

Investigation of Heavy Metal Hyperaccumulation at the Cellular Level: Development and Characterization of *Thlaspi caerulescens* Suspension Cell Lines^{1[OA]}

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The ability of *Thlaspi caerulescens*, a zinc (Zn)/cadmium (Cd) hyperaccumulator, to accumulate extremely high foliar concentrations of toxic heavy metals requires coordination of uptake, transport, and sequestration to avoid damage to the photosynthetic machinery. The study of these metal hyperaccumulation processes at the cellular level in *T. caerulescens* has been hampered by the lack of a cellular system that mimics the whole plant, is easily transformable, and competent for longer term studies. Therefore, to better understand the contribution of the cellular physiology and molecular biology to Zn/Cd hyperaccumulation in the intact plant, *T. caerulescens* suspension cell lines were developed. Differences in cellular metal tolerance and accumulation between the cell lines of *T. caerulescens* and the related nonhyperaccumulator, *Arabidopsis* (*Arabidopsis thaliana*), were examined. A number of Zn/Cd transport-related differences between *T. caerulescens* and *Arabidopsis* cell lines were identified that also are seen in the whole plant. *T. caerulescens* suspension cell lines exhibited: (1) higher growth requirements for Zn; (2) much greater Zn and Cd tolerance; (3) enhanced expression of specific metal transport-related genes; and (4) significant differences in metal fluxes compared with *Arabidopsis*. One interesting feature exhibited by the *T. caerulescens* cell lines was that they accumulated less Zn and Cd than the *Arabidopsis* cell lines, most likely due to a greater metal efflux. This finding suggests that the *T. caerulescens* suspension cells represent cells of the Zn/Cd transport pathway between the root epidermis and leaf. We also show it is possible to stably transform *T. caerulescens* suspension cells, which will allow us to alter the expression of candidate hyperaccumulation genes and thus dissect the molecular and physiological processes underlying metal hyperaccumulation in *T. caerulescens*.

The extreme heavy metal accumulation seen in metal hyperaccumulating plant species, *Thlaspi caerulescens*, is the result of physiological differences at the cellular, organ, and whole plant levels (Lasat et al., 1996, 1998; Küpper et al., 1999). These differences lead to the accumulation of zinc (Zn) and cadmium (Cd) in above-ground tissues to concentrations as high as 40,000 $\mu\text{g g}^{-1}$ Zn and 10,000 $\mu\text{g g}^{-1}$ Cd dry weight (DW). Most nonhyperaccumulator plant species only accumulate 100 to 300 $\mu\text{g g}^{-1}$ Zn and 0.1 to 10 $\mu\text{g g}^{-1}$ Cd foliar concentrations (Marschner, 1995; Kochian et al., 2002, and refs. therein). Differences in Zn and

Cd accumulation relative to nonhyperaccumulators are due, in part, to altered metal transport at a number of different sites within the plant, including the root surface, xylem loading within the root, and reabsorption of xylem-born metals by leaf cells (Lasat et al., 1996, 1998). Due to this altered transport, *T. caerulescens* maintains both higher shoot and lower root Zn concentrations relative to the related nonhyperaccumulator, *Thlaspi arvense*, when both are grown at both low and high Zn concentrations (Lasat et al., 1996). These differences in metal accumulation in roots versus shoots between *T. caerulescens* and related nonaccumulators indicates that metal accumulation and tolerance is a complex trait, requiring the coordinated function of different cells types, tissues, and organs.

Most studies of Zn/Cd hyperaccumulation in *T. caerulescens* have focused on investigations at the whole plant or organ levels, and, for example, have identified specialized sites of metal accumulation in the leaf. While high Zn and Cd concentrations are found in the *T. caerulescens* leaf, a significant fraction of Zn and Cd transported to the shoots appears to be sequestered in a soluble form within the vacuoles of nonphotosynthetic epidermal cells (Küpper et al., 1999; Frey et al., 2000; Ma et al., 2005). *T. caerulescens* grown hydroponically with a ¹⁰⁹Cd radiotracer established Cd localization at the leaf margins with additional Cd accumulation as spots across the adaxial and abaxial sides of the leaf blade. The fixed distribution of Cd over the course of the exposure period suggests that Cd preferentially accu-

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mulates in specific leaf regions and does not remobilize (Cosio et al., 2005). These organ level studies further promote the idea of localized sites of accumulation in *T. caerulescens* leaves rather than a generalized and non-specific accumulation of metals. However, it should be noted that although Zn/Cd accumulation to significantly higher concentrations occurs in *T. caerulescens* leaf epidermal cells, more of the total metal in the entire leaf is accumulated in leaf mesophyll cells by virtue of the fact that much more of the leaf volume is occupied by mesophyll cells.

Further advances into the molecular physiology of metal hyperaccumulation in *T. caerulescens* have suffered from the lack of an appropriate technique to study these processes at the cellular level. To date, the primary approach for studying cellular aspects of metal hyperaccumulation in this plant has been with protoplasts isolated from *T. caerulescens* leaves. These studies in *T. caerulescens* and a second metal-hyperaccumulating species, *Arabidopsis halleri*, have used protoplasts to characterize membrane transporters, to quantify short term fluxes, and for longer term viability assays (Lasat et al., 1998; Piñeros and Kochian, 2003; Cosio et al., 2004; Marques et al., 2004; Ma et al., 2005). Protoplast studies by Cosio et al. (2004) and Lasat et al. (1998) came to similar conclusions that transport properties of protoplasts did not match the accumulation phenotype seen in whole plants or leaves. There are major problems in using protoplasts for cellular studies of hyperaccumulation, including the significant physiological disruption arising from digesting cell walls to isolate protoplasts, the very low protoplast yields from root tissues, and the fragility of protoplasts, which severely limits the types of studies that can be conducted.

Along with the physiological properties of metal hyperaccumulation that have been identified primarily from whole plant studies, recent molecular progress in hyperaccumulation studies have also been made that could set the stage for a molecular dissection of metal hyperaccumulation at the cellular level if the proper experimental system was developed. Most notably, a number of metal transport-related genes have been found to be expressed to much higher levels in both *T. caerulescens* and *A. halleri* compared to nonhyperaccumulating plant species. This was first described by Pence et al. (2000) with the identification and characterization of the Zn/Cd transporter, *ZNT1*. High expression levels of *ZNT1* were seen in roots of *T. caerulescens* relative to *T. arvense* when plants were grown under low and sufficient Zn concentrations, and down-regulation of *ZNT1* expression in *T. caerulescens* was seen only following Zn accumulation at much higher Zn levels (Pence et al., 2000). *TcMTP1*, a vacuolar metal efflux transporter, has been shown to confer moderately higher levels of Zn accumulation when overexpressed in *Arabidopsis thaliana* and was also shown to be hyperexpressed in *T. caerulescens* relative to *T. arvense* (van der Zaai et al., 1999; Assunção et al., 2001). Additionally, *HMA4*, a P-type ATPase, was also shown to be expressed to

a much higher level in *T. caerulescens* roots relative to *Arabidopsis* (Bernard et al., 2004; Papoyan and Kochian, 2004). This transporter is localized to the plasma membrane of root xylem parenchyma cells in *Arabidopsis* and is thought to transport Zn/Cd into the xylem for transport from the roots to the shoots, and thus it has been suggested it may play a key role in the very efficient root-to-shoot translocation of Zn/Cd that is a hallmark of hyperaccumulation in *T. caerulescens* (Papoyan and Kochian, 2004).

More recent molecular studies of *T. caerulescens* have used genome-wide approaches, including EST library creation from root and shoot tissues and *Arabidopsis* GeneChips to examine genome-wide expression of shoot genes in *T. caerulescens* compared to *T. arvense* or *Arabidopsis* (Rigola et al., 2006; Hammond et al., 2006). van de Mortel et al. (2006), using an *Arabidopsis* microarray, found a number of metal-related genes and also a number of genes of unknown function up-regulated in *T. caerulescens* compared to *Arabidopsis*. As these molecular studies identify greater numbers of candidate hyperaccumulation genes, the need increases for a reasonable model system to study these processes at the cellular level. There is also a need for an effective transformation system of *T. caerulescens* cells to begin to identify and dissect the function of candidate metal transporter genes of interest.

