Cadmium (Cd) and zinc (Zn) are two widespread harmful heavy metals, but there is no cost-effective mean to remove them from the soil. Although phytoextraction using hyperaccumulator plants is seen as a promising technique, a lack of understanding of the basic physiological, biochemical, and molecular mechanisms involved in heavy metal hyperaccumulation prevents the optimization of the phytoextraction technique and its further commercial application. Therefore, a research priority is to gain basic information on the dynamics of metal movement into the cells, their final allocation, and their sink capacities in hyperaccumulating species.

*Thlaspi caerulescens* and *Arabidopsis halleri* are both plants able to hyperaccumulate Zn and Cd (Robinson et al., 1998; Bert et al., 2000). In *T. caerulescens*, Zn seems to be sequestered preferentially in vacuoles of epidermal cells in a soluble form (Küpper et al., 1999; Frey et al., 2000). In *A. halleri* leaves, Zn was found to be predominantly coordinated to malate (Sarret et al., 2002) and accumulated in the mesophyll cells (Küpper et al., 2000; Zhao et al., 2000). An important trait of hyperaccumulating species might be enhanced translocation of the absorbed metal to the shoot. Time course studies of Zn accumulation revealed that *T. caerulescens* exhibited a 10-fold greater Zn translocation to the shoot as compared with *Thlaspi arvense* (Lasat et al., 1996), which was correlated with a 5-fold increase of Zn in xylem sap (Lasat et al., 1998). These authors have performed compartmentation studies and have found that 65Zn uptake by leaf protoplasts is also stimulated in *T. caerulescens* compared with *T. arvense*. These physiological evidences indicate that Zn hyperaccumulation in *T. caerulescens* is caused, in part, by an increased transport at multiple sites along the metal absorption and translocation pathway. 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. Although vacuole and/or protoplast transport and storage of major elements have been studied for crop plants (Dietz et al., 1992), Lasat’s study is, to our knowledge, the only one trying to define the role of the leaf cells in uptake and storage of heavy metals in hyperaccumulator plants. Tolerance and accumulation in *T. caerulescens* also have been studied at the molecular level. Three ZIP-like (Grotz et al., 1998) Zn transporters mainly overexpressed in the roots and one ZAT-like (Van der Zaal et al., 1999) Zn transporter mainly expressed in the leaves have been described (Pence et al., 2000; Assunção et al., 2001). Comparison with *T. arvense* (nonaccumulator) indi-

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cated that metal regulation of gene expression was altered in the hyperaccumulator but not functionally different from the nonaccumulator (Lasat and Kochian, 2000).

Contrary to Zn, little information is available on Cd hyperaccumulation, and no putative high-affinity transporter gene has been identified in plants yet. In T. caerulescens, Cd has been found in the apoplasm and in the vacuole (Vázquez et al., 1992). It has been demonstrated that the physiological mechanism of Cd tolerance is not based on an enhanced synthesis of phytochelatins (Ebbs et al., 2002; Schat et al., 2002) but on a preferential compartmentation of the metal in the plant. Boominnathan and Doran (2003) reported, for example, the ability of T. caerulescens hairy roots to hold most of the Cd in the cell wall. It is generally believed that Cd uptake by plants represents opportunistic transport via cation channels for Ca and Mg or via a carrier for other divalent cations such as Zn, Cu, or Fe (Welch and Norvell, 1999). It also has been suggested that there exist common mechanisms of absorption and transport of Zn and Cd in T. caerulescens because Cd and Zn have a similar electronic structure (Baker et al., 1994). Bert et al. (2003) showed that in A. halleri, Cd and Zn accumulation were positively correlated, suggesting that the metals are taken up, at least to a certain degree, by the same transporter(s) or are controlled by common regulators. There is evidence that the Zn transporter, ZNT1, recently cloned from the Prayon ecotype of T. caerulescens, also can mediate transport of Cd, albeit with low-affinity (Pence et al., 2000). Zn was found in some cases to depress Cd uptake, indicating some kind of interaction between these metals (Lombi et al., 2000, 2001). However, because of differences in Cd uptake found between populations, it seems now that there might be differences between Cd and Zn for uptake and accumulation by T. caerulescens (Lombi et al., 2000).

From these early results, it seems that removal of heavy metals from metabolically active cellular sites and subsequent storage in inactive compartments is the key to heavy metal tolerance; therefore, vacuolar compartmentalization or cell wall binding could play a major role in tolerance and hyperaccumulation.

