The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*

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An integrated molecular and physiological investigation of the fundamental mechanisms of heavy metal accumulation was conducted in *Thlaspi caerulescens*, a Zn/Cd-hyperaccumulating plant species. A heavy metal transporter cDNA, *ZNT1*, was cloned from *T. caerulescens* through functional complementation in yeast and was shown to mediate high-affinity Zn^{2+} uptake as well as low-affinity Cd^{2+} uptake. It was found that this transporter is expressed at very high levels in roots and shoots of the hyperaccumulator. A study of *ZNT1* expression and high-affinity Zn^{2+} uptake in roots of *T. caerulescens* and in a related nonaccumulator, *Thlaspi arvense*, showed that alteration in the regulation of *ZNT1* gene expression by plant Zn status results in the overexpression of this transporter and in increased Zn influx in roots of the hyperaccumulating *Thlaspi* species. These findings yield insights into the molecular regulation and control of plant heavy metal and micronutrient accumulation and homeostasis, as well as provide information that will contribute to the advancement of phytoremediation by the future engineering of plants with improved heavy metal uptake and tolerance.

Recently, there has been considerable interest in the use of terrestrial plants as a green technology for the remediation of surface soils contaminated with toxic heavy metals. This technology, termed phytoremediation, uses plants to extract heavy metals from the soil and to concentrate them in the harvestable shoot tissue (1, 2). A major factor behind the interest in phytoremediation of metal-polluted soils has been the growing awareness of the existence of a number of metal-accumulating plant species. These plant species, called hyperaccumulators, are endemic to metalliferous soils and can accumulate and tolerate high levels of heavy metals in the shoot (3, 4). Among the best known hyperaccumulators is *Thlaspi caerulescens*. This member of the Brassicaceae family has attracted the interest of plant biologists for over a century because of its ability to colonize calamine and serpentine soils containing naturally elevated levels of heavy metals such as Zn, Pb, Cd, Ni, Cr, and Co. Certain ecotypes of *T. caerulescens* have been shown to accumulate up to 30,000 ppm Zn and 1,000 ppm Cd in their shoots without exhibiting toxicity symptoms (5). By comparison, normal foliar Zn concentrations are around 100 ppm, with 30 ppm considered adequate and 300–500 ppm considered toxic (6). Foliar Cd levels above 1 ppm usually are considered toxic.

The practical utility of many hyperaccumulators for phytoremediation may be limited, because many of these species, including *T. caerulescens*, are slow-growing and produce little shoot biomass, severely constraining their potential for large-scale decontamination of polluted soils (7). Transferring the genes responsible for the hyperaccumulating phenotype to higher shoot-biomass-producing plants has been suggested as a potential avenue for enhancing phytoremediation as a viable commercial technology (8, 9). Progress toward this goal has been hindered by a lack of understanding of the basic molecular, biochemical, and physiological mechanisms involved in heavy metal hyperaccumulation. The unique physiology of metal hyperaccumulators such as *T. caerulescens* makes them ideal model systems for studying the fundamental mechanisms that plants employ to absorb, tolerate, and hyperaccumulate toxic heavy metals. In this study, we investigated the physiological and molecular basis for plant heavy metal hyperaccumulation through an investigation of Zn transport and accumulation in *T. caerulescens* in comparison with a related nonaccumulator, *Thlaspi arvense*.

We previously conducted physiological studies that focused on the use of radiotracer flux techniques ($^{65}Zn^{2+}$) to characterize Zn transport and compartmentation in these two species (10, 11). These studies indicated that a number of Zn transport sites contribute to the hyperaccumulation trait in *T. caerulescens*. These sites include Zn influx across the root–cell plasma membrane, xylem-localized Zn loading, and reabsorption and storage of xylem-borne Zn in leaf mesophyll cells (10, 11). It was shown that root Zn absorption is mediated by a high-affinity Zn^{2+} transporter with a similar affinity for Zn^{2+} in the two *Thlaspi* species (Michaelis constants, K_m, for root Zn^{2+} uptake are 6 and 8 μM in *T. caerulescens* and *T. arvense*, respectively). However, there was a 5-fold larger V_{max} for root Zn uptake in *T. caerulescens* as compared with *T. arvense* (10). These findings suggest that the increased Zn uptake in *T. caerulescens* is caused by a higher density of Zn transporters in the root-cell plasma membrane (10).