While *T. caerulescens* has been extensively studied as a model metal hyperaccumulator species, it contains a number of reproductive and physiological traits that make it difficult to stably transform. Some of the less desirable traits in this species include a lengthy life cycle, extended requisite vernalization for flower induction, low fertility, and low transformation efficiency (Assunção et al., 2003; Peer et al., 2003). These traits limit the ability to functionally characterize genes of interest in the heavy metal hyperaccumulator using whole-plant-based methods.

To address the shortcomings of this field, specifically the study of the cellular aspects of metal hyperaccumulation, we describe here the creation and characterization of a *T. caerulescens* (Prayon) suspension cell line created for two purposes. The first is to examine the cellular behavior of *T. caerulescens* at both the physiological and molecular levels with regards to metal tolerance, transport, and accumulation relative to a cell line for the related nonaccumulator model species, *Arabidopsis*. The findings presented here show that *T. caerulescens* suspension cells are an appropriate model system for studying metal hyperaccumulation, as they share a number of key metal tolerance and transport features with the whole plant, including increased Zn requirement for growth, enhanced metal tolerance, increased expression of specific metal transport genes, and a different pattern of metal fluxes and accumulation compared with cells of *Arabidopsis*. We also show here that it is possible to stably transform *T. caerulescens* suspension cells, which will allow us in the future to alter the expression of candidate hyperaccumulation genes and thus begin to dissect the molecular and

physiological processes underlying metal hyperaccumulation in *T. caerulescens*.

RESULTS

Creation of Suspension Cell Lines

To date, there have been no published attempts to produce calli or suspension cells from *Thlaspi* species. Murashige and Skoog (MS) and Linsmaier and Skoog media have previously been used to successfully produce calli from other *Brassica* species, including *Arabidopsis* (Toriyama et al., 1987; Jain et al., 1988; Encina et al., 2001). In this study, *T. caerulescens* calli and suspension cells were produced from seeds germinated on MS media under both low and high Zn concentrations. Calli were subcultured and those cells showing vigorous growth were transferred to liquid MS media for experimentation. The subset of calli showing vigorous growth were also kept on solid media for long-term maintenance of suspension cell lines. *T. caerulescens* calli grown with Zn supplementation showed greater fresh weight production relative to cell lines grown under low Zn conditions.

Characterization of Suspension Cell Lines

Suspension Cell Growth Rates

Following creation of the *T. caerulescens* suspension cell lines, a time course experiment was conducted to examine growth rates of *T. caerulescens* cells in standard MS media (TcMS) and Zn supplemented MS media (TcZN) relative to *Arabidopsis* suspension cells grown on standard MS media (AtMS). The time course experiments were started with an initial cell density of 2.5 mg fresh weight per milliliter of media and spanned 24 d with whole flask replicates collected as sample time points every 3 d. As seen in Figure 1A, *Arabidopsis* suspension cell lines produced significantly higher fresh weight biomass compared to either *T. caerulescens* line. This growth difference between the *T. caerulescens* and *Arabidopsis* cell lines was smaller but still present when measured on a DW basis (Fig. 1B). A comparison in growth rates between the *T. caerulescens* cell lines shows that an increase in Zn media concentration from 30 to 300 μM Zn increased the cellular biomass produced (Fig. 1, A and B). For the initial study of metal accumulation as an indicator of both cellular growth and cellular accumulation of metals, suspension cell lines were started at identical fresh weights and total metal accumulation was calculated from the product of cellular metal concentration times cell biomass at the time points specified. When total Zn accumulation over the course of the 24-d experiment was determined, the TcMS and AtMS cell lines grown on 30 μM Zn exhibited similar, low rates of total Zn accumulation, while the TcZN cells grown on a much higher Zn concentration (300 μM Zn) accumulated greater amounts of Zn (Fig. 1C). The differences in cellular growth and Zn accu-

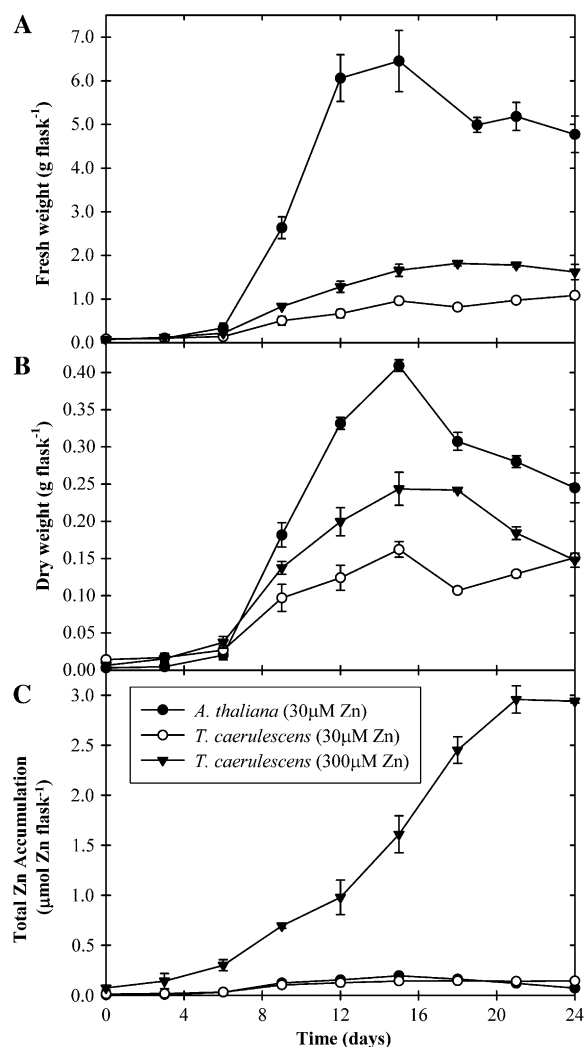


Figure 1. Time course for growth and metal (Zn) accumulation in suspension cell lines of *T. caerulescens* and *Arabidopsis*. For the growth and accumulation data depicted here, suspension cell lines were grown in liquid MS media as described in "Materials and Methods." AtMS had a total Zn concentration of 30 μM , while the Zn concentration in the *T. caerulescens* media was 30 or 300 μM Zn for the TcMS and TcZN media lines, respectively. A, Suspension cell growth over time as measured by fresh weight increase for the *Arabidopsis* cell line grown on standard MS media and for *T. caerulescens* cell lines grown on standard MS media or on MS media supplemented with ZnSO_4 to a final Zn concentration of 300 μM . B, Suspension cell growth over time as measured by DW increase. All the conditions are as described in the legend for Figure 1A. C, Time course for suspension cell Zn accumulation during the course of the 24-d growth experiment. Cellular growth and Zn accumulation were determined on a per flask basis for each set of suspension cells on the particular day. All data points are the mean \pm SE ($n = 3-4$).

mulation among the three lines suggest that like the intact plant, *T. caerulescens* cells require a higher level of Zn for optimal growth compared with nonaccumulator species (Levent et al., 2003). This is shown by the much lower rates of growth for *T. caerulescens* versus *Arabidopsis* cell lines on 30 μM Zn and the increase in growth

rate for *T. caerulescens* cells when the Zn concentration was increased from 30 to 300 μM .

