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Physiological Characterization of Root Zn^{2+} Absorption and Translocation to Shoots in Zn Hyperaccumulator and Nonaccumulator Species of *Thlaspi*[1]

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Radiotracer techniques were employed to characterize $^{65}Zn^{2+}$ influx into the root symplasm and translocation to the shoot in *Thlaspi caerulescens*, a Zn hyperaccumulator, and *Thlaspi arvense*, a nonaccumulator. A protocol was developed that allowed us to quantify unidirectional $^{65}Zn^{2+}$ influx across the root-cell plasma membrane (20 min of radioactive uptake followed by 15 min of desorption in a 100 μM $ZnCl_2$ + 5 mM $CaCl_2$ solution). Concentration-dependent Zn^{2+} influx in both *Thlaspi* species yielded nonsaturating kinetic curves that could be resolved into linear and saturable components. The linear kinetic component was shown to be cell-wall-bound Zn^{2+} remaining in the root after desorption, and the saturable component was due to Zn^{2+} influx across the root-cell plasma membrane. This saturable component followed Michaelis-Menten kinetics, with similar apparent Michaelis constant values for *T. caerulescens* and *T. arvense* (8 and 6 μM, respectively). However, the maximum initial velocity for Zn^{2+} influx in *T. caerulescens* root cells was 4.5-fold higher than for *T. arvense*, indicating that enhanced absorption into the root is one of the mechanisms involved in Zn hyperaccumulation. After 96 h 10-fold more ^{65}Zn was translocated to the shoot of *T. caerulescens* compared with *T. arvense*. This indicates that transport sites other than entry into the root symplasm are also stimulated in *T. caerulescens*. We suggest that although increased root Zn^{2+} influx is a significant component, transport across the plasma membrane and tonoplast of leaf cells must also be critical sites for Zn hyperaccumulation in *T. caerulescens*.

Recently, there has been an increased interest in the use of plants to decontaminate heavy-metal-polluted soils. In this process, called phytoremediation, higher plants are used to absorb contaminants from the soil into their roots and translocate them to shoots. Pollutants are subsequently removed by harvesting the above-ground tissues. This research area is lacking in basic information regarding the fundamental mechanisms employed by some plants to accumulate heavy metals in shoots.

There is a small number of plant species capable not only of growing in soils containing high levels of heavy metals, but also of accumulating high metal concentrations in shoots. The existence of these plants, designated hyperaccumulators by Brooks et al. (1977), indicates that the genetic potential exists for successful phytoremediation of metal-polluted soils. Such a hyperaccumulator is *Thlaspi caerulescens* J&C Presl, a member of the Brassicaceae family. Certain populations of *T. caerulescens* have been shown to accumulate and tolerate up to 40,000 μg g^{-1} Zn in their shoots (Chaney, 1993). Because for most plants optimal Zn concentration is between 20 and 100 μg g^{-1} (Mengel and Kirkby, 1987), the unique physiology of Zn tolerance and transport in *T. caerulescens* represents a very interesting experimental system for studying mechanisms of metal hyperaccumulation as they relate to phytoremediation.

Both tolerance to elevated Zn in the soil and high Zn accumulation in *T. caerulescens* have been previously reported (Rascio, 1977; Reeves and Brooks, 1983; Reeves and Baker, 1984). More recently, Brown et al. (1995) showed that from a culture solution *T. caerulescens* accumulated more than 25,000 μg g^{-1} Zn in the shoots before any yield reduction was observed. This study, however, provided little information on the mechanisms of uptake and translocation that result in Zn hyperaccumulation in *T. caerulescens*.

The topic of plant Zn transport has not been extensively studied. Several reports have dealt with general aspects of Zn uptake in roots and translocation to shoots (for reviews, see Kochian 1991, 1993). In time-dependent kinetic studies of root Zn absorption in barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.), an initial rapid component of accumulation was observed, followed by a slower linear phase of accumulation (Veltrup, 1978; Santa Maria and Cogliatti, 1988). It was suggested that the initial phase represented rapid entry into root-free space and binding to the cell walls and that the subsequent slower phase was due to transport across the plasma membrane into the cytoplasm (Santa Maria and Cogliatti, 1988). Studies of the concentration-dependent kinetics of root Zn absorption over a 0 to 10 μM concentration range showed that Zn uptake followed Michaelis-Menten kinetics, with K_m values of 3 and 1.5 μM for barley and maize (*Zea mays* L.), respectively (Veltrup, 1978; Mullins and Sommers, 1986).

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Abbreviation: MC, methanol-chloroform.

The existence of this saturable transport component suggests that Zn transport into the cytosol is via a protein-mediated transport system with a fairly high affinity for Zn.

There is currently little basic information concerning mechanisms of Zn uptake in roots and translocation to shoots in hyperaccumulator species at metal concentrations relevant to soils from contaminated sites. In this research, we employed radiotracer flux techniques to characterize root $^{65}Zn^{2+}$ influx and its translocation to the shoot in hydroponically grown seedlings of *T. caerulescens* and a related species, *Thlaspi arvense* L., which is not a Zn hyperaccumulator.