An important trait of hyperaccumulating plant species is 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 *T. arvense* (10), which was correlated with a 5-fold increase in xylem sap Zn (11). Additionally, leaf $^{65}Zn^{2+}$ uptake at Zn concentrations representative of those found in *T. caerulescens* xylem sap (≈1 mM Zn^{2+}) demonstrated that there was a 2-fold greater leaf Zn accumulation in *T. caerulescens* (11). This physiological evidence indicates that Zn hyperaccumulation in *T. caerulescens* is caused, in part, by increased Zn transport at multiple sites along the Zn absorption and translocation pathway. However, the underlying basis for this increased transport cannot readily be elucidated through purely physiological investigations. Hence, we are integrating molecular and physiological studies of Zn transport and hyperaccumulation in *T. caerulescens* to understand the basic mechanisms underlying this complex trait.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF133267).

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In this paper, we report on the cloning and characterization of a Zn/Cd transport cDNA, *ZNT1*, from *T. caerulescens*. Investigation of ZNT1 transport properties by using expression in yeast as a heterologous system showed that the transporter mediates high-affinity Zn^{2+} uptake as well as low-affinity Cd^{2+} uptake. Studies on the effect of varying plant Zn status on both *ZNT1* expression and high-affinity Zn^{2+} uptake into roots of the two *Thlaspi* species indicated that Zn hyperaccumulation in *T. caerulescens* is caused, in part, by an alteration in the regulation of Zn transporters by plant Zn status. This alteration results in an increased Zn transporter gene expression and a concomitant enhanced Zn^{2+} uptake and transport in the hyperaccumulating plant species.

Materials and Methods

Plant Material and Culture. *T. caerulescens* ecotype Prayon (provided by A. J. M. Baker, University of Sheffield, U.K.) and *T. arvense* (Crucifer Genetics Cooperative, University of Wisconsin, Madison) seeds were germinated and grown in modified Johnson's nutrient solution [macronutrients 1.2 mM KNO_3/0.8 mM $Ca(NO_3)_2$/0.1 mM $NH_4H_2PO_4$/0.2 mM $MgSO_4$ and micronutrients 50 μM KCl/12.5 μM H_3BO_3/1 μM $MnSO_4$/1 μM $ZnSO_4$/0.4 μM $CuSO_4$/0.1 μM Na_2MoO_4/0.1 μM $NiSO_4$]. The solution was supplemented with 1 mM Mes (2-[*N*-morpholino]ethanesulfonic acid) buffer, pH 6.0, and 5 μM Fe-EDDHA (*N*,*N*′-ethylenediamine-di(*O*-hydroxyphenylacetic acid). The nutrient solution was aerated and replaced weekly. Plants were grown in a greenhouse (18–22°C) without artificial light supplementation.

To compensate for a greater growth rate in *T. arvense*, seedlings of *T. caerulescens* and *T. arvense* were grown for 50 and 40 days, respectively, before treatments were induced. Plants were transferred to Zn-deficient (0 μM $ZnSO_4$), Zn-replete (1 μM $ZnSO_4$), or Zn-excess (10 or 50 μM $ZnSO_4$) modified nutrient solution for 14 days. Zn-excess medium contained 10 μM $ZnSO_4$ and 50 μM $ZnSO_4$ for *T. arvense* and *T. caerulescens,* respectively, because of the differential Zn sensitivity exhibited by the two species.

Yeast Growth Conditions. The yeast Zn transport-deficient double-mutant ZHY3 (*MATα ade6 can1 his3 leu2 trp1 ura3 zrt1*::*LEU2 zrt2*::*HIS3*) (12, 13) and its parent strain DY1457 (*MATα ade6 can1 his3 trp1 ura3*), containing the yeast expression vector pFL61 or the *T. caerulescens* Zn transport cDNA *ZNT1* in pFL61 (pZNT1), were grown on supplemented minimal medium (14) amended with 0.1% Casamino acids, 20 mg/liter adenine, 20 mg/liter tryptophan, and 10 μM Fe-EDTA. Low-Zn medium, which permitted growth of the parent strain but prohibited visible growth of ZHY3, contained 650 μM $ZnSO_4$ and 1 mM EDTA. High-Zn medium, required for visible growth of ZHY3, contained 2 mM $ZnSO_4$ and 1 mM EDTA.

Cloning of a Zn Transport cDNA, *ZNT1*, Through Functional Complementation of ZHY3. A cDNA library was constructed with combined poly(A)$^+$ RNA from roots and shoots of *T. caerulescens* seedlings grown on both Zn-deficient and Zn-replete nutrient solutions. The cDNA was synthesized by using the Superscript Choice System (GIBCO/BRL), then ligated with *Bst*XI/*Eco*RI adapters into the bifunctional yeast/*Escherichia coli* expression plasmid vector pFL61 (15). This vector contains a yeast phosphoglycerate kinase promoter and a uracil selection marker.