Long-Term Growth in Response to a Range of Zn Concentrations

When TcZN and AtMS suspension cells were grown for 10 d in MS media with Zn concentrations varying from 30 to 1,500 μM Zn, the TcZN cells exhibited no significant changes in growth rate, while the growth of AtMS suspension cells was inhibited at all Zn concentrations greater than 30 μM (Fig. 2A). AtMS cells grown in MS media with 1,500 μM Zn showed a 2-fold increase in growth relative to the starting cellular concentration, but this was still a 96% reduction in growth relative to Arabidopsis suspension cells grown without additional Zn. There was a lower Zn concentration in TcZN cells relative to AtMS cells when cells were grown at Zn concentrations greater than 300 μM (Fig. 2B). However, when total cellular Zn accumulation was considered on a per flask basis, the TcZN cells showed greater Zn accumulation at the two highest Zn concentrations (1,000 and 1,500 μM Zn). As seen in Figure 2C, when grown in media containing 1,500 μM Zn, the TcZN cells accumulated 7.8 μmol Zn per flask, while the AtMS cells only accumulated 3.2 μmol Zn per flask. This is primarily due to differences in biomass at the highest Zn levels, because as shown later, AtMS cells actually accumulate more Zn on a per cell basis, but because their growth is significantly inhibited on 1,500 μM Zn, they produce much less biomass than the TcZN cells.

Long-Term Growth in Response to a Range of Cd Concentrations

When cells were grown for 10 d in MS media containing Cd concentrations between 0 and 50 μM , both TcZN and TcMS suspension cells exhibited a significant increase in growth with increasing Cd levels, while the AtMS suspension cells showed a significant inhibition of growth (>50% inhibition at 50 μM Cd; Fig. 3A). At all Cd concentrations, the Arabidopsis suspension cells maintained a higher cellular Cd concentration than either *T. caerulescens* line (Fig. 3B). When total Cd accumulation was determined (cell concentration times total biomass), even with a 50% decrease in biomass, the AtMS cells still accumulated greater total amounts of Cd compared to either *T. caerulescens* line (Fig. 3C).

Short-Term Cd Influx and Efflux

Based on the final fresh weights presented in Figures 2 and 3, the *T. caerulescens* suspension cells appear to better tolerate higher Zn and Cd concentrations but accumulate lower Zn and Cd concentrations relative to the Arabidopsis-derived suspension cells. The lower metal accumulation seen in *T. caerulescens*-derived suspension cells could be due to decreased influx, increased efflux, or a combination of both. To test these

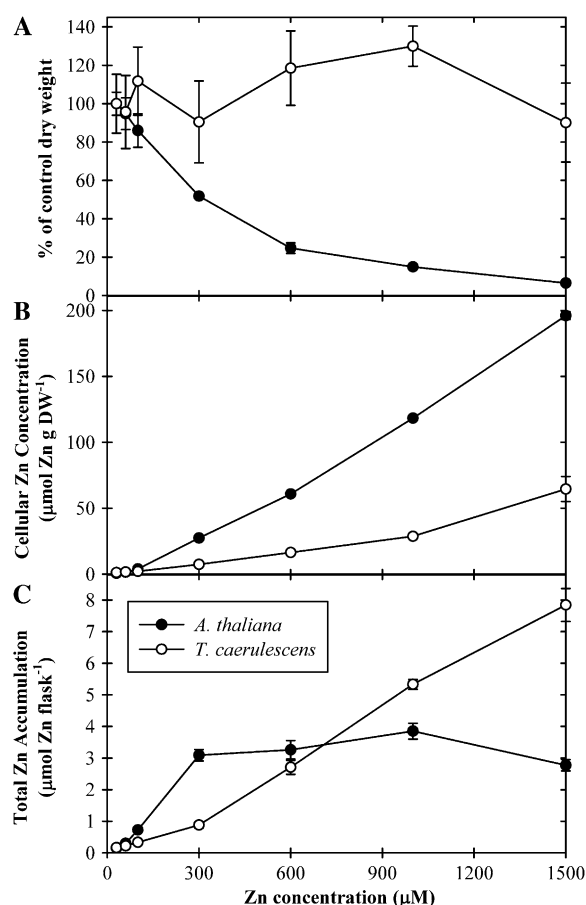


Figure 2. The influence of increasing Zn concentration in the liquid growth media (ranging 30–1,500 μM Zn) on cell growth and Zn accumulation for TcZN and AtMS suspension cells. A, Suspension cell growth presented as the percent of biomass (DW) accumulation at the specific Zn concentration relative to growth on the standard MS media where the Zn concentration was 30 μM . B, Cell Zn accumulation as determined by changes in suspension cell Zn concentration for TcZN and AtMS suspension cells in response to growth on 30 to 1,500 μM Zn. C, Total Zn accumulation for TcZN and AtMS suspension cells in response to growth on 30 to 1,500 μM Zn based on the amount of Zn accumulated on a per flask basis. All data points are the mean \pm SE ($n = 2-3$).

hypotheses, short-term Cd accumulation treatments that should approximate Cd influx and Cd efflux were examined in the TcZN and AtMS suspension cell lines. To examine cellular Cd fluxes, a 5-min time course study was completed. From these data (Fig. 4A), similar initial rates of Cd influx are seen in the *T. caerulescens* and Arabidopsis suspension cells. For the Cd efflux experiments, both suspension cell lines were allowed to accumulate Cd for 20 h prior to the efflux period. At the start of the efflux period, the AtMS cells had accumulated 177 ± 4 nmol Cd/g DW, while the TcZN cells had accumulated 131 ± 4 nmol Cd/g DW. After a 5-min efflux period into Cd-free media, Arabidopsis cells had maintained a Cd concentration of 168 ± 2 nmol Cd/g DW (a loss of 5%), while the *Thlaspi* cells exhibited a significantly greater Cd efflux, with a loss of 30% of

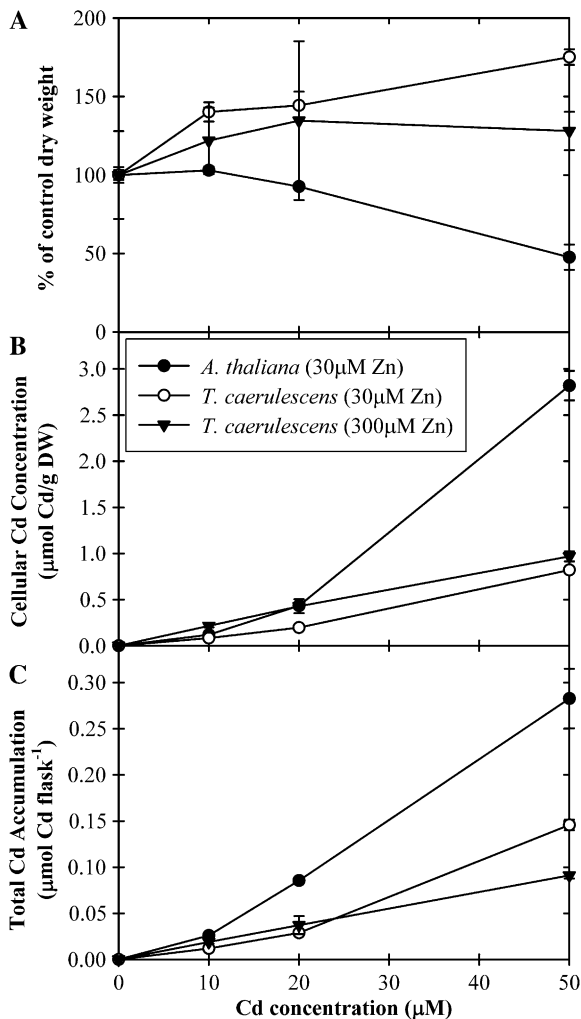


Figure 3. The influence of increasing Cd concentration in the liquid growth media (ranging 0–50 μM Cd) on cell growth and Cd accumulation for TcZN and AtMS suspension cells. A, The influence of increasing Cd concentrations on suspension cell growth presented as the percent of biomass (DW) accumulation at the specific Cd concentration relative to growth on the standard MS media without Cd. B, Cell Cd accumulation as determined by changes in suspension cell Cd concentration for TcZN and AtMS suspension cells in response to growth on 0 to 50 μM Cd. C, Total Cd accumulation for TcZN and AtMS suspension cells in response to growth on 0 to 50 μM Cd based on the amount of Cd accumulated on a per flask basis. All data points are the mean \pm SE ($n = 2$ –3).

their total accumulated Cd after the 5-min efflux period, a final cellular Cd concentration of 92.5 ± 5 nmol Cd/g DW (Fig. 5).