MATERIALS AND METHODS

Seeds of *Thlaspi arvense* were obtained from the Crucifer Genetics Cooperative (University of Wisconsin, Madison). *Thlaspi caerulescens* seeds were obtained from plants growing near a Zn/Cd smelter in Prayon, Belgium (Vázquez et al., 1992). Seeds of *T. arvense* and *T. caerulescens* were surface-sterilized in 0.5% NaOCl and immersed in drops of 0.7% agarose (w/v) placed on a 1-mm^2 nylon mesh. The mesh containing the seeds was floated on deionized water in a 5-L black plastic tub. After 5 d, deionized water was replaced with a nutrient solution containing the following macronutrients (mM): Ca^{2+}, 0.8; K^+, 1.2; Mg^{2+}, 0.2; NH_4^+, 0.1; NO_3^-, 2.0; PO_4^{3-}, 0.1; SO_4^{2-}, 0.2, and micronutrients (μM): BO_3^{2-}, 12.5; Cl^-, 50; Cu^{2+}, 0.5; Fe^{3+}-*N,N'*-ethylenebis[2-(2-hydroxyphenyl)-glycine], 10.0; MoO_4^{2-}, 0.1; Mn^{2+}, 1.0; Ni^{2+}, 0.1; Zn^{2+}, 1.0. The solution was buffered at pH 5.5 with 1 mM Mes-Tris. At this time the nylon mesh was covered with black polyethylene beads to minimize illumination of the growth solution. Seedlings were grown in a growth chamber at 24/15°C (light/dark, 16:8 h) under a light intensity of 300 μmol photons m^{-2} s^{-1}.

Determination of Zn Concentrations in Roots and Shoots of *T. caerulescens* and *T. arvense*

Twenty-two-day-old seedlings grown in normal nutrient medium (containing 1 μM Zn) were transferred to tubs filled with nutrient solution containing 1, 25, 50, or 100 μM Zn^{+2}. After 10 d, plants were harvested and roots were desorbed for 15 min in a solution containing 5 mM Mes-Tris buffer (pH 6.0), 5 mM $CaCl_2$, and 100 μM $CuCl_2$ (to remove apoplasmic Zn^{2+}) and separated from shoots. Shoots and roots were oven-dried at 65°C for 3 d. Dried shoot and root material was ground and a 0.2-g aliquot was digested overnight in 1 mL of double-distilled HNO_3 in a heat block at 120°C. Subsequently, samples were dissolved in 0.75 mL of HNO_3:$HClO_4$ (1:1, v/v) and incubated at 220°C until dry. Samples were then redissolved in 10 mL of 5% HNO_3 and analyzed for elemental composition with a trace analyzer emissions spectrometer (model ICAP 61E, Thermo-Jarrell Ash, Waltham, MA).

Determination of Relative Chlorophyll Content

Seedlings of both *Thlaspi* species were grown as described above for 22 d and then transferred to fresh nutrient medium containing 1, 25, 50, or 100 μM $ZnSO_4$. After 10 d of growth in this nutrient medium, the relative amount of chlorophyll in the older leaves was measured with a chlorophyll meter (model SPAD-502, Minolta, Tokyo, Japan).

Radiotracer ($^{65}Zn^{2+}$) Uptake Experiments

Thirty-one- to 35-d-old seedlings were used in all radiotracer studies. One day prior to the uptake experiment, groups of four seedlings were bundled together and their roots immersed in aerated pretreatment solution consisting of 2 mM Mes-Tris buffer (pH 6.0) and 0.5 mM $CaCl_2$.

Time Course of $^{65}Zn^{2+}$ Desorption from Roots

Bundles of four seedlings were immersed with their roots in a 1-L beaker filled with aerated pretreatment solution. One minute prior to initiation of radioactive uptake, 10 μM $ZnCl_2$ was added to the solution. Radiotracer uptake was initiated by the addition of $^{65}ZnCl_2$ (1.4 μCi L^{-1}). After 20 min, the radioactive uptake solution was replaced with an ice-cold desorption solution containing 5 mM Mes-Tris (pH 6.0), 5 mM $CaCl_2$, and 100 μM $ZnCl_2$. At specific time intervals, one bundle of each *Thlaspi* species was harvested, and its roots excised. The roots were then blotted to remove adhering solution and weighed. Root radioactivity was measured using a gamma counter (model 5530, Packard Instruments, Downers Grove, IL). Following removal of each pair of bundles, the desorption solution was vacuum-withdrawn and replaced with fresh desorption solution.

Effect of Desorption on $^{65}Zn^{2+}$ Accumulation in Intact Roots and Root-Cell-Wall Preparations of *T. caerulescens*

To study the efficacy of our desorption procedure in removing cell-wall-bound Zn^{2+}, time-course studies for the accumulation of $^{65}Zn^{2+}$ were conducted with either roots of intact *T. caerulescens* seedlings or with morphologically intact root-cell-wall preparations. These cell-wall preparations were obtained by immersing roots of intact *T. caerulescens* seedlings in a MC (2:1, v/v) solution for 3 d. We have previously shown in maize (*Zea mays* L.) roots that this treatment yields lipid-free root-cell-wall preparations that generally maintain the same shape and size of an intact root (Hart et al., 1992). Subsequent to the MC treatment, the root-cell-wall preparations were washed in a number of changes of deionized water for 12 h. Roots of either intact or MC-treated seedlings were incubated in radiolabeled uptake solution (10 μM $^{65}Zn^{2+}$, specific activity 1.4 μCi L^{-1}, 0.5 mM $CaCl_2$, and 2 mM Mes-Tris, pH 6.0) for time periods between 2 and 45 min, and then either briefly rinsed in deionized water (undesorbed) or desorbed in 1 L of desorption solution for 15 min. Subsequently, roots or root-cell-wall preparations were excised, blotted, and weighed, and ^{65}Zn was quantified via gamma detection.