The goal of this work was to understand some of the mechanisms involved in Cd and Zn uptake at the mesophyll protoplast level. Special attention was directed to the differences between T. caerulescens population “Ganges,” A. halleri, both hyperaccumulator species, and T. caerulescens population “Prayon” accumulating Cd at a lower degree. More in detail, we assessed: (a) if differences in uptake at the plant level could be explained by an enhanced transport at the cell level, (b) if Cd and Zn compartmentation in T. caerulescens leaves was a passive or carrier-mediated mechanism and how it differed between T. caerulescens ecotypes and A. halleri, and (c) the rate of metal uptake in protoplasts and their storage kinetics for the three plants tested. Thus, metal uptake experiments on mesophyll protoplasts were performed overtime, and at various concentrations of Cd, the effects of plant pretreatment with metals, the effects of low temperature and several divalent cations, and the effect of a competitor on protoplast uptake were assessed. Emphasis was put on Cd although Zn was also studied for comparison.

RESULTS
Viability and Homogeneity of Protoplast Samples

The percentage of viable protoplasts was determined by staining with fluorescein diacetate (FDA). Protoplast viability ranged between 80% and 95%. In addition, sizes of protoplasts were measured to assess homogeneity between the different samples. Protoplasts were similarly distributed for all the plants tested and the different treatments. Most of the protoplasts were found in the surface classes between 1,000 and 2,000 μm² (39% ± 6% of protoplast no.) and between 2,000 and 4,000 μm² (41% ± 4%). Only 7% ± 4% were smaller than 1,000 μm² and 13% ± 8% bigger than 4,000 μm².

Effects of Plant Pre-Exposure to Cd and Zn

To determine whether Zn and Cd accumulation in protoplasts was modified by exposure of the plant to heavy metals, we investigated the effect of plant pretreatment with Cd or Zn on the time-dependent kinetics of heavy metal apparent uptake in protoplasts. The concentration of Cd and Zn measured in leaves and the aerial biomass of T. caerulescens “Ganges” and A. halleri are presented in Table I. No phytotoxic effect was visible on leaves of both pre-treated plants. No effect of Zn pre-exposure of the plants was observed on the Zn uptake capacities of protoplasts from T. caerulescens “Ganges,” but protoplasts extracted from plants grown in 10 μM Cd solution accumulated 2.8 times more Cd than protoplasts extracted from plants grown in 5 μM Cd (Fig. 1). On the contrary, protoplasts extracted from A. halleri grown in 10 μM Cd solution accumulated 2.1 times less Cd than protoplasts extracted from plants grown in 5 μM or no Cd (Fig. 1). The differences were statistically significant for both plants (Student’s t test P < 0.001).

Cd and Zn Accumulation in Protoplasts of the Two Ecotypes of T. caerulescens and A. halleri

Plants were grown in absence of metals in the nutrient medium to avoid pre-exposure effects. When starting the study, we calculated kinetic constants for different times of exposure to find the most representative time of incubation for the calculations. Based on the time-dependent experiment (Fig. 1), calculations of the Michaelis-Menten parameters...
$V_{\text{max}}$ and $K_m$ were done at $t = 5$ min (linear accumulation rate phase) and at $t = 30$ min (beginning of the plateau). After 5 min of incubation, *T. caerulescens "Ganges"* exhibited for Cd a $V_{\text{max}}$ of $0.83 \pm 0.07 \mu\text{M min}^{-1} 0.25 \mu\text{L}^{-1}$ protoplasts and a $K_m$ of $2.58 \pm 1.03 \mu\text{M}$. For Zn, the $V_{\text{max}}$ was $1.915 \pm 0.3 \mu\text{M min}^{-1} 0.25 \mu\text{L}^{-1}$ protoplasts and $K_m$ was $5.053 \pm 3.14 \mu\text{M}$. Results at 30 min were a $V_{\text{max}}$ of $0.50 \pm 0.08 \mu\text{M Cd min}^{-1} 0.25 \mu\text{L}^{-1}$ protoplasts and a $K_m$ of $9.68 \pm 5.78 \mu\text{M}$. $V_{\text{max}}$ of $0.27 \pm 0.03 \mu\text{M Zn min}^{-1} 0.25 \mu\text{L}^{-1}$ protoplasts, and a $K_m$ of $4.01 \pm 1.90 \mu\text{M Zn}$. Because of high sd, there were no significant differences in the calculated $K_m$ between the two times of incubation, and $V_{\text{max}}$ values were in the same order of magnitude. In addition, at 30 min, reproducibility was better. Then, we decided to perform all the measurements and calculations at 30 min, although the accumulation rate was not linear.