To identify a Zn transport cDNA, we used functional complementation of ZHY3's inability to grow on low-Zn medium. ZHY3 was transformed with the *T. caerulescens* cDNA library, and 350,000 transformants were screened for growth on low-Zn medium. Plasmids that restored ZHY3 growth on low-Zn medium were isolated (Qiagen), subcloned into pGEM-5Zf(+) (Promega) for sequencing (ABI Prism; Perkin–Elmer), and retransformed into ZHY3 to confirm functional complementation. 5-Fluoroorotic acid treatment, which causes loss of plasmids from the yeast, confirmed that ZHY3 growth on low-Zn medium was plasmid-dependent (16).

Multiple alignments were performed by using the CLUSTAL method (LASERGENE software; DNAstar, Madison, WI). TMPRED (European Molecular Biology Network, Swiss node, www.ch.embnet.org/software/TMPRED_form.html; ref. 17) was used to predict ZNT1 protein structure and transmembrane domains.

Yeast Metal Uptake Studies. ZHY3 yeast strains containing plasmids pFL61 or pZNT1 were grown to mid-log phase in minimal medium (14) amended with 0.1% Casamino acids, 20 mg/liter adenine, 20 mg/liter tryptophan, and 10 μM Fe-EDTA. Cells were harvested, and aliquots of the cell suspension were mixed with equal volumes of a radiolabeled $^{65}Zn^{2+}$ or $^{109}Cd^{2+}$ solution. Uptake solution contained 10 mM Mes (pH 6.0), 2% glucose, and $ZnCl_2$ or $CdCl_2$ at concentrations ranging from 1 to 80 μM. After an uptake period of 3 min, the cells were centrifuged through a silicone oil/dinonyl phthalate pad into a droplet of 40% perchloric acid. ^{65}Zn or ^{109}Cd content of the pellet then was determined by γ detection and converted to Zn^{2+} or Cd^{2+} influx values.

Cloning of *ZNT1-arvense*. *ZNT1-arvense* was isolated by reverse transcription–PCR with *T. arvense* root and leaf RNA by using the Superscript II protocol (GIBCO/BRL). Second-strand synthesis used the forward primer 5′-GAT/C TTT/C ATG GGG CAN CAG/A TA-3′ and the reverse primer 5′-CCT TCG AAA/G AAC/T TGG/A TGG/A AA-3′. These degenerate primers were designed to a conserved region of *ZNT1* and the similar *Arabidopsis* spp. Zn transporter, *ZIP4* (18). The resulting partial cDNA was sequenced and designated *ZNT1-arvense*.

Northern Analysis. Total RNA was isolated from roots and shoots of *T. caerulescens* and *T. arvense* grown on Zn-deficient, Zn-replete, and Zn-excess nutrient solution. Samples were denatured, separated by denaturing agarose gel electrophoresis, and transferred to nylon membranes (Hybond N$^+$; Amersham Pharmacia). Equal loading of RNA in each lane was confirmed by ethidium bromide staining of the ribosomal subunits. Probes were labeled with [α-^{32}P]dCTP by random hexamer primers. After hybridization at 65°C, the nylon membranes were washed twice for 15 min at 65°C in a low-stringency wash solution [2× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/0.1% SDS]. After autoradiography, membranes were stripped for 30 min with 0.5% SDS at 100°C.

Concentration-Dependent Zn^{2+} Uptake Kinetics in Plants. Roots of intact *T. caerulescens* or *T. arvense* seedlings were immersed in 80 ml of pretreatment solution (2 mM Mes·Tris, pH 6.0/0.5 mM $CaCl_2$) in individual Plexiglas wells of an uptake apparatus (19). Subsequently, Zn^{2+} was added as $ZnCl_2$ to each uptake well to yield a final Zn concentration between 0.5 and 100 μM 1 min before the addition of 0.08 μCi (1 Ci = 37 GBq) of $^{65}ZnCl_2$. After a 20-min uptake period, radioactive solutions were vacuum-withdrawn, and wells were refilled with ice cold desorption solution (100 μM $ZnCl_2$/5 mM $CaCl_2$/2 mM Mes·Tris, pH 6.0). After a 15-min desorption period to remove cell-wall-bound ^{65}Zn, seedlings were harvested and their roots were excised, blotted, and weighed. ^{65}Zn was quantified by γ detection.