Concentration-Dependent Kinetics of $^{65}Zn^{2+}$ Influx

Roots of four intact *T. caerulescens* or *T. arvense* seedlings were immersed in 80 mL of pretreatment solution (2 mM

Mes-Tris, pH 6.0, and 0.5 mM $CaCl_2$) in individual Plexiglas wells of an uptake apparatus (Hart et al., 1992). Subsequently, Zn 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 of $^{65}ZnCl_2$. After a 20-min uptake period, radioactive solutions were vacuum-withdrawn and wells were refilled with ice-cold desorption solution. Following a 15-min desorption period, seedlings were harvested and their roots excised, blotted, and weighed, and ^{65}Zn was quantified via gamma detection.

Long-Term Time Course of $^{65}Zn^{2+}$ Accumulation in *T. caerulescens* and *T. arvense* Roots and Shoots

Roots of four seedlings of the two *Thlaspi* species were immersed in 1 L of aerated uptake solution containing 10 μM $^{65}Zn^{2+}$ (1.4 μCi). At different times, one bundle of each species was harvested and the roots were desorbed for 15 min and then separated from shoots. The excised roots were blotted, roots and shoots were weighed, and ^{65}Zn was quantified via gamma detection.

RESULTS

After 10 d of growth in solutions containing different Zn^{2+} concentrations (1–100 μM), *T. arvense* accumulated more Zn^{2+} in roots, whereas most of the Zn was translocated to the shoot in *T. caerulescens*. From the growth solution containing 100 μM Zn^{2+}, *T. caerulescens* shoots accumulated 4.6-fold more Zn compared with *T. arvense*, whereas 1.9-fold more Zn accumulated in roots of *T. arvense* than in *T. caerulescens* (Table I). The enhanced Zn translocation did not affect the chlorophyll content of *T. caerulescens* shoots. In contrast, lower Zn concentrations in *T. arvense* shoots resulted in dramatic leaf chlorosis, as indicated by a significant reduction in relative chlorophyll content (Table I). Aside from chlorosis, no significant differences were observed between *T. arvense* plants and *T. caerulescens* plants grown at 1 or 100 μM Zn^{2+} (data not shown).

In uptake experiments, binding to the cell wall can confound the estimation of Zn^{2+} transport into the cytosol. To develop an experimental protocol for measuring influx into the root symplasm, desorption of Zn^{2+} from intact *T. caerulescens* and *T. arvense* roots was monitored over time following a 20-min exposure to uptake solution containing 10 μM radioactive Zn. Desorption curves were similar for the two *Thlaspi* species (Fig. 1). At the end of a 45-min desorption period in a solution containing high levels of nonlabeled Zn^{2+} (100 μM) and Ca^{2+} (5 mM), 85% of the $^{65}Zn^{2+}$ accumulated during the uptake period was desorbed into the external solution. Zn release into the external solution was initially very rapid, and was followed by a slower phase of desorption. A majority of the Zn desorption (95%) occurred during the first 15 min.

The efficacy of the desorption regime at removing cell-wall Zn^{2+} was tested further by investigating the time course of Zn accumulation in living roots and lipid-free, morphologically intact root-cell-wall extracts prepared by immersing intact roots in MC for 3 d (Fig. 2). In intact, undesorbed roots Zn^{2+} accumulation was biphasic (Fig. 2A). The initial rapid phase (first 5 min of accumulation) presumably was due to diffusion into the free space and to binding to root-cell walls. The slower linear phase of accumulation over the subsequent 40 min is believed to represent transport across the plasma membrane. In desorbed, intact *T. caerulescens* roots Zn^{2+} accumulation was linear and passed near the origin; the slope of this line was similar to that of the slower phase for accumulation in undesorbed roots (Fig. 2A). These results provide circumstantial evidence that the 15-min desorption period primarily removes the initial rapid phase representing cell-wall-bound Zn^{2+}. Zn accumulation in undesorbed root-cell walls was linear (Fig. 2B) and exceeded Zn^{2+} accumulation in undesorbed, intact roots (Fig. 2A). The 15-min desorption treatment removed most of this accumulated Zn, leaving a small linear accumulation. Thus, it appears that a significant proportion of the root apoplasmic Zn was removed by desorption. Consequently, in the subsequent time- and concentration-dependent experiments, a 15-min desorption regime was employed following radioactive uptake to remove most of the root-cell-wall ^{65}Zn and to more accurately quantify Zn transport into the root symplasm.

Accumulation of Zn^{2+} was significantly different in desorbed intact roots of the two *Thlaspi* species. After a 3-h absorption period, 2.2-fold more Zn^{2+} accumulated in the roots of *T. caerulescens* compared with *T. arvense* (Fig. 3).