Transporter Gene Expression in *T. caerulescens* and *Arabidopsis* Cell Lines

Changes in expression of Zn/Cd transporter genes in *T. caerulescens* suspension cells over a range of metal concentrations in the growth media were studied. We were interested in the expression of genes under low, sufficient, and excess Zn media concentrations and if

the expression changed with the addition of Cd to the media. Standard MS media imposed Zn deficiency and limited the growth of *T. caerulescens* suspension cells, as evidenced in Figure 1, A and B, but not for *Arabidopsis* suspension cells. To this end, the GeoChem PC speciation program (Parker et al., 1995) was used to analyze the effect of EDTA as a Zn^{2+} chelator to limit Zn^{2+} activity and create an MS media for *Arabidopsis* that would impose Zn deficiency conditions. It was determined that an EDTA concentration of 120 μM would lower the Zn^{2+} activity to levels approximately 10% of the activity of standard MS media, an activity level that should impose Zn deficiency conditions. When 120 μM EDTA was added to AtMS media, *Arabidopsis* suspension cell growth was reduced by 70% and total Zn accumulation by 50% compared to cells grown on standard MS (data not shown). Based on these results, EDTA was added to a final concentration of 120 μM to create a low (deficient) Zn medium for *Arabidopsis* suspension cells.

To examine the molecular behavior of these *T. caerulescens* suspension cells, the expression of three previously studied Zn transporters implicated in Zn hyperaccumulation and tolerance for *T. caerulescens* was examined along with their closest *Arabidopsis* orthologs: the P-type ATPase *HMA4* (Tc:AY486001; At:AJ297264), the cation diffusion facilitator family member *MTP1* (Tc:AY483146; At:NM_180128), and the ZIP family member, *ZNT1* (Tc:AF133267; At:NM_100972). The *Arabidopsis* actin gene *ACT2* (NM_112764) and its closest *T. caerulescens* ortholog were used as controls. To limit differential hybridization, the gene-specific PCR primers used to study transporter gene expression and the actin control exactly matched

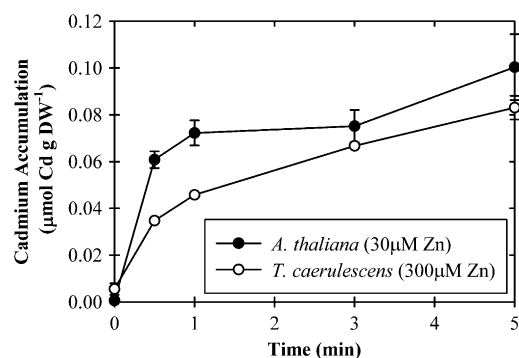


Figure 4. Time course for Cd influx from *T. caerulescens* and *Arabidopsis* suspension cells. Seven-day-old cells growing on TcZN or AtMS media (for *T. caerulescens* or *Arabidopsis* cells, respectively) were sampled just before addition of Cd to the uptake solution, and CdCl_2 was added to a final concentration of 10 μM . The 10-mL cell aliquots were then taken each minute for the 5-min uptake period. At the specified time points, sampled cells were vacuum filtered, desorbed to remove cell wall Cd, dried, weighed, and Cd content determined by ICP-AES. Three separate biological flasks were quantified for each line. Data points represent mean of time points taken from three different flasks \pm SE ($n = 3$).

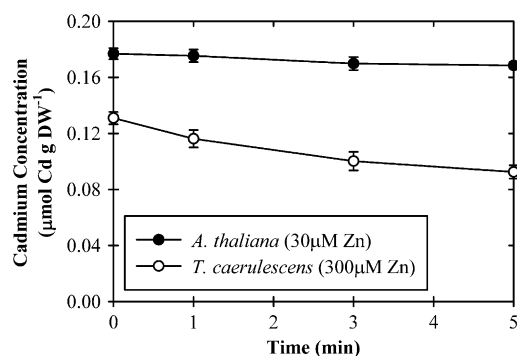


Figure 5. Time course of Cd efflux from *T. caerulescens* and Arabidopsis suspension cells. After a 20-h incubation in TcZN or AtMS media (for the *T. caerulescens* or Arabidopsis cells, respectively) containing 10 µM CdCl₂, the 7-d-old cells were washed then resuspended in Cd-free MS media, and 10-mL aliquots were taken at each minute over the 5-min efflux period. Aliquoted cells were vacuum filtered, briefly washed to remove solution adhering to the cells, dried, weighed, and Cd content determined by ICP-AES. Three separate biological flasks were quantified for each line. Data points represent mean of time points taken from three different flasks ± SE (n = 3).

both the *T. caerulescens* and the Arabidopsis ecotype Landsberg *erecta* (*Ler*) coding sequences.

Based on the semiquantitative reverse transcription (RT)-PCR results shown in Figure 6, both constitutive and Zn status-dependent differences were seen in the expression of these metal transporters in *T. caerulescens* and Arabidopsis suspension cells. *HMA4* showed high transcription levels independent of cellular Zn or Cd status in the TcZN cell lines compared with the AtMS cells. Transcription of *MTP1* also showed higher constitutive expression in TcZN suspension cells relative to AtMS cells; both sets of *MTP1* expression patterns were not affected by Zn or Cd status. This mirrors the *MTP1* expression pattern seen in Arabidopsis in response to a range of Zn concentrations, as shown previously by both van der Zaai et al. (1999) and Kobae et al. (2004). Both *HMA4* and *MTP1* have previously been shown to be highly expressed in the intact *T. caerulescens* plant compared with related nonhyperaccumulator species (Assunção et al., 2001; Papoyan and Kochian, 2004).

The expression of *ZNT1* was found to be highly expressed in *T. caerulescens* suspension cells, and expression in both species was repressed as cellular Zn status increased (Fig. 6). As expression of the actin gene, used as an internal standard, was significantly greater in Arabidopsis cells, the normalization of *ZNT1* expression to actin would show a more pronounced up-regulation of *ZNT1* in TcZN relative to AtMS suspension cells (Fig. 6). High expression of *ZNT1* under low-to-sufficient Zn conditions in *T. caerulescens* plants compared with *T. arvense* has been previously documented (Pence et al., 2000). Overall, the gene expression patterns seen in these suspension cells follow the general expression patterns previously demonstrated in *T. caerulescens* and Arabidopsis plants. The high expression of transporter genes in suspension cells of

T. caerulescens relative to Arabidopsis confirms that the pattern of high expression commonly observed in whole plants is also seen at the cellular level.

Transformation of Suspension Cell Lines

T. caerulescens suspension cells were transformed with *A. tumefaciens* harboring the pCambia vector 1303-containing reporter gene consisting of cytosolic GUS fused with GFP under the control of a 35S promoter. As shown in Figure 7, 3 weeks after transformation, both GFP and GUS expression was observed in the cytoplasm of transformed cells, demonstrating successful transformation and stable gene expression of the reporter genes in these suspension cell lines.

DISCUSSION

In this study, suspension cell lines were developed for the metal hyperaccumulator, *T. caerulescens*, to investigate whether the extreme metal tolerance and accumulation phenotype seen in this plant is a characteristic seen at the single cell level, or whether hyperaccumulation requires the additional functional coordination of different cells, tissues, and organs in the plant. From the physiological and molecular studies conducted here comparing suspension cell lines derived from *T. caerulescens* and the related nonaccumulator, Arabidopsis, it is clear that a number of the whole plant traits are expressed at the cellular level, including increased tolerance to Zn and Cd, a higher Zn requirement for growth, increased expression of metal tolerance genes, and altered patterns of Zn/Cd fluxes and accumulation.