The accumulation in protoplasts was shown to be concentration dependent for the three plants tested. Because $^{109}\text{Cd}$ and $^{65}\text{Zn}$ uptake was measured during a short period only (30 min), the results mainly represent unidirectional influxes. The concentration-dependent accumulation kinetics for $^{109}\text{Cd}$ and $^{65}\text{Zn}$ was characterized by smooth non-saturating curves. The curves could be mathematically resolved into linear and saturable components (Fig. 2) by applying a modified hyperbolic function defined as:

\[ y = \left(\frac{a \times x}{b + x}\right) + (c \times x) \]

**Figure 1.** Time-dependent uptake of 10 $\mu\text{M Cd}$ in *T. caerulescens "Ganges"* (A) or *A. halleri* (B) mesophyll protoplasts. Plants were pre-exposed for 7 weeks to 0 (triangles), 5 (circles), or 10 (squares) $\mu\text{M Cd}$ during growth in hydroponics. C, Time-dependent uptake of 10 $\mu\text{M Zn}$ in *T. caerulescens "Ganges"* mesophyll protoplasts. Plants were pre-exposed for 7 weeks to 0 (triangles), 50 (circles), or 500 (squares) $\mu\text{M Zn}$ during growth in hydroponics. Error bars do not extend outside some data symbols. Error bars = means $(n = 4) \pm \text{SD}$.
where \( x = \) Cd or Zn concentration in the incubation medium in micromolar; \( y = \) Cd or Zn accumulation rate in protoplasts in micromolar per minute per 0.25 \( \mu \text{L} \) of protoplasts; and \( a, b, \) and \( c \) were parameters determined by the curve fitting algorithm to best fit the data points. In all cases, the model fitted closely the experimental data as demonstrated by the \( R^2 \) values for the fitted curves ranging from 0.961 to 0.996 (Table II). As calculated by Lasat et al. (1996) and Lombi et al. (2001, 2002) subtractions of the regression line plotted through high-concentration points leaves out the saturating Michaelis-Menten curve, which allows determination of the kinetic constants \( K_m \) and \( V_{\text{max}} \) (Table II). The remaining saturable component is the result of carrier-mediated transport across the plasma membrane. There were no significant differences in the calculated kinetic constants between the plants studied: Mesophyll protoplasts of the three plants showed the same affinity and, when not pre-incubated, the same capacity to transport Cd or Zn into the cell.

**Effect of Low Temperature**

We investigated the effect of low temperature on heavy metal uptake in protoplasts to further determine whether Cd and Zn accumulation in protoplasts was caused by movement across the plasma membrane into the cytosol or by binding of heavy metal to negatively charged sites associated with the external face of the plasma membrane. The uptake was strongly inhibited when performed at ice temperature (2°C) for both Cd and Zn for the three plants: After 30 min of incubation with 10 \( \mu \text{M} \) Cd or Zn, the cold treatment decreased total accumulation of Cd or Zn by at least 50% compared with the controls (Figs. 3 and 4).

**Effect of Competitors**

To determine whether Cd accumulation in protoplasts was because of opportunistic transport via channels or carriers for other cations, we investigated the effects of several cations and of a specific inhibitor of Ca channel, verapamil, on the kinetics of Cd and Zn apparent uptake in protoplasts. Figure 4 summarizes the results for the three plants. The addition of 100 \( \mu \text{M} \) verapamil had no effect on the apparent uptake of Cd or Zn in the “Ganges” ecotype (data not shown). In this ecotype, addition of 50 \( \mu \text{M} \) Cd\(^{2+}\) or 50 \( \mu \text{M} \) Ca\(^{2+}\) decreased Zn transport by 40% (Student’s \( t \) test \( P < 0.0001 \)) and 15% \( (P < 0.001) \), respectively, whereas only addition of 50 \( \mu \text{M} \) Zn\(^{2+}\) decreased Cd transport by 20% \( (P < 0.01) \). However, 200 \( \mu \text{M} \) Ca\(^{2+}\) had also a 15% \( (P < 0.001) \) inhibitory effect on Cd\(^{2+}\) uptake. Fifty micromolar Cd\(^{2+}\) inhibited Zn uptake by 15% \( (P < 0.05) \) in T. caerulescens “Prayon,” but 50 \( \mu \text{M} \) Zn\(^{2+}\), 50 \( \mu \text{M} \) Ca\(^{2+}\), and 50 \( \mu \text{M} \) Mg\(^{2+}\) inhibited Cd uptake by respectively 30% \( (P < 0.0001) \), 15% \( (P < 0.0001) \), and 25% \( (P < 0.001) \). A competition between Cd and Zn was also observed for A. halleri: 50 \( \mu \text{M} \) Cd\(^{2+}\) reduced Zn accumulation by 15% \( (P < 0.001) \) and 50 \( \mu \text{M} \) Zn\(^{2+}\) reduced Cd accumulation by 10% \( (P < 0.001) \).