Results and Discussion

We initiated a molecular characterization of plant heavy metal hyperaccumulation by cloning a Zn transporter cDNA from *T. caerulescens* through functional complementation in yeast. The *Saccharomyces cerevisiae* mutant ZHY3 is defective in the

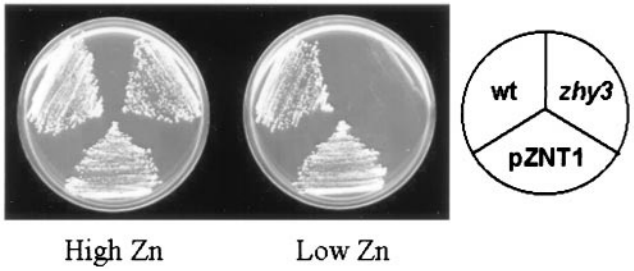

Fig. 1. Functional complementation of Zn transport in yeast by *ZNT1*. A yeast *zrt1 zrt2* mutant (zhy3) lacks both high- and low-affinity Zn transporters, and unlike the wild type (wt), requires high-Zn medium for growth. The *ZNT1* cDNA ligated into the yeast expression vector pFL61 (denoted pZNT1) restores growth of zhy3 on low-Zn medium (10). Yeast was grown on supplemented minimal medium (14) amended with 0.1% Casamino acids, 20 mg/liter adenine, 20 mg/liter tryptophan, 1 mM EDTA, and 10 μM Fe-EDTA. High- and low-Zn media included 1 mM and 650 μM $ZnSO_4$, respectively.

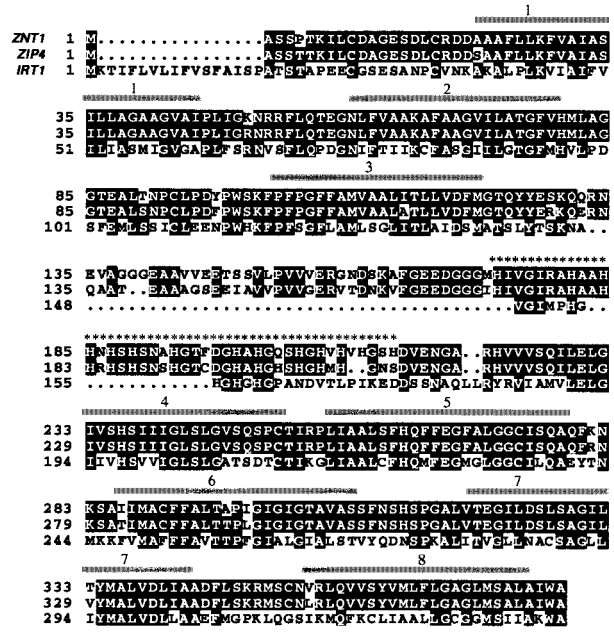

Fig. 2. Sequence identity among *ZNT1*, *ZIP4*, and *IRT1*. The deduced amino acid sequence of *ZNT1* (GenBank accession no. AF133267) is aligned with the *ZIP4* (GenBank accession no. U95973) and *IRT1* (GenBank accession no. U27590) members of the *ZIP* gene family, by using the CLUSTAL method in LASERGENE software (DNAstar, Madison, WI). The predicted peptide encoded by *ZNT1* exhibits 88% sequence identity to *ZIP4* and 34% identity to *IRT1*; shaded areas indicate regions of identity to *ZNT1*. The asterisks above the sequence alignment identify the histidine-rich region located in a putative cytoplasmic domain, and the gray bars indicate the eight potential transmembrane domains predicted by TMPRED (17).

high- and low-affinity Zn transporters, ZRT1 and ZRT2, respectively (12). Consequently, ZHY3 has a much higher Zn requirement for growth than does the parental wild-type yeast (12, 13). The ZHY3 strain was transformed with a *T. caerulescens* cDNA library constructed in the yeast expression vector pFL61. Screening of 350,000 yeast transformants for growth on low-Zn medium resulted in the identification of seven clones that were able to restore growth on low-Zn medium. Of these seven, nucleotide sequencing identified five as representing the same 1.2-kb cDNA, which subsequently was designated *ZNT1* (for Zn transporter) and is the focus of this study.