It has previously been demonstrated that intact plants of *T. caerulescens* are Zn inefficient in that they require more Zn for normal biomass production compared with the related nonaccumulator plants (Levent et al., 2003). In this study, comparisons between *T.*

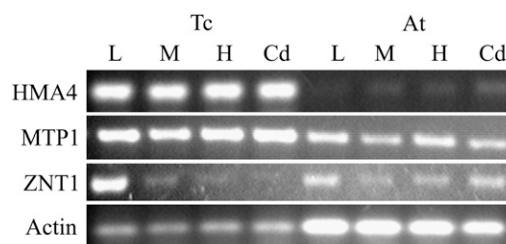


Figure 6. Semiquantitative RT-PCR analysis of metal transporter (*HMA4*, *MTP1*, and *ZNT1*) gene expression in TcZN and AtMS suspension cells grown in MS media that varied in Zn or Cd. For the Cd treatments, 10 µM CdCl₂ was included in the growth solution. See “Materials and Methods” for details concerning the media composition. For each of two biological replicates collected, RNA was isolated from 80 mL of suspension cells grown in a 250-mL flask for 7 d. RNA from each biological replicate was used to run three replicate PCR reactions. The images shown are representative of the results obtained from each biological replicate. Actin expression was used as a control.

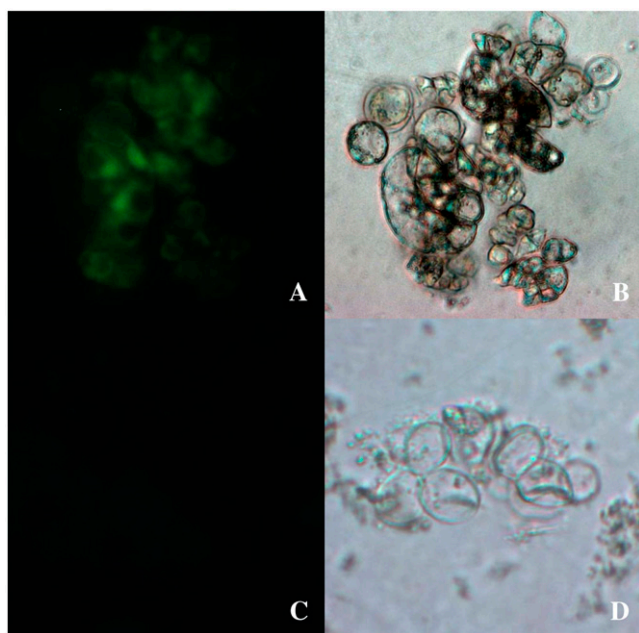


Figure 7. Bright field and fluorescence images of *T. caerulescens* suspension cells transformed via *A. tumefaciens* (C58) infiltration with the binary vector pCAMBIA 1303, which contains the reporter gene GUS:GFP under the control of the 35S promoter (A and B) and untransformed control cells (C and D). GFP expression is seen in A, while GUS expression (blue) is seen in the same cells in B. Transformed cultures were grown for 3 weeks on transformation-selective media prior to assaying for the reporter gene.

caerulescens and *Arabidopsis* suspension cells demonstrate this high Zn requirement is also seen at the cellular level. As seen in the time course experiments, *Arabidopsis* cells grew better on standard MS media (a total Zn concentration of $30\ \mu\text{M}$) than the *T. caerulescens* cell lines subcultured on low Zn ($30\ \mu\text{M}$) or high Zn ($300\ \mu\text{M}$) media (Fig. 1). Zn content data from Figure 1 showed that both the *Arabidopsis* and *Thlaspi* cell lines grown on unsupplemented MS media ($30\ \mu\text{M}$ Zn) had similar, low Zn content that remained unchanged after approximately 12 d of growth (Fig. 1C). However, when the *Thlaspi* cell lines were grown on high Zn media, Zn accumulation by the TcZN cells increased until day 21 to levels approximately 50-fold greater than what was seen in either the TcMS or AtMS lines. This increase in Zn accumulation by the TcZN line suggests that extra Zn present in the media continues to be taken up by the cells even after the cells enter the stationary growth phase. The differences in biomass and Zn concentrations and content between *T. caerulescens* and *Arabidopsis* suspension cells suggest underlying differences in the homeostatic regulation of Zn at the cellular level.

Longer term studies examining cell growth and Zn/Cd accumulation demonstrated increased metal tolerance in *T. caerulescens* suspension cell lines relative to the *Arabidopsis* cell lines. These studies spanned 10 d and included metal concentrations in the MS media

ranging from 30 to $1,500\ \mu\text{M}$ Zn or Cd concentrations ranging from 0 to $50\ \mu\text{M}$ (Figs. 2 and 3). Even at the highest Zn concentration of $1,500\ \mu\text{M}$, the *T. caerulescens* suspension cells showed no significant decrease in growth, while the *Arabidopsis* cell line was strongly inhibited, with a greater than 90% inhibition of cell growth. In contrast, when grown in Cd-enriched media, the *T. caerulescens* cell lines exhibited a stimulation of growth that was as great as 50% to 75% at the highest Cd concentration ($50\ \mu\text{M}$) relative to the Cd-free media. Growth of the *Arabidopsis* suspension cell line, in response to $50\ \mu\text{M}$ Cd, was inhibited by more than 50% (Fig. 3). These differences between the cell lines suggest that these *T. caerulescens* suspension cells contain the mechanisms for increased metal tolerance seen in the tissues of *T. caerulescens* plants where the metal is not hyperaccumulated (e.g. the roots) and may be a useful system for the dissection of that aspect of metal hyperaccumulation tolerance trait.

Changes in metal transport at different sites in *T. caerulescens* plants compared with the related non-accumulator *T. arvensis* have been well documented. Lasat et al. (1996, 1998) showed that for Zn transport these transport alterations in *T. caerulescens* include: (1) greater Zn uptake into the root; (2) less Zn accumulation in the vacuole of root cortical cells, which presumably provides a large mobile pool of Zn for radial movement to the root xylem; (3) greater xylem loading of Zn and associated larger xylem transport to the shoot; and (4) larger Zn uptake into leaf cells, which presumably reflects uptake into leaf epidermal cells, where a significant fraction of the Zn is stored. Differences in Zn and Cd transport were also observed in *T. caerulescens* suspension cells compared with the *Arabidopsis* cell line. As depicted in Figures 2 and 3, *T. caerulescens* cells demonstrated significantly lower Zn and Cd accumulation. The increased metal accumulation in *Arabidopsis* cell lines was not an artifact of cell damage due to greater metal toxicity in *Arabidopsis*, because even at lower Zn and Cd levels where minimal toxicity was observed, the *Arabidopsis* cell lines still accumulated more Zn and Cd. For example, at the lowest Zn levels where little or no toxicity was observed in *Arabidopsis* cell lines, they still maintained as much as a 2-fold greater Zn accumulation (data not shown). With regards to Cd accumulation, when cells were grown in media containing $20\ \mu\text{M}$ Cd, which did not inhibit *Arabidopsis* cell growth, the *Arabidopsis* cells still accumulated 2- to 3-fold more Cd than did *T. caerulescens* cells (Fig. 3). As long-term Zn and Cd accumulation studies reflect a net flux due to metal influx minus metal efflux, these differences in net Zn and Cd accumulation were further studied via short-term Cd accumulation and efflux studies over the first 5 min of uptake and efflux. This would allow a comparison of unidirectional Cd influx and efflux in the two cell lines. The difference in Zn media concentrations precluded examination of unidirectional Zn flux in these cells. The short-term time course for Cd accumulation (Fig. 4) indicated that the time course

is biphasic, with an initial rapid uptake over the first minute, which presumably is dominated by Cd entry and binding in the cell wall (see, for example, Hart et al. [1998]). Subsequently, over the next 4 min, a slower linear phase of Cd uptake was seen that is presumed to be dominated by Cd influx across the plasma membrane (Hart et al., 1998). Based on this analysis, it appears that there might be a slight increase in Cd influx for Arabidopsis relative to *T. caerulescens* suspension cells. However, there is a much bigger difference in Cd efflux between Arabidopsis and *Thlaspi* suspension cells (Fig. 5), as *T. caerulescens* suspension cells had released nearly 30% of the Cd accumulated prior to the efflux period, while the Arabidopsis cell line lost only approximately 5% of the accumulated Cd.