**Table II.** Parameters of the Michaelis-Menten model for Cd and Zn absorption by mesophyll protoplasts \((n = 4, \text{ SD in parentheses})\)

<table>
<thead>
<tr>
<th>Plant Used for Protoplast Extraction</th>
<th>( V_{\text{max}} ) ( \mu \text{M} \text{ min}^{-1} ) 0.25 ( \mu \text{L} ) protoplasts</th>
<th>( K_m ) ( \mu \text{M} )</th>
<th>( R^2 )</th>
<th>( V_{\text{max}} ) ( \mu \text{M} \text{ min}^{-1} ) 0.25 ( \mu \text{L} ) protoplasts</th>
<th>( K_m ) ( \mu \text{M} )</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. caerulescens “Ganges”</td>
<td>0.50 (0.08)</td>
<td>9.68 (5.78)</td>
<td>0.976*</td>
<td>0.27 (0.03)</td>
<td>4.01 (1.90)</td>
<td>0.996*</td>
</tr>
<tr>
<td>T. caerulescens “Prayon”</td>
<td>1.15 (0.14)</td>
<td>4.83 (2.11)</td>
<td>0.977*</td>
<td>0.53 (0.08)</td>
<td>12.30 (5.80)</td>
<td>0.961*</td>
</tr>
<tr>
<td>A. halleri</td>
<td>0.78 (0.10)</td>
<td>4.81 (2.13)</td>
<td>0.983*</td>
<td>0.05 (0.01)</td>
<td>3.35 (4.42)</td>
<td>0.983*</td>
</tr>
</tbody>
</table>

*2\( \alpha < 0.001 \).*

DISCUSSION

Effects of Plant Pretreatment

Laboratory studies using nutrient solutions are the only possible experiments allowing determination of the precise metal concentration in the growth medium. In soil, it is necessary to base the discussion on total metal concentration or one of a variety of measures of the available metal concentration. Because of numerous parameters controlling soil metal bioavailability, the link between metal available to plant in soil and plant uptake is not obvious. On the other hand, in soils, the metals are present from the beginning and during the plant growth and may have a significant effect on plant uptake ability. To date, most studies in nutrient solution have examined Cd transport at unrealistically high concentrations of metal and very short-time exposure, creating experimental conditions far from the soil conditions and limiting the possible impact of pre-exposure (Sanità di Toppi and Gabbrielli, 1999). Therefore, it is difficult to compare hydroponics studies with soil studies. We chose long-time exposure and Cd concentrations in the range of concentrations that can be found in soil solution of contaminated soils. In addition, concentrations were chosen to avoid visible phytotoxic symptoms, like necrosis or chlorosis, and no phytotoxic symptoms were observed on any of the treated plants compared with the controls except a loss in biomass (Table I).

*T. caerulescens* “Ganges” showed a higher uptake capacity in mesophyll protoplasts when exposed to 10 μM Cd during growth. It seems that a mechanism was switched on in the treated plant above a determined threshold because 5 μM Cd did not trigger an increased uptake. This mechanism may allow a faster removal of heavy metals from metabolically active cellular sites and subsequent storage in inactive compartments. No such effect was observed with Zn, which could indicate that either the mechanism for Zn uptake is regulated differently, or the threshold has not been reached at 500 μM Zn. Further experiments with Zn would be needed to clarify this point; however, this was not the purpose of the present work. *A. halleri*, on the contrary, showed a decreased transport into protoplasts in Cd-treated plants, indicating the establishment of a mechanism of Cd avoidance. Moreover, *A. halleri* seemed to be more affected than “Ganges” by Cd pretreatments, as shown by the larger loss in biomass (Table I). An explanation to the establishment of a mechanism of Cd avoidance in *A. halleri* could be that a plant accumulating at a high concentration of Cd in the mesophyll cells would risk damaging its photosynthesis apparatus (Krupa and Baszynski, 1995; Horváth et al., 1996; Geiken et al., 1998). These results suggest that “Ganges” and *A. halleri* have a different mechanism regulating adaptation to Cd, despite the fact that mesophyll cells of the leaves have a similar constitutive Cd transport capacity as discussed below.
Cd and Zn Uptake by the Two Ecotypes of *T. caerulescens* and *A. halleri*