Expression of *ZNT1* in ZHY3 restored growth on low-Zn medium to that of the parental wild-type yeast (Fig. 1). The predicted ORF for *ZNT1* is 379 aa in length and demonstrates significant sequence identity with the *Arabidopsis* genes *ZIP4* and *IRT1*, which encode putative Zn and Fe transporters, respectively (Fig. 2; refs. 16 and 20). These *Arabidopsis* genes are members of the recently discovered ZIP (for ZRT/IRT-like proteins) family of micronutrient transport proteins (21). ZNT1 shares the structural features exhibited by other members of this family, including eight putative transmembrane domains and a highly hydrophilic cytoplasmic region predicted to reside between transmembrane domains three and four. This putative cytoplasmic domain contains a series of histidine repeats, which may define a metal-binding region for the transporter. Other micronutrient/heavy metal transporters in this gene family, including IRT1 and ZIP4, contain similar histidine-rich regions between the third and fourth membrane-spanning domains (18, 20, 22–24). The similarities in predicted amino acid sequence and protein structure between ZNT1 and other members of the ZIP family suggest that ZNT1 is an integral membrane protein that mediates Zn^{2+} transport across the cell membrane.

To test the hypothesis that ZNT1 is a Zn transport protein, *ZNT1* was expressed in yeast (ZHY3), and radiotracer ($^{65}Zn^{2+}$ and $^{109}Cd^{2+}$) flux techniques were used to determine the concentration-dependent kinetics of Zn^{2+} and Cd^{2+} influx mediated by ZNT1 in ZHY3. Yeast expressing *ZNT1* exhibited biphasic concentration-dependent kinetics for $^{65}Zn^{2+}$ influx that were smooth and nonsaturating (Fig. 3 *Upper*). In ZHY3 expressing the empty pFL61 vector, where Zn^{2+} uptake is caused by low levels of transport activity mediated by other yeast ion transporters (e.g., Ca^{2+} channels), these cells yielded linear Zn^{2+} transport kinetics. To determine the contribution by ZNT1 to the complex Zn^{2+} transport kinetics depicted in Fig. 3 *Upper*, the linear Zn transport caused by residual activity in ZHY3 was subtracted from the overall transport kinetics. This method yielded saturable Zn uptake that conformed to Michaelis–Menten kinetics with a K_m of 7.5 μM (Fig. 3 *Upper*). As shown in Fig. 3 *Lower*, ZNT1 also mediates a low-affinity Cd^{2+} influx in yeast that follows first-order (linear) transport kinetics. It has often been speculated (25, 26) that Cd^{2+} enters and is transported in plants by endogenous Zn transporters; here we provide direct evidence for this idea. The kinetic properties for Zn^{2+} and Cd^{2+} uptake mediated by *ZNT1* in yeast is similar to what we have seen previously for Zn^{2+} and Cd^{2+} uptake in *T. caerulescens* roots. That is, Zn^{2+} uptake systems in yeast and roots are both saturable, with very similar K_m values (10), whereas Cd^{2+} influx is nonsaturable in both systems. These results are consistent with the hypothesis that *ZNT1* encodes a root plasma membrane Zn^{2+}/Cd^{2+} transporter.

A 5-fold increase in the V_{max} for root Zn^{2+} influx in *T. caerulescens* as compared with *T. arvense* in an earlier study led us to speculate that there are a greater number of Zn transporters per unit area of root-cell plasma membrane in the hyperaccumulator (10). To test this speculation further, the expression of *ZNT1* was examined by Northern analysis with RNA isolated from roots and shoots of both *Thlaspi* species. *ZNT1* transcript abundance was dramatically higher in roots and shoots of *T. caerulescens* grown under Zn-sufficient and -deficient conditions as compared with *T. arvense* (Fig. 4), which is consistent with the hypothesis that Zn hyperaccumulation in *T. caerulescens* is caused, in part, by increased expression of Zn transporters in the root and shoot. When *T. arvense* total RNA was probed with the full-length *T. caerulescens ZNT1* cDNA, almost no signal was detected in roots or shoots of ±Zn-grown plants (Fig. 4). To ensure that the apparent difference in *ZNT1* transcript abundance observed between the two *Thlaspi* species was not caused by sequence divergence between *ZNT1* and its homolog in *T. arvense*, we cloned a gene-specific probe for the *T. arvense*