When gene expression is compared between the two cell lines, the high expression of *HMA4* in the *T. caerulescens* cells is striking and may offer an explanation for the decreased metal accumulation in the *T. caerulescens* suspension cells. It has been shown in several studies that *HMA4* in Arabidopsis and *T. caerulescens* is a plasma membrane transporter that mediates the efflux of Zn, Cd, and other heavy metals (Hussain et al., 2004; Papoyan and Kochian, 2004; Verret et al., 2004). In whole plants, *HMA4* is expressed in the root vasculature, and a role in xylem loading of metals has been proffered (Hussain et al., 2004; Verret et al., 2004). This suggests that the suspension cells derived from *T. caerulescens*, while expected to be undifferentiated, might reflect the transport properties of xylem parenchyma cells. It is also possible that a general characteristic of *T. caerulescens* cells not located at critical sites for metal influx (such as the root and the leaf epidermis) are to exclude metals from the cells to more efficiently move the metal to the shoot, which is a hallmark of metal hyperaccumulators. The lower rates of Zn and Cd accumulation combined with the greater short-term Cd efflux rates and higher constitutive levels of *HMA4* expression (Fig. 6) in these *T. caerulescens* cells does suggest a general tendency toward excluding Zn and Cd, along with the ability to tolerate higher concentrations. Considering that high levels of Zn/Cd accumulation only appear to occur in specific *T.*

caerulescens leaf epidermal and mesophyll cells, the ability to take up the heavy metals from the soil and transport them to storage cells requires that a great number of intermediary cells in the transport pathway from the root to the leaf epidermis do not retain the Zn or Cd, but rather keep it in a mobile pool to facilitate the very efficient root-to-shoot metal transport. Nonaccumulator species such as Arabidopsis typically sequester heavy metals in the root to prevent the excess heavy metal from reaching the shoot and leaves. One of the most sensitive processes negatively affected by heavy metals is photosynthesis, and it appears that nonaccumulators limit metal availability to photosynthetic machinery by sequestration in roots or in the stem of the shoot. The Zn and Cd transport properties of the Arabidopsis suspension cells appears to reflect this feature.

Recent research efforts have focused on comparing genome-wide changes between metal hyperaccumulator species relative to related nonaccumulator species. Using an Affymetrix Arabidopsis microarray, transcript profiling of roots and shoots of the hyperaccumulators *A. halleri* and *T. caerulescens* to Arabidopsis and *T. arvense* found higher levels of expression for a number of metal tolerance and accumulation genes in the hyperaccumulator species relative to the nonhyperaccumulator species (Becher et al., 2004; Weber et al., 2004; Hammond et al., 2006). Metal transporters, including members of the ZIP, NRAMP, cation diffusion facilitator, and P-type ATPase families, were found to be up-regulated in these studies of hyperaccumulators. This altered regulation of gene expression seen at the whole plant level is also present at the cellular level, as evidenced by the up-regulation of transporter genes in the *T. caerulescens* suspension cell lines presented here. Understanding the factors involved in the transcriptional regulation of candidate hyperaccumulation genes, including the roles of promoter elements and transcription, will be an important future area of investigation.

The use of *T. caerulescens* in genetic studies as a model metal-hyperaccumulating plant species has been limited by a number of factors, including a lengthy life cycle, a long period of vernalization to induce

Table 1. Forward and reverse specific primers used for the RT-PCR amplification of the Zn transporter cDNAs, the size of the amplified fragment, and annealing temperatures

Gene	Primer Sequences	Fragment Size	PCR Cycle	T _{annealing}
		bp		°C
<i>HMA4</i>	(Forward) 5'-GAAGAAGTTGAAGTAGATGAG-3' (Reverse) 5'-GATTGCTGGAGTATAGTACTGAGAACATTT-3'	332	33	58
<i>MTP1</i>	(Forward) 5'-AGGCAGACTTACGGGTTCTTCA-3' (Reverse) 5'-TCCTCCAATCATAACACCAAC-3'	503	20	58
<i>ZNT1</i>	(Forward) 5'-ATCCTCTGTGATGCTGGCGAATC-3' (Reverse) 5'-CAGGGCTATGCGAGTTGAAAGA-3'	924	25	58
<i>ACT</i>	(Forward) 5'-GAAGAACTACGAGCTACCTGATG-3' (Reverse) 5'-GATCCTCCGATCCAGACACTGTA-3'	320	22	58

flowering, low fertility, and a low transformation efficiency (Assunção et al., 2003; Peer et al., 2003). One possible method to better understand the role and contribution of specific genes to heavy metal hyperaccumulation may be through the transformation of suspension cells and, when informative, regeneration of plants from transformed tissue culture cells for studies in planta. The findings presented here show that it is possible to stably and effectively transform *T. caerulescens* suspension cells. Using this suspension cell system, it will now be possible to explore the role of various candidate genes for heavy metal hyperaccumulation and tolerance by altering their expression (both elevating and suppressing) and studying the resultant change to cellular metal tolerance and transport. The cell suspension experimental system may also be useful to further the study of cis and trans factors that regulate gene expression. Transformation of suspension cells has been previously reported (Menges and Murray, 2004; Berger et al., 2007) and *A. halleri* plants regenerated from calli were shown to maintain their heavy metal accumulation phenotype (Dal Corso et al., 2005). The findings from this report and other laboratories suggest the feasibility of such an approach.

In conclusion, this study describes the creation and characterization of *T. caerulescens* suspension cells. From this investigation, it was found that many of the traits associated with metal hyperaccumulation in intact *T. caerulescens* plants are also expressed at the cellular level. These include a greater requirement of Zn for normal growth, greater tolerance to the heavy metals Zn and Cd, altered cellular transport of heavy metals, and high expression of certain key metal transporter genes. It is intriguing that *T. caerulescens* cells actually accumulated less Zn and Cd than did Arabidopsis cells under the same conditions. This appears to be due to the increased metal efflux, possibly through the Zn and Cd transporter, HMA4. We propose that these suspension cells represent a portion of the heavy metal transport pathway that lies between the initial entry of the metals into the root epidermis, and their final storage in the leaf epidermis, as a hallmark of this long-distance pathway in *T. caerulescens* is the effective maintenance of the metals in a mobile pool for efficient root-to-shoot transport (Lasat et al., 1998). Furthermore, these *T. caerulescens* suspension cell lines may be a useful system for the further study of basic cellular tolerance and hyperaccumulation by manipulating the expression of potential candidate hyperaccumulation genes.

MATERIALS AND METHODS

Generation of Suspension Cell Lines

Thlaspi caerulescens (Prayon) seeds were sterilized with a 5% bleach solution for 5 min, washed with sterile 18 mΩ water, and then sown on either low or high Zn-containing MS plates made up of MS salts and vitamins, 3% (w/v) Suc, 0.7% (w/v) phytagar (Gibco BRL), 0.5 to 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0 to 1.0 mg/L kinetin with pH 5.8 (adjusted with KOH). The total Zn concentration (30 μM) in the low Zn media came from the Zn included in the MS salts. To produce high Zn MS plates, an additional 270 μL/L of 1 M

ZnSO₄ solution was added to the media (final Zn concentration of 300 μM). The seeds germinated on the plates and were incubated in the dark at 25°C until callus formation occurred. Two months after sowing, calli produced primarily from the hypocotyl or roots were divided, and vigorous calli were selected to be subcultured onto fresh plates. Media used for calli growth was the same as that used in calli-inducing plates except the agar concentration was increased to 0.8%. One month after the first subculture, large and vigorous callus aggregates were selected and transferred to liquid culture media. The liquid culture media was identical to the media used for the MS plates without agar. Suspension cells were then regularly subcultured over a 6-month period before the start of experiments. Arabidopsis (*Arabidopsis thaliana*; Ler) suspension cells were provided by the laboratory of Dr. Roger Innes (Indiana University).