Table I. *Zn accumulation and relative chlorophyll content in T. arvense and T. caerulescens seedlings exposed for 10 d to different Zn^{2+} levels*

Tubs containing 22-d-old seedlings grown on nutrient medium containing 1 μM Zn were refilled with nutrient solution containing 1, 25, 50, or 100 μM Zn. After 20 d of growth, Zn concentration in roots and shoots was determined by emissions spectrometry. Relative chlorophyll content was determined at harvest using a chlorophyll meter. The results are presented as means ± SE (n = 8–27).

Zn Concentration	*T. arvense*			*T. caerulescens*		
	Roots	Shoots	Chl[a]	Roots	Shoots	Chl
μM	μmol Zn^{2+} g^{-1} dry wt		%	μmol Zn^{2+} g^{-1} dry wt		%
1	—[b]	3	100	—	10	100
25	54 ± 6	12 ± 2	53 ± 8	19 ± 1	20 ± 3	98 ± 12
50	54 ± 8	14 ± 2	32 ± 5	25 ± 3	40 ± 5	102 ± 8
100	90 ± 11	15 ± 3	18 ± 4	48 ± 8	70 ± 3	104 ± 8

[a] Chl, Relative chlorophyll content. [b] —, No data.

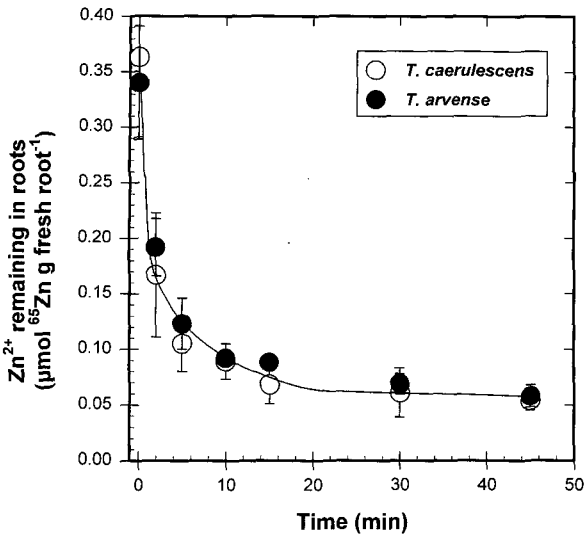

Figure 1. Time course of Zn^{2+} desorption from *T. arvense* and *T. caerulescens* roots. Roots were immersed in a solution containing 2 mM Mes-Tris (pH 6.0), 0.5 mM $CaCl_2$, and 10 μM $^{65}Zn^{2+}$ (1.4 μCi/L). After 20 min, the radioactive uptake solution was replaced with ice-cold desorption solution containing 5 mM Mes-Tris (pH 6.0), 5 mM $CaCl_2$, and 100 μM $ZnCl_2$. At different time intervals, one bundle of each *Thlaspi* species was harvested, the roots were excised, blotted, and weighed, and the gamma activity was counted. Data points and error bars represent means ± SE of four replicates.

During the time course of this experiment, no ^{65}Zn was translocated from roots to shoots (data not shown).

Concentration-dependent uptake kinetics for $^{65}Zn^{2+}$ influx were characterized by a nonsaturating curve that approached linearity at external concentrations above 20 μM (Fig. 4A). This uptake curve was graphically resolved into a saturable and a linear component (Fig. 4B). The apparent K_m values (derived from Lineweaver-Burk data transformations) for the saturable components were 8 and 6 μM for *T. caerulescens* and *T. arvense*, respectively. The V_{max} values were 270 and 60 nmol Zn^{2+} g^{-1} fresh weight h^{-1} for *T. caerulescens* and *T. arvense*, respectively, indicating that Zn^{2+} influx in *T. caerulescens* roots was more than 4-fold higher compared with influx into *T. arvense* roots. The first-order rate coefficients for the linear components were 3.57 and 3.15 nmol Zn^{2+} g^{-1} fresh weight h^{-1} μM^{-1} for *T. arvense* and *T. caerulescens*, respectively. When the same 20-min exposure to radiolabeled uptake solution followed by 15 min of desorption was employed to look at the concentration-dependent kinetics for ^{65}Zn binding in MC-treated roots, $^{65}Zn^{2+}$ binding was linear (Fig. 5), with a slope nearly identical to that of the linear component for desorbed, intact roots (cf. Figs. 4B and 5).

Long-term time-course studies of $^{65}Zn^{2+}$ accumulation in roots and translocation to shoots showed that there were dramatic differences in Zn partitioning between the two *Thlaspi* species. At the end of a 96-h uptake period, 29% more radioactive Zn accumulated in roots of *T. arvense* compared with *T. caerulescens*. In contrast, Zn translocation to the shoot was approximately 10-fold greater in *T. caerulescens* compared with *T. arvense* (Fig. 6).

DISCUSSION

Quantification of $^{65}Zn^{2+}$ Influx into the Root Symplasm

The development and evolution of phytoremediation into an effective technology for the cleanup of heavy-metal-polluted soils requires an understanding of the basic mechanisms for absorption of metals into the roots and subsequent translocation to shoots. *T. caerulescens*, which can accumulate Zn in shoots to concentrations exceeding 4% dry weight (Chaney et al., 1993), represents an intriguing model system for studying mechanisms of metal hyperaccumulation. These mechanisms probably involve transport regulation at several different sites within the plant, including influx across root-cell plasma membrane, transport within the root symplasm, unloading into the xylem, long-distance translocation in the xylem (and possibly phloem), transport across the leaf-cell plasma membrane, and storage in the leaf-cell vacuole. In this study we have conducted, to our knowledge, the first physiological characterization of root Zn influx and translocation in *T. caerulescens* compared with *T. arvense*, a related nonaccumulator species.