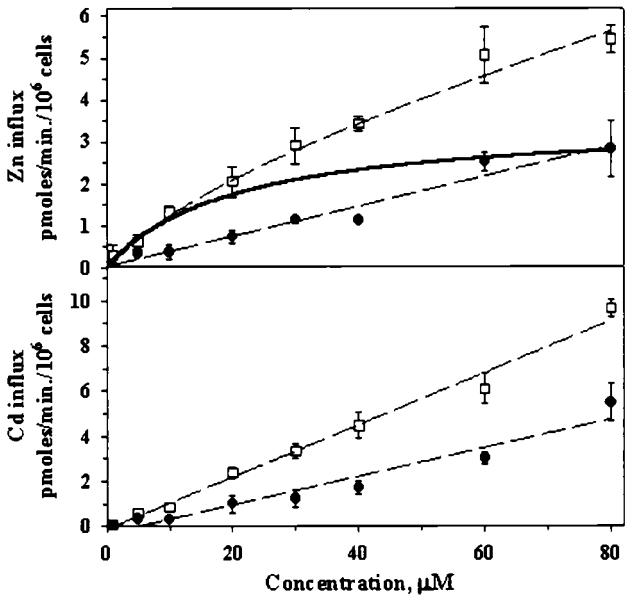

Fig. 3. ZNT1 mediates Zn and Cd transport when expressed in yeast. (*Upper*) ZNT1-mediated Zn influx kinetics (denoted by solid line) were determined by the subtraction of residual Zn uptake in ZHY3 transformed with pFL61 (●) from the complex Zn influx kinetics exhibited by ZHY3 transformed with pFL61:*ZNT1* (□). The resolved curve followed classical Michaelis–Menten kinetics for Zn influx. The K_m of 7.5 μM and V_{max} of 2.2 pmol of Zn per min per 10^6 cells were determined graphically by Lineweaver–Burke analysis of the uptake data. (*Lower*) The concentration-dependent kinetics of Cd influx did not conform to Michaelis–Menten kinetics, and a saturable component could not be resolved. Cd influx was enhanced by the presence of *ZNT1* (□) as seen in comparison to residual Cd uptake in ZHY3 transformed with pFL61 (●). Error bars represent SE, and $n = 6–10$.

homolog of *ZNT1*, designated *ZNT1-arvense*. When the Northern blot was rehybridized with the *ZNT1-arvense* probe, the same large difference in *ZNT1* transcript abundance between the two *Thlaspi* species was observed (Fig. 4). The *ZNT1-arvense* probe did detect a clear, although faint, signal from *T. arvense* root and shoot tissue only in Zn-deficient seedlings. The same pattern was

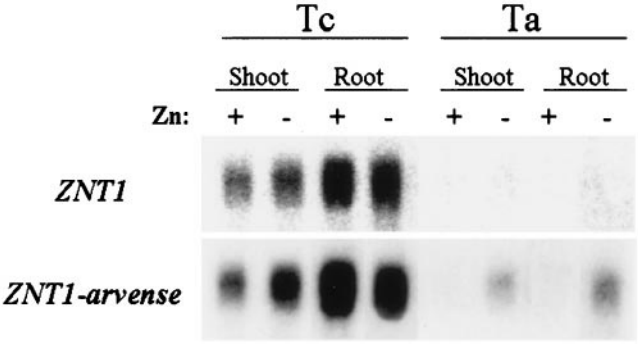

Fig. 4. ZNT1 expression in *T. caerulescens* and *T. arvense*. Total RNA was isolated from roots and shoots of *T. caerulescens* (Tc) and *T. arvense* (Ta) grown for 14 days in a modified Johnson's solution with 0 (−) or 1 (+) μM Zn. The Northern blot, equally loaded with 7 μg of total RNA per lane, depicts the extremely high *ZNT1* transcript abundance in *T. caerulescens* roots and shoots when probed with the full-length cDNA of *ZNT1*. Visualization of rRNA indicated that total RNA was equally loaded (data not shown). A subsequent probing with a gene-specific 0.4-kb fragment of the *ZNT1* homolog from *T. arvense* (*ZNT1-arvense*) revealed that *ZNT1* is expressed in the nonaccumulator under Zn deficiency in both the roots and shoots. Signals from both probes indicate a transcript size of ≈1.2 kb.