Characterization of Suspension Cell Lines

Culture Conditions

Both *T. caerulescens* and Arabidopsis suspension cells were grown on modified MS media of the following compositions: TcZN consisted of MS salts and vitamins, 3% Suc, 1 mg/L 2,4-D, 1 mg/L kinetin, and 270 μL/L 1 M ZnSO₄ for a final Zn concentration of 300 μM, pH 5.8; TcMS consisted of MS salts and vitamins, 3% Suc, 1 mg/L 2,4-D, and 0.5 mg/L kinetin, pH 5.8, with a final Zn concentration of 30 μM; AtMS consisted of MS salts, Gamborg vitamins, 3% Suc, and 1 mg/L 2,4-D, pH 5.7, with a final Zn concentration of 30 μM. All suspension cell cultures were shaken at 120 rpm at 20°C to 24°C in the dark using a rotating shaker. Unless otherwise stated, all cell cultures were grown in 125-mL Erlenmeyer flasks containing 30 mL of media. At harvest, cells were vacuum filtered through Whatman No. 40 paper in a Büchner funnel and washed briefly with 18 mΩ distilled water and then a wash solution of 5 mM CaCl₂ and 3% Suc. The fresh weight from each flask was determined after filtration, cells were lyophilized, and the DW was determined before the cells were then transferred to quartz tubes for metal analysis by inductively coupled plasma-atomic emission spectrometry (ICP-AES; ICAP 61E Trace Analyzer; Thermo-Jarrell Ash).

Time Course Growth Curves

Growth curve studies were run with a starting cellular concentration of 2.5 mg/mL fresh weight cells. Cells from replicate flasks were harvested every 3 d starting on day 0 and continuing until day 24; all cell lines were harvested and handled as outlined above unless specified.

Long-Term Zn and Cd Tolerance

Long-term studies examining tolerance and accumulation of Cd and Zn were initiated at a cell density of approximately 3.3 mg/mL fresh weight cells and grown for 10 d. Cell lines were treated with a range of Zn and Cd concentrations (30–1,500 μM Zn and 0–50 μM Cd) with filter-sterilized heavy metal stocks added directly to flasks following media autoclaving (to avoid metal precipitation).

Short-Term Cd Influx and Efflux

TcZN and AtMS suspension cell lines for both experiments were subcultured in 250-mL Erlenmeyer flasks containing 80 mL total volume and an initial cell density of approximately 3.3 mg/mL fresh weight cells. On day 6, cells for Cd efflux measurements were treated with Cd (10 μM CdCl₂ final concentration) to begin a 20-h Cd loading period prior to Cd efflux measurements. Subsequently, the Cd efflux experiments were initiated by vacuum filtration of cells, which were then washed with 25 mL Cd-free suspension cell media and then resuspended in 60 mL of Cd-free suspension cell media. Ten-milliliter aliquots were taken at specified times, vacuum filtered using Whatman No. 40 filter paper in Büchner funnels, washed with 10 mL of 5 mM CaCl₂, collected, frozen, and lyophilized. DWs were determined and the samples were analyzed by ICP-AES for Cd content. Cd influx experiments were conducted on day 7 in uptake media containing 30 μM ZnCl₂ with the zero time point taken immediately before Cd treatment. After exposure of cells to the same medium containing 10 μM CdCl₂, 10-mL samples were

collected at the specified time points to determine Cd uptake rates. Cd influx was terminated by vacuum filtration of the cells followed by a wash with 10 mL of 5 mM CaCl₂, after which they were vacuum filtered again and lyophilized. DWs were determined and samples were analyzed by ICP-AES for Cd content.

Molecular Characterizations

TcZN and AtMS suspension cells were subcultured into 250-mL Erlenmeyer flasks containing 80 mL of total culture volume and an initial cellular concentration of 3.3 mg/mL fresh weight cells. The TcZN and AtMS suspension cells were grown in either low, standard, or high Zn media or standard media containing 10 μM Cd (Zn concentration for low, standard, and high Zn TcZN media: 30, 300, and 1,000 μM Zn, respectively; Zn concentration for low, standard, and high Zn AtMS media: 30 μM Zn with 120 μM EDTA, 30 μM, and 300 μM Zn, respectively). Suspension cells were harvested on day 7 by vacuum filtration through Miracloth (Calbiochem), washed once with 18 mΩ water, and then flash frozen in liquid N₂. RNA was isolated using Trizol following the manufacturer's directions (Invitrogen), except that RNA isolation from *T. caerulescens* cells 1.2 M NaCl and 0.8 M citrate with equal volume isopropanol was added to prevent sugar precipitation. Isolated RNA was treated with DNaseI amplification grade (Invitrogen) and then run on a QIAGEN RNeasy column. First-strand complementary DNA (cDNA) was produced with 500 ng of total RNA using the Invitrogen first-strand synthesis kit following the manufacturer's directions with random hexamers. cDNAs were cleaned using the QIAquick PCR purification kit (QIAGEN) to remove digested RNA and unincorporated dNTPs before proceeding with PCR. The PCR was run with 25 ng cDNA per reaction and a primer concentration of 10 μM for each gene-specific primer set (Table I). Primers were designed for homologous regions within the genes of interest for both *T. caerulescens* (Prayon) and *Arabidopsis* (*Ler*), and PCR products ranged in length from 300 to 1,000 bp. The primers were designed from the following National Center for Biotechnology Information identified sequences: *HMA4* (Tc:AY486001; At:AJ297264), *MTP1* (Tc:AY483146; At:NM_180128), and *ZNT1* (Tc:AF133267; At:NM_100972). The *Arabidopsis* housekeeping gene *ACT2* (NM_112764) and its closest *T. caerulescens* ortholog were used as controls. Initial control reactions showed that under the chosen conditions, all PCR products were in the linear amplification range (data not shown). The experiments were repeated using three biological replicates, and duplicate RT-PCR experiments were conducted for each gene from each biological replicate.

Transformation of Suspension Cell Lines

The vector pCambia 1303 containing the reporter gene GUS:GFP under the control of the 35S promoter was transformed into the *Agrobacterium tumefaciens* C38 strain and grown for 48 h at 30°C on Luria broth plates containing 50 μg/L kanamycin and 50 μg/L rifampicin. Individual colonies of transformed *A. tumefaciens* C38 were picked and grown to a final OD₆₀₀ of 0.6 in 10 mL of Luria-Bertani media containing kanamycin and rifampicin at 30°C and spun at 250 rpm. Bacteria were then spun down at 4,400 rpm for 5 min and remaining media was removed. The *A. tumefaciens* pellet was washed once with TcZN media to remove residual antibiotics before being resuspended in 2 mL of TcZN media and added to a 125-mL Erlenmeyer flask containing 30 mL of newly subcultured *T. caerulescens* cells in TcZN media. The *A. tumefaciens*-inoculated flask grew under standard conditions for 72 h. After 72 h of cocultivation, *T. caerulescens* cells were washed twice with TcZN media. Over the next 21 d, the transformed *T. caerulescens* suspension cells were selected for on TcZN media containing 50 μg/mL hygromycin and 100 μg/mL timentin, replaced every 7 d. At the end of 3 weeks, cultures were assayed for β-glucuronidase activity and GFP expression. A 1-mL sample was taken from transformed cells, residual media was removed, and cells were placed in 1 mL of a solution containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-GlcUA), 100 mM potassium phosphate buffer, pH 7.0, 0.5 mM K₂Fe(CN)₆, 0.5 mM K₂Fe(CN)₆, 10 mM EDTA, and 0.1% Tween 20. Tissue was incubated at 37°C for 3 to 4 h, and blue calli were selected for observation using GFP fluorescence using a Axiovert 100 with AxioCam (Zeiss) with the filter set 18 (BP 390–420, FT 425, LP 450) epifluorescence to confirm presence of the GFP protein.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AJ297264 (*AtHMA4*), AY486001 (*TcHMA4*), NM_180128 (*AtMTP1*), AY483146 (*TcMTP1*), NM_100972 (*AtZNT1/ZIP4*), AF133267 (*TcZNT1*), and NM_112764 (*AtACT2*).