To quantify Zn influx across the root-cell plasma membrane (the entry point for Zn accumulation in the plant) and translocation to shoots, we conducted radiotracer stud-

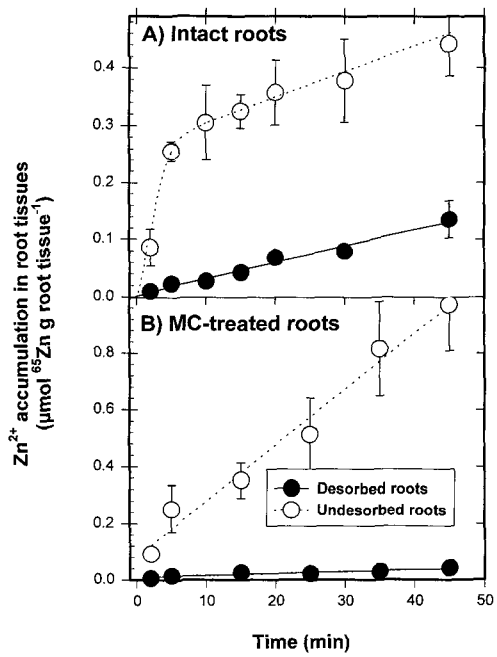

Figure 2. Effect of desorption on Zn^{2+} accumulation in intact (A) and MC-treated (B) *T. caerulescens* roots. Bundles of four intact seedlings or MC-treated roots were exposed to the uptake solution containing 2 mM Mes-Tris (pH 6.0), 0.5 mM $CaCl_2$, and 10 μM $^{65}Zn^{2+}$ (1.4 μCi/L). At the end of the incubation periods shown, roots were either rinsed with deionized water, excised, blotted, and weighed, and the gamma activity counted, or they were desorbed for 15 min in 5 mM Mes-Tris (pH 6.0), 5 mM $CaCl_2$, and 100 μM $ZnCl_2$ before being separated from the shoots, blotted, and weighed, and their gamma activity counted. Data points and error bars represent means ± SE of four replicates.

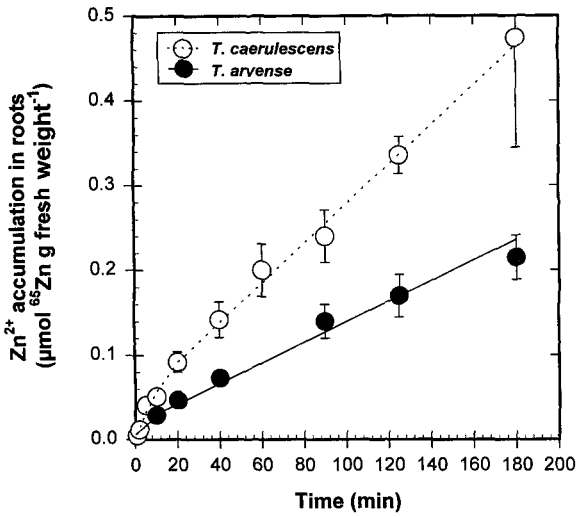

Figure 3. Time course of Zn^{2+} accumulation in *T. arvense* and *T. caerulescens* roots. Bundles of four seedlings were immersed with roots in uptake solution containing 2 mM Mes-Tris (pH 6.0), 0.5 mM $CaCl_2$, and 10 µM $^{65}Zn^{2+}$ (1.4 µCi/L). Following the appropriate incubation period, roots were desorbed for 15 min in a 5 mM Mes-Tris (pH 6.0), 5 mM $CaCl_2$, and 100 µM $ZnCl_2$ solution. Following desorption, roots were separated from shoots, blotted, and weighed, and their gamma activity was counted. Data points and error bars represent means ± SE of four replicates.

ies using $^{65}Zn^{2+}$. A major difficulty with this method is separating true uptake (Zn transported across the plasma membrane into the cytosol) from Zn^{2+} bound to negatively charged sites associated with the cell wall. Binding of $^{65}Zn^{2+}$ to the cell wall can represent a substantial fraction of the metal accumulated in roots (Marschner, 1995). The confounding effect of cell wall binding can be eliminated to some degree by desorbing cell-wall-bound $^{65}Zn^{2+}$ from roots following radioactive treatment. After a 20-min exposure to a radiolabeled uptake solution, 15 min of incubation in a desorption solution containing 100 µM nonradioactive $ZnCl_2$ and 5 mM $CaCl_2$ caused a loss of 85% of the accumulated radioactive Zn from intact roots. Little additional Zn^{2+} was removed from roots by desorption periods longer than 15 min (Fig. 1).