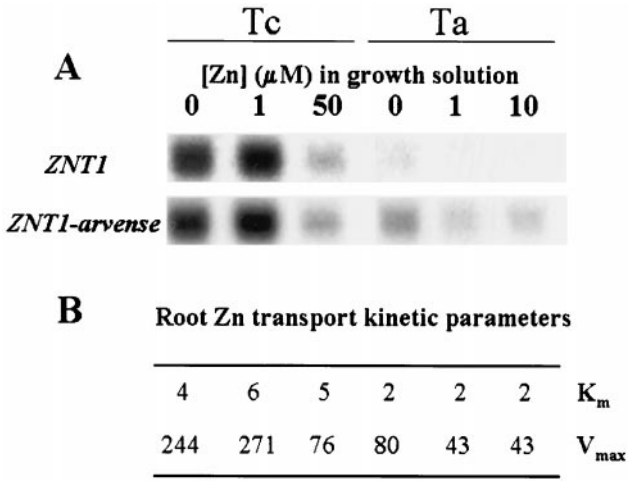

Fig. 5. Influence of varying plant Zn status on *ZNT1* expression in *T. caerulescens* and *T. arvense*. *T. caerulescens* (Tc) and *T. arvense* (Ta) were grown for 14 days in a modified Johnson's solution containing 0, 1, or 10 μM Zn (for *T. arvense*) and 50 μM Zn (for *T. caerulescens*). (*A*) Total RNA was isolated from roots and shoots. The Northern blot, equally loaded with 20 μg of total RNA per lane, is shown probed with *ZNT1* from *T. caerulescens* or the *ZNT1* homolog from *T. arvense*. (*B*) Radiotracer studies of unidirectional $^{65}Zn^{2+}$ influx in roots of *T. caerulescens* and *T. arvense* grown under the different Zn concentrations were performed. The K_m and V_{max} values were determined for saturable Zn^{2+} uptake from the resulting concentration-dependent kinetics [after subtraction of the nonsaturating uptake component that we previously had shown to be root cell-wall-bound ^{65}Zn that remained after desorption of radiolabel (10)]. The units for K_m and V_{max} are μM and pmol of Zn absorbed per 10^6 cells per min, respectively.

seen when Northern blots also were hybridized with a gene-specific probe for *ZNT1* from *T. caerulescens* (data not shown).

Several important pieces of information can be gleaned from the Northern analysis data presented in Fig. 4. First, the ZNT1 Zn transporter is expressed to much higher levels in both roots and shoots of *T. caerulescens*, and this response may play a key role in Zn hyperaccumulation. Second, in the nonaccumulator, *T. arvense*, Zn transporters are expressed to very low levels in Zn-sufficient plants. Imposition of Zn deficiency induces an increased expression of these transporters, facilitating enhanced Zn absorption. Similar regulation of Zn transporter expression recently was described in *Arabidopsis* (18). In *T. caerulescens*, Zn transporters are expressed to very high levels, irrespective of the plant Zn status (i.e., Zn-deficient *and* adequate plants). Apparently, there is an alteration in the perception of plant Zn status in *T. caerulescens*, resulting in increased Zn transporter gene expression and greater Zn uptake.

To investigate the relationship among plant Zn status, root Zn uptake, and expression of Zn transport genes in more detail, the kinetics of root Zn^{2+} uptake and expression of *ZNT1* in roots of the two *Thlaspi* species both were determined for seedlings grown in a range of Zn levels. Because *ZNT1* expression in *T. caerulescens* did not respond to changes in plant Zn status for growth on 0 vs. 1 μM Zn, seedlings were also grown on higher Zn concentrations to see whether plant Zn levels needed to be elevated to down-regulate Zn transporter expression. Thus, plants were grown under Zn-deficient (0 μM), Zn-replete (1 μM), or Zn-excess (10 or 50 μM for *T. arvense* and *T. caerulescens*, respectively) conditions.

Both the Northern analysis data and root Zn influx (root Zn transport kinetic parameters: K_m and V_{max}) for the Zn-deficient, Zn-sufficient, and high-Zn-grown *Thlaspi* plants are summarized in Fig. 5. A close correlation between *ZNT1* expression (Fig. 5*A*) and the V_{max} for root Zn^{2+} influx (Fig. 5*B*) was found in both

Thlaspi species. In *T. arvense*, growth on adequate (1 μM) or high (10 μM) Zn had no effect on the low level of root *ZNT1* expression or on the small root Zn^{2+} influx that was observed (V_{max} of 43 nmol/g per h). Only when *T. arvense* plants were made Zn-deficient was a moderate increase in *ZNT1* expression and root Zn^{2+} influx seen (increase in V_{max} to 80 nmol/g per h). Quantification of root transcript abundance from the data in the Northern blot presented in Fig. 5 indicated that Zn deficiency caused a 2-fold increase in *T. arvense* mRNA abundance, which correlates with the 2-fold enhancement of V_{max}.