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LITERATURE CITED

- Assunção AGL, Martins PD, De Folter S, Vooijs R, Schat H, Aarts MGM (2001) Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. *Plant Cell Environ* **24**: 217–226
- Assunção AGL, Schat H, Aarts MGM (2003) *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. *New Phytol* **159**: 351–360
- Becher M, Talke IN, Krall L, Krämer U (2004) Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. *Plant J* **37**: 251–268
- Berger B, Stracke R, Yatusевич R, Weisshaar B, Flüge UI, Gigolashvili T (2007) A simplified method for the analysis of transcription factor-promoter interactions that allows high-throughput data generation. *Plant J* **50**: 911–916
- Bernard C, Roosens N, Czernic P, Lebrun M, Verbruggen N (2004) A novel CPX-ATPase from the cadmium hyperaccumulator *Thlaspi caerulescens*. *FEBS Lett* **569**: 140–148
- Cosio C, DeSantis L, Frey B, Diallo S, Keller C (2005) Distribution of cadmium in leaves of *Thlaspi caerulescens*. *J Exp Bot* **56**: 765–775
- Cosio C, Martinoia E, Keller C (2004) Hyperaccumulation of cadmium and zinc in *Thlaspi caerulescens* and *Arabidopsis halleri* at the leaf cellular level. *Plant Physiol* **134**: 716–725
- Dal Corso G, Borgato L, Furini A (2005) In vitro plant regeneration of the heavy metal tolerant and hyperaccumulator *Arabidopsis halleri* (Brassicaceae). *Plant Cell Tissue Organ Cult* **82**: 267–270
- Encina CL, Constantin M, Botella J (2001) An easy and reliable method for establishment and maintenance of leaf and root cell cultures of *Arabidopsis thaliana*. *Plant Mol Biol Rep* **19**: 245–248
- Frey B, Keller C, Zierold K, Schulin R (2000) Distribution of Zn in functionally different leaf epidermal cells of the hyperaccumulator *Thlaspi caerulescens*. *Plant Cell Environ* **23**: 675–687
- Hammond JP, Bowen HC, White PJ, Mills V, Pyke KA, Baker AJM, Whiting SN, May ST, Broadley MR (2006) A comparison of the *Thlaspi caerulescens* and *Thlaspi arvense* shoot transcriptomes. *New Phytol* **170**: 239–260
- Hart JJ, Welch RM, Norvell WA, Sullivan LA, Kochian LV (1998) Characterization of cadmium binding, uptake and translocation in intact seedlings of bread and durum wheat cultivars. *Plant Physiol* **116**: 1413–1420
- Hussain D, Haydon MJ, Wang Y, Wong E, Sherson SM, Young J, Camakaris J, Harper JE, Cobbett CS (2004) P-Type ATPase heavy metal transporters with roles in essential zinc homeostasis in *Arabidopsis*. *Plant Cell* **16**: 1327–1339
- Jain RK, Chowdhury JB, Sharma DR, Friedt W (1988) Genotypic and media effects on plant regeneration from cotyledon explant cultures of some Brassica species. *Plant Cell Tissue Organ Cult* **14**: 197–206
- Kobae Y, Uemura T, Sato MH, Ohnishi M, Mimura T, Nakagawa T, Maeshima M (2004) Zinc transporter of *Arabidopsis thaliana* AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis. *Plant Cell Physiol* **45**: 1749–1758
- Kochian LV, Pence NS, Letham DLD, Piñeros MA, Magalhaes JV, Hoekenga OA, Garvin DF (2002) Mechanisms of metal resistance in plants: aluminum and heavy metals. *Plant Soil* **247**: 109–119
- Küpper H, Jie ZF, McGrath SP (1999) Cellular compartmentation of zinc in leaves of the hyperaccumulator *Thlaspi caerulescens*. *Plant Physiol* **119**: 305–312
- Lasat MM, Baker AJ, Kochian LV (1996) Physiological characterization of root

- Zn²⁺ absorption and translocation to shoots in Zn hyperaccumulator and nonaccumulator species of *Thlaspi*. *Plant Physiol* **112**: 1715–1722
- Lasat MM, Baker AJ, Kochian LV** (1998) Altered Zn compartmentation in the root symplasm and stimulated Zn absorption into the leaf as mechanisms involved in Zn hyperaccumulation in *Thlaspi caerulescens*. *Plant Physiol* **118**: 875–883
- Levent O, Cakmak I, Kochian LV** (2003) Shoot biomass and zinc/cadmium uptake for hyperaccumulator and non-accumulator *Thlaspi* species in response to growth on a zinc-deficient calcareous soil. *Plant Sci* **164**: 1065–1071
- Ma JF, Ueno D, Zhao FJ, McGrath SP** (2005) Subcellular localisation of Cd and Zn in the leaves of a Cd-hyperaccumulating ecotype of *Thlaspi caerulescens*. *Planta* **220**: 731–736
- Marques L, Cossegal M, Bodin S, Czernic P, Lebrun M** (2004) Heavy metal specificity of cellular tolerance in two hyperaccumulating plants, *Arabidopsis halleri* and *Thlaspi caerulescens*. *New Phytol* **164**: 289–295
- Marschner H** (1995) Mineral Nutrition of Higher Plants, Ed 2. Academic, San Diego
- Menges M, Murray JAH** (2004) Cryopreservation of transformed and wild-type *Arabidopsis* and tobacco cell suspension cultures. *Plant J* **37**: 635–644
- Papoyan A, Kochian LV** (2004) Identification of *Thlaspi caerulescens* genes that may be involved in heavy metal hyperaccumulation and tolerance: characterization of a novel heavy metal transporting ATPase. *Plant Physiol* **136**: 3814–3823
- Parker DR, Norvell WA, Chaney RL** (1995) GEOCHEM-PC: a chemical speciation program for IBM and compatible personal computers. In RH Loeppert, AP Schwab, S Goldberg, eds, Chemical Equilibrium and Reaction Models. Soil Science Society of America, American Society of Agronomy, Madison, WI, pp 253–269
- Peer WA, Mamoudian M, Lahner B, Reeves RD, Murphy AS, Salt DE** (2003) Identifying model metal hyperaccumulating plants: germplasm analysis of 20 Brassicaceae accessions from a wide geographical area. *New Phytol* **159**: 421–430
- Pence NS, Larsen PB, Ebbs SD, Letham DL, Lasat MM, Garvin DF, Eide D, Kochian LV** (2000) The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. *Proc Natl Acad Sci USA* **97**: 4956–4960
- Piñeros MA, Kochian LV** (2003) Differences in whole-cell and single-channel ion currents across the plasma membrane of mesophyll cells from two closely related *Thlaspi* species. *Plant Physiol* **131**: 583–594
- Rigola D, Fiers M, Vurro E, Aarts MGM** (2006) The heavy metal hyperaccumulator *Thlaspi caerulescens* expresses many species-specific genes as identified by comparative EST analysis. *New Phytol* **170**: 753–766
- Toriyama K, Kameya T, Hinata K** (1987) Ability of callus growth and shoot regeneration in the wild species of Brassicaceae. *Plant Tissue Culture Letters* **4**: 75–78
- van de Mortel J, Villanueva LA, Schat H, Kwekkeboom J, Coughlan S, Moerland PD, Ver Loren van Themaat E, Koornneef M, Aarts MGM** (2006) Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana* and the related metal hyperaccumulator *Thlaspi caerulescens*. *Plant Physiol* **142**: 1127–1147
- van der Zaal BJ, Neuteboom LW, Pinas JE, Chardonnens AN, Schat H, Verkleij JA, Hooykaas PJ** (1999) Overexpression of a novel Arabidopsis gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. *Plant Physiol* **119**: 1047–1055
- Verret F, Gravot A, Auroy P, Leonhardt N, David P, Nussaume L, Vavasseur A, Richaud P** (2004) Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. *FEBS Lett* **576**: 306–312
- Weber M, Harada E, Vess CV, van Roepenack-Lahaye E, Clemens S** (2004) Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. *Plant J* **37**: 269–281