To correctly interpret our experimental results, it is important to determine the source of the Zn^{2+} removed during desorption. Desorbed Zn^{2+} could have originated from the cell walls and/or from the cytosol via efflux across the plasma membrane back into the external solution. In compartmentation studies with *T. aestivum*, half-times for Zn^{2+} efflux from the cell wall and cytoplasm were estimated to be 3.6 and 36 min, respectively (Santa Maria and Cogliatti, 1988). If time constants for Zn^{2+} efflux are similar for wheat and *T. caerulescens*, our results suggest that most of the Zn removed during the 15-min desorption period originated from the cell wall. Furthermore, because all higher plant cells maintain a large, negative-inside electropotential across the root-cell plasma membrane, Zn^{2+} efflux must be an active, metabolically dependent process. Consequently, to reduce the active efflux of Zn^{2+} from the cytoplasm the desorption solution was maintained at 2°C. Based on our experimental results and on these theoretical considerations, we conclude that most of the desorbed ^{65}Zn originated from the root-cell walls.

Studies of the accumulation of several different cations in roots have demonstrated that time-dependent cation uptake kinetics are biphasic, with an initial rapid component followed by a slower, linear phase of uptake (Veltrup, 1978; Körner et al., 1986; Zhang and Taylor, 1989; Hart et al., 1992). The rapid component has generally been interpreted to represent accumulation in the apoplasm, whereas the slower, linear phase is thought to be due to transport across the plasma membrane. The time-dependent kinetics for $^{65}Zn^{2+}$ uptake in undesorbed intact *T. caerulescens* roots could also be resolved into two components (Fig. 2A). With root-cell-wall preparations (MC-treated roots), however, accumulation (binding) was monophasic and rapid, and

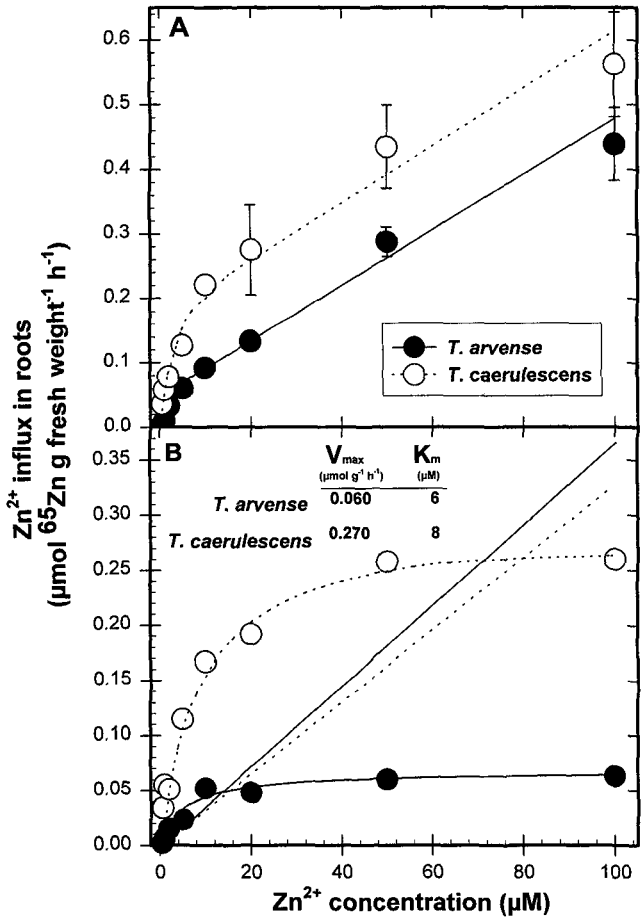

Figure 4. Concentration-dependent kinetics of Zn^{2+} uptake into roots of intact *T. arvense* and *T. caerulescens* seedlings. Roots were immersed in a solution containing 2 mM Mes-Tris (pH 6.0), 0.5 mM $CaCl_2$, and $^{65}Zn^{2+}$ (1.4 µCi/L) at the concentrations shown. Following a 20-min uptake period, roots were desorbed in an ice-cold solution containing 5 mM Mes-Tris (pH 6.0), 5 mM $CaCl_2$, and 100 µM $ZnCl_2$ for 15 min. Roots were then excised, blotted, and weighed, and their gamma activity was counted. A, Overall kinetic curve for Zn^{2+} influx. Data points and error bars represent means ± SE of four replicates. B, Resolution of overall kinetic curves into saturable and linear components.

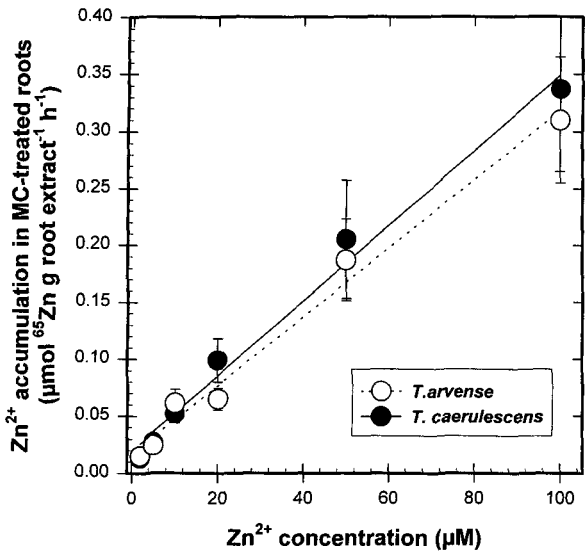

Figure 5. Concentration-dependent kinetics of Zn^{2+} binding in MC-treated roots of *T. arvense* and *T. caerulescens*. MC-treated roots were immersed in a solution containing 2 mM Mes-Tris (pH 6.0), 0.5 mM $CaCl_2$, and $^{65}Zn^{2+}$ (1.4 μCi/L) at the concentrations shown. Following a 20-min uptake period, roots were desorbed for 15 min, excised, blotted, and weighed, and their gamma activity was counted. Data points and error bars represent means ± SE of four replicates.

increased linearly for up to 45 min; no evidence for a second, slower component of accumulation was observed. These results support the hypothesis that in intact roots, the slower second phase represents Zn accumulation in the root symplasm.