The saturable nature of Cd and Zn accumulation in this study suggests that they are both taken up in protoplasts via a carrier-mediated system. There were no significant differences in the calculated kinetic constants between the three plants. In the three cases, mesophyll cells were equally capable of transporting and accumulating the two metals. Nevertheless, *A. halleri* is known to hyperaccumulate Cd and Zn in the mesophyll cells (Küpper et al., 2000; Zhao et al., 2000), whereas *T. caerulescens* preferentially hyperaccumulates Zn in the epidermal cells (Frey et al., 2000). Moreover, *T. caerulescens* “Prayon” does not hyperaccumulate Cd in the field (Lombi et al., 2000). Calculated kinetic constants were also similar for Cd and Zn in the three plants, again not reflecting the differences of accumulation known in “Prayon” ecotype for Zn and Cd. Because experimental procedures and conditions have a considerable influence on the kinetics of ion uptake, $V_{\text{max}}$ and $K_m$ reported by different authors are not strictly comparable. However, the $K_m$s estimated here for Zn in mesophyll protoplasts were in the same order of magnitude as those reported by several authors (ranging from 0.3–8 μM) for whole plants or roots of *T. caerulescens* (Lasat and Kochian, 1997; Pence et al., 2000; Lombi et al., 2001, 2002; Kochian et al., 2002). $V_{\text{max}}$ values are even more difficult to compare because of the different units of measure used. However, we found a higher $V_{\text{max}}$ value for “Prayon” than for “Ganges” for Zn uptake, whereas Lombi et al. (2001) reported the opposite for roots of the same plants. This difference may indicate that different mechanisms underlie in the different steps along the way of the metal from the soil to the leaf cells, indicating that the mechanism responsible for metal uptake in hyperaccumulator plants is probably very complex.

Lombi et al. (2001, 2002) are the only authors who calculated Michaelis-Menten parameters for Cd in *T. caerulescens*. They reported values for apparent $K_m$s between 0.18 and 1.21 μM for Cd in roots of “Ganges” and “Prayon.” However, the studies were performed on 40-d-old seedlings of *T. caerulescens*, thereby limiting the comparison with our work that was performed on leaf cells of 12-week-old plants. Besides, it is questionable whether 40-d-old plants had reached their plain maturity because they have a very slow growth. Flowering usually takes place after at least 14 weeks in controlled conditions. Bovet et al. (2003) showed that results obtained on Cd transport in Arabidopsis could not be extrapolated from seedlings to mature plants.

The physiological evidence obtained from this study (similar behavior at the protoplast level) suggests the existence of regulation mechanisms before the protoplast plasma membrane that direct the metals to their final location in plants. The cell wall could act as a selective barrier, considering its capacity to interact with metal ions and the presence of many enzymes at its surface (Wang and Evangelou, 1995). In mycorrhizal fungi, Cd was found to be bound to negatively charged sites associated with the cell wall such as cellulose, cellulose derivatives, or to the outer pigmented layer of the cell wall (Galli et al., 1994; Turnau et al., 1994; Blaudez et al., 2000). Nickel was also found to be associated with cell wall pectates in *Hybanthus floribundus* (Salt and Krämer, 2000). Metal binding to cell wall as a possible heavy metal tolerance mechanism has been proposed for various plants and metals (for review, see Wang and Evangelou, 1995). The importance of Cd binding to cell wall and the limitation of its subsequent translocation into shoots is well known for root cells of non-hyperaccumulator plants (Wagner, 1993; Grant et al., 1998) and was described recently in *T. caerulescens* hairy roots (Boominathan and Doran, 2003). There are large variations in the retention of Cd in cells between plant species (Guo et al., 1995) or genotypes of a given species (Florjin and Van Beusichem, 1993; Cakmak et al., 2000): different binding capacity to the cell wall has been proposed by these authors as an explanation for the differences in Cd uptake and distribution. Polyvalent cations are more likely to interact with cell walls than monovalent cations because of their stronger electrostatic attraction to cell wall negative charge sites. Most heavy metals are divalent or trivalent cations and, therefore, are expected to undergo absorption/exchange reactions with wall surfaces before they move to their final location (Wang and Evangelou, 1995). Thus, it is likely that variation in binding to leaf cell walls could explain the differences of heavy metal allocation described for the three plants studied here. Nevertheless, because this study is based on short-term experiments because of the short life of extracted protoplasts, we cannot totally exclude that in the long term differences might appear, originating from the required time for synthesis of heavy metals complexing molecules or to reach the equilibrium between free and bound heavy metals, for example.

Effects of Cold and Competitors on Cd and Zn Uptake

Zhao et al. (2002) assumed that metabolically dependent uptake would be negligible at low temperatures. Thus, the difference between results obtained at room temperature and on ice would represent the metabolically dependent uptake of Cd and Zn. In our experiment, Cd and Zn accumulation in protoplasts was strongly decreased at low temperature for the three plants, suggesting that it was a metabolically mediated process. This result, together with the concentration-dependent results, confirms a carrier-mediated pathway for both metals.