The responses of root Zn uptake to changes in plant Zn status in *T. caerulescens* were found to be qualitatively similar to the responses in *T. arvense* when seedlings were grown in a wide range of Zn concentrations in the nutrient solution (0 to 50 μM Zn). That is, for *T. caerulescens* seedlings grown in 0 and 1 μM Zn, a very high level of *ZNT1* expression as well as a considerably larger V_{max} for root Zn^{2+} influx (V_{max} values of 244 and 271) were found in comparison with *T. arvense*. However, when *T. caerulescens* seedlings were grown on 50 μM Zn^{2+} (which is comparable to levels of available Zn^{2+} in soil solution for Zn-contaminated soils), a significant down-regulation in *ZNT1* expression and reduction in root Zn^{2+} uptake were observed (V_{max} was reduced to 76 nmol/g per h and there was a 6-fold reduction in root mRNA abundance). Although growth on 50 μM Zn reduced *ZNT1* expression and Zn uptake in *T. caerulescens*, they were still 4-fold and 2-fold higher, respectively, than in Zn-sufficient *T. arvense*. Thus, it seems that an alteration in the regulation of Zn transport by Zn status, and not a constitutive increase in Zn transporter gene expression, plays a role in Zn hyperaccumulation.

These findings provide insights into the molecular regulation of heavy metal hyperaccumulation in plants. As in *T. arvense*, recent studies with other nonaccumulating plant species have revealed that increased transcription of Zn and Fe transporters is caused by Zn or Fe deficiency (18, 20, 27). However, in *T. caerulescens*, heavy metal hyperaccumulation is correlated with dramatically increased Zn transport and *ZNT1* expression in both roots and shoots. What is not currently understood are the molecular mechanisms by which Zn transporter gene expression is regulated by plant Zn status and how these regulatory mechanisms are altered in *T. caerulescens*. In yeast, where cellular Zn homeostasis is controlled by the regulation of high- and low-affinity Zn^{2+} transporters (ZRT1 and ZRT2), the molecular basis for Zn-dependent regulation of Zn transport only now is beginning to be elucidated (28, 29). Both *ZRT1* and *ZRT2* are members of the *ZIP* transporter gene family and share sequence and structural similarities with *ZNT1*. In yeast, there seems to be an elegant regulatory mechanism that links cellular Zn^{2+} activity as the primary signal, a Zn-responsive transcriptional activator protein, ZAP1, and Zn-responsive elements in the promoters of *ZRT1*, *ZRT2*, and *ZAP1* to regulate cellular Zn levels. A similar Zn-responsive regulatory scheme has not been elucidated in higher plants, but given the response of plant Zn transport genes such as *ZNT1* to changes in plant Zn status, it is likely to exist.

If a Zn-responsive regulatory scheme similar to that in yeast exists in higher plants, how might it be altered to cause the enhanced Zn transporter gene expression and Zn hyperaccumulation observed in *T. caerulescens*? One possibility involves a mutation in a putative Zn-responsive transcriptional activator, which would alter Zn-dependent down-regulation of *ZNT1* expression. Such a mutation in *ZAP1* has been isolated in yeast (28, 29). The semidominant mutant allele, *ZAP1–1*^{up}, results from a substitution of a serine for a cysteine residue in the N-terminal region and causes a high level of expression of the yeast Zn transporters under Zn-replete conditions. Thus, specific alterations in Zn-responsive elements possibly play an important role in heavy metal hyperaccumulation in *T. caerulescens*.

In summary, we present here an integrated molecular and physiological analysis of heavy metal hyperaccumulation in higher plants. In this study, we show that an important component of the Zn hyperaccumulation trait in *T. caerulescens* involves an overexpression of a Zn transporter gene, *ZNT1*, in root and shoot tissue. In *T. caerulescens* roots, it was demonstrated that this increased gene expression is the basis for the increased Zn^{2+} uptake from the soil, and it is likely that the same mechanism underlies the enhanced Zn^{2+} uptake into leaf cells. In the future, it will be important for researchers to elucidate the regulatory components linking plant Zn status to *ZNT1* gene expression and to understand how alterations in this pathway contribute to the heavy metal hyperaccumulation in *T. caerulescens*. By continuing to elucidate the molecular basis for heavy metal transport in this model Zn/Cd hyperaccumulator, researchers should be able to generate the tools that will ultimately allow them to engineer high-biomass metal-hyperaccumulating plants for the purpose of phytoremediation of contaminated soils.

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