At the end of a 45-min uptake period, significantly more ^{65}Zn accumulated in undesorbed root-cell-wall preparations of *T. caerulescens* compared with undesorbed intact roots (Fig. 2). Presumably, this increased accumulation in undesorbed MC-treated roots is due to movement of uptake solution into root regions normally bound by the plasma membrane, with subsequent binding of Zn^{2+} to normally unavailable and now exposed cell-wall sites. In contrast, less Zn^{2+} accumulated in desorbed MC-treated roots compared with desorbed intact roots. Because in the root-cell-wall preparations all of the $^{65}Zn^{2+}$ was apoplasmically bound, most of this adsorbed Zn was easily removed by desorption.

After a 180-min uptake period followed by the 15-min desorption period, *T. caerulescens* roots accumulated twice as much $^{65}Zn^{2+}$ as those of *T. arvense* (Fig. 3). For both species, Zn^{2+} uptake during the first 10 min was characterized by a rapid linear phase, whereas uptake during the subsequent 170 min was represented by a second, slower linear phase. We interpret the initial rapid component to represent cell-wall-associated Zn^{2+} not removed by the desorption treatment. The existence of a tightly bound cell-wall fraction that is difficult to desorb has been previously demonstrated for Ca^{2+} (Spanswick and Williams, 1965) and Zn^{2+} (Peterson, 1969). Our results indicate that the slower linear phase of accumulation that dominates the uptake curve at exposures longer than 10 min is primarily due to Zn^{2+} transport into the symplasm, with a minor component due to undesorbed cell-wall ^{65}Zn. Because the rate of uptake was linear from 10 to 180 min, we chose a 20-min uptake period to investigate concentration-dependent accumulation of ^{65}Zn in roots. This relatively short uptake period allowed us to investigate uptake while minimizing the possibility of ^{65}Zn efflux across the plasma membrane back into the external solution.

Based on the results presented in Figures 1 to 3, a 20-min radioactive uptake followed by a 15-min desorption was used to quantify $^{65}Zn^{2+}$ uptake, and primarily reflected unidirectional Zn influx across the root-cell plasma membrane.

To simulate Zn levels in the soil solution from a Zn-contaminated site, concentration-dependent studies of $^{65}Zn^{2+}$ influx were conducted from uptake solutions with metal levels ranging up to 100 μM. In both species, uptake kinetics were characterized by smooth nonsaturating curves (Fig. 4A) that could be graphically resolved into saturable and linear components (Fig. 4B). In studies of putrescine (DiTomaso et al., 1992) and paraquat (Hart et al., 1992) influx into maize roots, which yielded similar nonsaturating transport kinetics, the linear component was demonstrated to represent cell-wall-bound radiolabel remaining after desorption. We also hypothesized that the linear component for Zn^{2+} influx shown in Figure 4B rep-

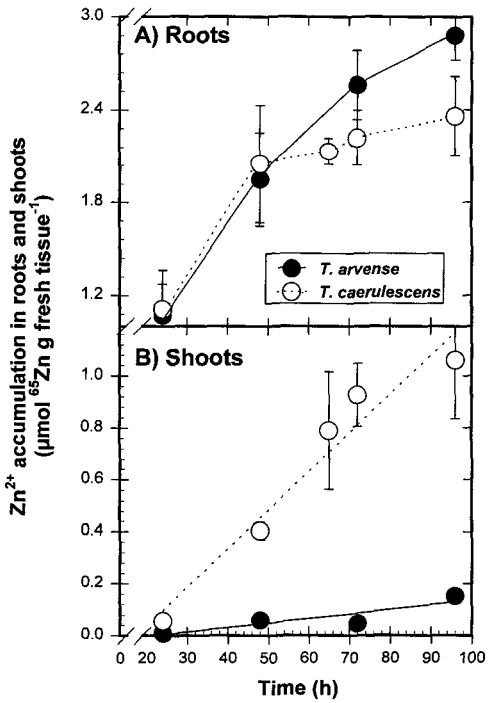

Figure 6. Long-term time course of Zn^{2+} accumulation in roots (A) and shoots (B) of *T. arvense* and *T. caerulescens*. Roots of intact seedlings were immersed in a 2 mM Mes-Tris (pH 6.0), 0.5 mM $CaCl_2$, and 10 μM $^{65}Zn^{2+}$ (1.4 μCi/L) solution. Following the incubation periods shown, roots were desorbed in an ice-cold solution containing 5 mM Mes-Tris (pH 6.0), 5 mM $CaCl_2$, and 100 μM $ZnCl_2$ for 15 min. Roots were then excised and blotted, and both roots and shoots were weighed and their gamma activity was counted. Data points and error bars represent means ± SE of four replicates.

resents cell-wall-bound $^{65}Zn^{2+}$ remaining after desorption. To test this hypothesis, we measured concentration-dependent $^{65}Zn^{2+}$ binding in root-cell-wall preparations (MC-treated roots) at the end of a 20-min exposure to radiolabeled Zn, followed by a 15-min desorption. In these root preparations, the concentration-dependent kinetics for Zn^{2+} binding was linear, with no evidence for a saturable component (Fig. 5). This linear component was almost identical to the linear component for Zn^{2+} influx in intact roots (Fig. 4B), providing evidence that the latter represents cell-wall-bound ^{65}Zn not removed by desorption.