It has been shown that verapamil at micromolar concentrations inhibited voltage-dependent Ca and Cd influx in animal cells (Hinkle et al., 1987), but the
effects reported in plant studies seem inconsistent (Pineros and Tester, 1997): Ca influx into protoplasts from *Physcomitrella patens* (Schumaker and Gizinski, 1993), carrot (*Daucus carota*; Graziana et al., 1988), *Amaranthus tricolor* (Rengel and Elliot, 1992), and tobacco (*Nicotiana tabacum*; Volotovski et al., 1998) and into plasma membrane vesicles isolated from oat (*Avena sativa*) roots (Gonzalez et al., 1999) was reduced upon exposure to verapamil, although up to 100 μM verapamil did not block the calcium influx into plasma membrane vesicles isolated from wheat roots or oat seedlings (Huang et al., 1994; Babourina et al., 2000). Furthermore, in aquatic plants, it was found to inhibit Ca uptake but to increase Cd uptake (Karez et al., 1990; Tripathi et al., 1995). In epidermal peels of Arabidopsis, 250 μM verapamil was found to inhibit the stomatal closure induced by Cd (Perfus-Barbeoch et al., 2002). Interpretation of these pharmacological results is difficult as the effect of verapamil on other experimental variables (e.g., membrane potential) is unknown (Pineros and Tester, 1997; Babourina et al., 2000). In this study, verapamil at 100 μM was found to have no effect on the accumulation of Cd or Zn by “Ganges.”

Cd could be taken up in plants by carriers or cation channels for other cations such as Zn
2+, Fe2+, Ca2+, or Mg2+ (Welch and Norvell, 1999). There are numerous studies showing inhibitory effects of those divalent cations on Cd uptake by higher plants or algae (Smeyer-Verbeke et al., 1978; Cataldo et al., 1983; Karez et al., 1990; Costa and Morel, 1993; Tripathi et al., 1995; Hart et al., 2002). In nonaccumulating plants, Clemens et al. (1998) showed that a Ca transport pathway could be involved in the uptake of Cd, albeit with low affinity. Members of the ZIP gene family were shown to be capable of transporting transition metals including Fe, Zn, Mn, and Cd (Guerinot, 2000). The Fe transporters such as IRT1 (ZIP) and Nramp have been shown to be able to transport several metals including Cd in Arabidopsis (Korshunova et al., 1999; Thomine et al., 2002) and in *T. caerulescens* (Zhao et al., 2002). All these data are consistent with the reciprocal uptake inhibition between Cd and Zn in the two ecotypes of *T. caerulescens* and *A. halleri* that we observed in our work. We also observed an inhibition by Ca2+ and Mg2+ for “Prayon,” whereas “Ganges” was less sensitive to Ca2+ and not at all to Mg2+. Although a definite type of transporter could not be discriminated from our experiment, it seems that we are in the presence of different Cd transporters for the two ecotypes of *T. caerulescens*. In “Prayon,” Cd could be transported by a Zn and Ca pathway, whereas in “Ganges,” Cd could be transported mainly by a different pathway than Zn and Ca. Considering the multiple effects observed with the different cations tested here, however, it is likely that more than one single transport system are carrying Cd into the cell.

**CONCLUSION**

The main objectives of the study were to characterize the uptake at the leaf cell level of Cd and Zn in two contrasting ecotypes of *T. caerulescens* and in *A. halleri*. In particular, the kinetics of Cd and Zn uptake, the effects of Ca2+, Ca2+ channel blocker, and several other divalent cations, and the impact of plant pre-exposure to metals on Cd and Zn uptake in mesophyll protoplasts were investigated.

Despite the similarity between Cd and Zn in their electronic structure, it seems that there are differ-

**Figure 5.** Purified mesophyll protoplasts from leaves of 12-week-old *T. caerulescens* “Ganges.”

**Figure 6.** Relation between the microliters of protoplasts measured with the 3H-water method and the total number of protoplasts per milliliter counted with a hemocytometer. Error bars do not extend outside some data symbols. Error bars = means (n = 4) ± SD.
ences between these metals in terms of their accumulation by the plants. Nevertheless, we found that Zn depressed Cd uptake and reciprocally in the three plants, indicating some kind of interaction between the uptake mechanisms of these two metals. This study demonstrated that the cellular uptake of Cd and Zn in leaf cells is a carrier-mediated mechanism in hyperaccumulating plants and that differences in uptake between *T. caerulescens* “Ganges,” *T. caerulescens* “Prayon,” and *A. halleri* cannot be explained by different transport capacities at the protoplast level in leaves. Therefore, there must exist regulation mechanisms before the plasma membrane of the leaf cell that direct the metals to their final location in plants. The cell wall could play a role in this regulation. The accumulation capacities of protoplasts were nevertheless modified after the plants’ pre-exposure to Cd, indicating that above a determined Cd concentration threshold, different transport mechanisms were induced in leaf cells.

**MATERIALS AND METHODS**

**Plant Materials and Culture**

The plants studied were *Thlaspi caerulescens* ecotype “Prayon” (Belgium) known for Zn hyperaccumulation (Lombi et al., 2000), *T. caerulescens* ecotype “Ganges” (southern France) and *Arabidopsis halleri* (northern France), both known for Zn and Cd hyperaccumulation (Robinson et al., 1998; Bert et al., 2000; Dahnani-Muller et al., 2001). Seeds were germinated in the dark on filters moistened with deionized water. Three-week-old seedlings were transferred to a 1-L pot (four plants per pot) filled with modified one-quarter-strength Hoagland nutrient solution (Sigma, St. Louis) supplemented with 20 μM Fe-HBED (Strem Chemical, Newburyport, MA). Plants were allowed to grow 2 weeks in hydroponics medium with 100% percoll medium) and further with a layer of sorbitol medium cushion (500 mM Sorbitol, 10 mM CaCl₂, and 20 mM MES, pH 6 solubilized in Percol [Sigma]). The supernatant was discarded, and the layer of mesophyll protoplasts was resuspended in the residual liquid. Perocel medium (100%) was added to the protoplast mix to obtain a final mixture of 50% percoll medium (1:1 [v/v] sorbitol medium with 100% percoll medium), which was overlayed with 40% percoll medium (32 [v/v] sorbitol medium with 100% percoll medium) and further with a layer of sorbitol medium. The gradient was centrifuged at 4000 g for 5 min. The protoplasts were collected from the upper interface. All centrifugation steps were performed at 4 °C. Protoplasts were kept shortly in test tubes on ice until the uptake experiments were performed. A typical preparation of protoplasts is shown in Figure 5.

**Concentration of Cd and Zn in Plants**

The total concentration of Cd and Zn in leaves of *A. halleri* and *T. caerulescens* “Ganges” and “Prayon” pretreated with Cd (5 and 10 μM) and *T. caerulescens* “Ganges” pretreated with Zn (50 and 500 μM) and in control plants was measured. Four different 12-week-old plants grown in hydroponics (1 pot) were harvested and dried at 80°C for 1 week. Plants were individually weighted and hot-digested in 65% (w/v) HNO₃ suprapur (Fluka, Buchs, Switzerland) and 70% (w/v) HClO₄ pro analysis (Fluka). The concentrations in plants were then measured by ICP-AES (Perkin Elmer Plasma 2000, Perkin Elmer, Wellesley, MA).

**Preparation and Purification of Mesophyll Protoplasts**

Mesophyll protoplasts were prepared from leaves of eight different 12-week-old plants (two pots). Abaxial sides of leaves were peeled and placed in a cell wall-digesting medium composed of sorbitol medium (500 mM sorbitol, 10 mM MES, and 10 μM CaCl₂ [pH 5.3], 0.75% (w/v) Cellulase Y-C (Kikkoman, Tokyo), and 0.075% (w/v) Pectolyase Y-23 (Kikkoman). The leaves were incubated for 2 to 4.5 h at 30°C until digestion was judged satisfactory but had not reached the epidermal cell layer yet. The resulting suspension was centrifuged at 400g for 7 min on top of a 100% percoll medium cushion (500 mM Sorbitol, 10 mM CaCl₂, and 20 mM MES, pH 6 solubilized in Percol [Sigma]). The supernatant was discarded, and the layer of mesophyll protoplasts was resuspended in the residual liquid. Perocel medium (100%) was added to the protoplast mix to obtain a final mixture of 50% percoll medium (1:1 [v/v] sorbitol medium with 100% percoll medium), which was overlayed with 40% percoll medium (32 [v/v] sorbitol medium with 100% percoll medium) and further with a layer of sorbitol medium. The gradient was centrifuged at 4000 g for 5 min. The protoplasts were collected from the upper interface. All centrifugation steps were performed at 4 °C. Protoplasts were kept shortly in test tubes on ice until the uptake experiments were performed. A typical preparation of protoplasts is shown in Figure 5.

**Determination of Protoplast Viability**

The percentage of viable protoplasts in the stock was determined by staining with FDA (Fluka) as described by Lasat et al. (1998). A stock solution of 7.2 mM FDA dissolved in acetone was prepared. Protoplasts were incubated for 10 min in 36 μM FDA (final concentration) and inspected using a fluorescence microscope. Protoplasts showing bright fluorescence were counted as viable.