Zn^{2+} Transport Properties in *T. arvense* and *T. caerulescens*

The kinetics of Zn uptake into root cells of *T. caerulescens* and *T. arvense* were dominated by a single saturable component (Fig. 4B). Whereas the apparent K_m values were not significantly different, the V_{max} for Zn^{2+} influx was 4.5-fold greater in *T caerulescens* compared with *T. arvense*. These results suggest that in both *Thlaspi* species, transport across the plasma membrane is mediated by proteins with similar Zn^{2+} affinities, but that the capacity for influx is much greater in *T. caerulescens* roots. A significantly larger V_{max} value suggests that there is a higher density of Zn transporters per unit membrane area in *T. caerulescens* roots. A stimulation of Zn transport at the root cell plasma membrane of *T. caerulescens* is consistent with the observation that this species has a higher requirement for Zn (Brown et al., 1995). It should be mentioned that in this study Zn uptake was investigated over a concentration range up to 100 μM. However, *T. caerulescens* is known to accumulate Zn from solutions containing as much as 3 mM Zn without signs of toxicity (Brown et al., 1995). This raises the possibility that a different transport system with lower Zn affinity may operate at higher Zn concentrations.

Results presented here indicate that a characteristic of the hyperaccumulator *T. caerulescens* is enhanced Zn influx into the root symplasm. However, enhanced transport of Zn into root cells may not necessarily be associated with greater translocation to shoots. For example, we previously observed that although paraquat (a divalent cation) accumulation was not reduced in roots of an herbicide-resistant wild barley (*Hordeum glaucum* Steud.) biotype, its translocation to shoots was negligible (M.M. Lasat, unpublished results). Furthermore, we found that the herbicide was sequestered in the root vacuole of the resistant biotype and was therefore unavailable for long-distance transport.

In the current investigation, long-term accumulation studies from solutions containing 10 μM $^{65}Zn^{2+}$ (Fig. 6) or 25, 50, and 100 μM nonradioactive Zn (Table I) demonstrated that considerably more Zn accumulated in *T. arvense* roots compared with those of *T. caerulescens*. These results are somewhat unexpected because both short-term (Fig. 3) and concentration-dependent uptake experiments (Fig. 4) indicate that there was a greater Zn influx into the *T. caerulescens* root symplasm compared with *T. arvense*. During the time course of these experiments, no Zn was translocated from the root to shoot. In long-term studies, however, uptake into the plant was influenced by both shoot and root transport processes. The Zn^{2+} accumulation data depicted in Figure 6 indicate a dramatic difference in Zn partitioning between the two *Thlaspi* species. At the end of a 96-h uptake period, *T. arvense* retained more Zn in the roots than *T. caerulescens*, whereas the hyperaccumulator translocated considerably more Zn to the shoots. Similarly, long-term exposure to high Zn^{2+} concentrations resulted in greater metal accumulation in the shoots of *T. caerulescens* compared with *T. arvense* (Table I). These results indicate that Zn transport sites other than influx into the root are also altered in *T. caerulescens* and contribute to the dramatic increase in translocation to the shoot.

It is very likely that a second important transport site for Zn hyperaccumulation (in addition to influx into the root symplasm) is reabsorption of xylem-borne Zn into leaf cells and storage in the vacuole. This is supported by results from x-ray microanalysis of shoot tissue, which indicated that *T. caerulescens* prevents toxic Zn concentrations in the cytoplasm by sequestering the metal in the vacuoles of epidermal and subepidermal leaf cells as crystals of uncertain structure and chemical composition (Vázquez et al., 1992, 1994). Also, Ernst (1968) and Mathys (1977) proposed that excess Zn in the leaf-cell cytoplasm could be bound by malate and shuttled into the vacuole. Along these lines, Thurman and Rankin (1982) have shown that exposure to high Zn induced greater levels of organic acids in Zn-tolerant but not in nontolerant *Deschampia caespitosa* clones. To date, however, the mechanisms of Zn transport across the plasma membrane and tonoplast in *T. caerulescens* leaves have not been studied.

Results presented in this paper indicate that there is a complex alteration of Zn transport processes in *T. caerulescens*, resulting in Zn hyperaccumulation. Although enhanced influx into the root symplasm is a significant aspect of hyperaccumulation, it cannot entirely account for the enhanced Zn translocation to the shoot. It is likely that plasma membrane and tonoplast Zn transport in leaf cells are also critical sites for Zn^{2+} hyperaccumulation in *T. caerulescens*, and they will therefore be the focus of future research in our laboratory.

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