**Uptake Experiment with Protoplasts. Concentration and Time Dependence**

As a first step, we studied the concentration-dependent kinetics of Zn and Cd. The protoplasts were diluted 1:4 (v/v) with betaine medium (500 mM, 10 mM CaCl₂, and 20 mM MES [pH 5.5]) before the uptake experiment. In standard conditions, the pH of the incubation medium had a strong effect on the uptake. Several pHs (4.5, 5.0, 5.5, 6.2, 6.6, and 7.6) were tested for the betaine medium because it determines the final pH during incubation. Both Cd and Zn uptake into protoplasts was maximal at pH 5.5 (data not shown). In further experiments, pH was maintained at 5.5 with MES buffer, which is known to have minimal metal-complexing ability.

The time course of 65Zn and 109Cd uptake was initiated by the addition of ZnCl₂ spiked with 18.5 kBq ml⁻¹ 65ZnCl₂ (NEN Life Science Products, Boston) for a final total Zn concentration of 3.4, 6.6, 9.6, 19.2, 28.2, 37.1, and 45.8 μM, or CdCl₂ spiked with 18.5 kBq ml⁻¹ 109CdCl₂ (NEN Life Science Products) for a final total Cd concentration of 1.6, 3.2, 6.3, 9.6, 14.3, 19.3, 28.9, 38.3, 47.9, 96.1, and 186.8 μM. Uptake was measured until t = 120 min for *T. caerulescens* “Ganges” (Fig. 1). Because the accumulation rate reached a plateau after 30 min (Fig. 1), uptake was further measured only at t = 1, 2, 5, 10, and 30 min for all the samples. At various time intervals, a 100-μL aliquot of the spiked protoplast suspension was sampled and placed on top of a discontinuous gradient consisting of 200 μL of silicon oil (AR 200, Fluka) on top of 30 μL of 40% (w/v) percoll medium added in a 400-μL microcentrifuge tube. The microcentrifuge tubes were immediately centrifuged using a bench-top microcentrifuge (model 5417R, Eppendorf, Hamburg, Germany) at high speed for 20 s to pellet the protoplasts through the silicon oil layer. After centrifugation, tubes were frozen at −20°C. Tips containing the frozen protoplast pellets were cut off and placed in counting vials, and 4 mL of scintillation solution (Ultima Gold LSC-cocktail, Packard Bioscience, Meriden, CT) was added. The uptake of 65Zn and 109Cd was quantified via scintillation counting (Packard, Tri-Carb, liquid scintillation analyzer). The values obtained for t = 1 min incubation were considered as a background coming from the thin film of spiked solution sticking to the protoplast’s surface. These values were systematically subtracted from the following measures. All the fittings were calculated using SigmaPlot (SPSS, Inc., Chicago).

The protoplast volume collected after silicon oil centrifugation was quantified with ³H-water (Hartmann Analytic, Braunschweig, Germany). One hundred microliters of the concentrated protoplast solution were incubated for 10 min with 400 μL of betaine medium and 18.5 kBq ³H-water. The protoplasts were pelleted and quantified via scintillation counting as described before. The method allowed a quick estimation of number of protoplasts in the preparation. The results obtained by this method and the total number of protoplast per milliliter in the concentrated preparation counted with a hemocytometer were compared and are presented in Figure 6. All the calculations were reported for 0.25 μL of protoplasts, which corresponds to 4.8×10⁹ protoplasts ml⁻¹. In addition, because with this method the rate of uptake into cells is proportional to the surface area of the protoplasts, we
measured (with the software MetaView Imaging System, Universal Imaging Corporation, Westchester, PA) the diameter of the protoplasts to estimate their surface (4πr²).

Effect of Plant Cd and Zn Pre-Exposure on Protoplast Uptake

The effect of Cd²⁺ (5 and 10 μM) or Zn²⁺ (50 and 500 μM) during growth of plants on heavy metal uptake in protoplasts was investigated in *Thlaspi caerulescens* “Ganges.” The effect of plant pre-exposure to Cd²⁺ (5 and 10 μM) on Cd uptake in protoplasts was also studied in *A. halleri* to assess if Cd had a similar effect on the other Cd-hyperaccumulating plant. Experiments were carried out as described above for time-dependent uptake in mesophyll protoplasts. This experiment was not carried out with “Prayon” ecotype.

Effect of Different Protoplast Treatments on Zn and Cd Uptake

Several inhibitors/competitors (100 μM verapamil, 50 and 200 μM Ca²⁺, 50 and 200 μM Cd²⁺, 50 and 200 μM Mg²⁺, and 50 and 200 μM Zn²⁺, or on ice) were added in the standard assay medium. Cd or Zn uptake in mesophyll protoplasts extracted from non-pre-exposed plants was measured as explained above. Verapamil was only tested on *T. caerulescens* “Ganges.” Calcium and Mg²⁺ competition were not tested on *A. halleri.*

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LITERATURE CITED


Hyperaccumulation of Cadmium and Zinc