Biometals (2011) 24:51–58
DOI 10.1007/s10534-010-9373-6

Characterization of zinc transport by divalent metal transporters of the ZIP family from the model legume *Medicago truncatula*

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Received: 16 July 2010 / Accepted: 31 August 2010 / Published online: 16 September 2010

Abstract To understand how plants from the Fabaceae family maintain zinc (Zn) homeostasis, we have characterized the kinetics of three Zn transporting proteins from the ZIP family of divalent metal transporters in the model legume *Medicago truncatula*. Of six ZIP's studied, MtZIP1, MtZIP5 and MtZIP6 were the only members from this family determined to transport Zn and were further characterized. MtZIP1 has a low affinity for Zn with a K_m of 1 μM as compared to MtZIP5 and MtZIP6 that have a higher affinity for Zn with K_m of 0.4 μM and 0.3 μM, respectively. Zn transport by MtZIP1 was more sensitive to inhibition by copper (Cu) concentrations than MtZIP5 and MtZIP6, because 3 μM Cu inhibited Zn transport by 80% in MtZIP1 while 5 μM Cu was required to achieve the same inhibition of Zn transport in MtZIP5 and MtZIP6. Cadmium (Cd) had a greater effect on the ability of MtZIP1 to transport Zn than MtZIP5 and MtZIP6, because at a concentration of 3 μM Cd, the Zn transport by MtZIP1 was inhibited 55% and the transport of Zn by MtZIP5 and MtZIP6 was inhibited by 20–30%. However, only MtZIP6 transported Cd at higher rates than those observed in the control plasmid pFL61, demonstrating a low affinity for Cd based on a K_m of 57 μM. These results suggest that *Medicago truncatula* has both high and low affinity Zn transporters to maintain Zn homeostasis and that these transporters may function in different compartments within the plant.

Keywords Zinc · Membrane transport · Kinetics · Cadmium · Copper · Medicago truncatula

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Introduction

Zinc is an essential divalent metal required by plants that is obtained from the soil and redistributed throughout the plant to maintain optimal growth (Broadley et al. 2007; Hacisalihoglu et al. 2001). The ability of plants to maintain Zn homeostasis is extremely important due to the participation of Zn in many diverse and essential processes (Coleman 1992; McCall et al. 2000; Vallee and Auld 1990). These processes are inhibited if an organism is unable to acquire sufficient Zn; however, Zn can be detrimental to organisms when present in excess. Therefore, Zn concentration must be maintained by Zn transporters that are responsible for uptake, efflux and compartmentalization within the plant.

The function of Zn in biological processes, including those of plants, is that it serves a structural role within proteins, where it acts as a "borderline" Lewis acid that can accept electrons from several amino acids including histidine, aspartate, glutamate, cysteine, serine, threonine, and tyrosine (Lipscomb and Strater 1996). These Zn-protein interactions can be divided into two functions, one that serves a catalytic role and a second that serves a structural role. The catalytic role of Zn in proteins is its ability to form a tridentate active site coordinating with three amino acid residues, such that the remaining site of the Zn atom can be filled with a substrate to complete the coordination sphere of the active site (Lipscomb and Strater 1996; Vallee and Auld 1990). The structural role of Zn in many proteins is its ability to form stable tetrahedral complexes, most commonly with the side chains of cysteine and histidine and occasionally with aspartate and glutamate (Berg and Shi 1996; Coleman 1992; Lipscomb and Strater 1996), such as in the Zn finger proteins that are involved in the regulation of gene expression (McCall et al. 2000). Zn is important to these types of processes because it does not change redox state, making it more stable than several other divalent metals.

In many organisms, including plants, there are several different transporters that maintain Zn homeostasis; these include transporters from several families including ZIPs (Eng et al. 1998), CDFs (Haney et al. 2005), NRAMPs (Cellier et al. 1995), and heavy metal ATPases (Hall and Williams 2003). A majority of these transporters have affinity for multiple divalent metals as substrates for transport. One of these families is the divalent metal ZIP transporters (ZRT-, IRT-like proteins), named for their sequence similarity to the Zn regulated transporters (ZRT) and Fe regulated transporters (IRT) from yeast (Eide et al. 1996; Zhao and Eide 1996a, 1996b). The family consists of over 50 identified members from several taxonomic groups that include the agriculturally important crop families: Brassicaceae, Solanaceae, Fabaceae, and Poaceae. These proteins are classified into two subfamilies, one of which has similarity to the ZIP's identified from humans and a second that is composed primarily of ZIP's from plants (Guerinot 2000). These membrane-localized transporters have 7–8 transmembrane domains with a variable region that contains several histidine residues that are thought to bind the divalent metal substrate and facilitate transport. Although, most ZIP transporters appear to be localized to the plasma membrane, several have amino terminal signal peptides that predict they could be localized to other membranes (Grotz et al. 1998; López-Millán et al. 2004; Moreau et al. 2002).

To better understand the role of the ZIP family of divalent metal transporters in Zn homeostasis, we have characterized the biochemical transport properties of several members of this family in *Medicago truncatula*. We have determined the kinetic constants for Zn transport and have analyzed the effects of other divalent metals (Cd and Cu) on Zn transport rates of the *Medicago* ZIPs. This information provides additional knowledge that in combination with expression, localization, and functional data will lead to a better understanding of how these genes and their products interact to maintain Zn homeostasis in *Medicago truncatula*.

Materials and methods

Yeast strain and culture conditions

The *Saccharomyces cerevisiae* strain ZHY3 (MATα ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2 zrt2::HIS3) that has loss of function of both the high affinity (ZRT1) and low affinity (ZRT2) transporters for Zn (Zhao and Eide 1996b) was used to examine Zn transport of ZIP proteins from *Medicago truncatula*. Yeast cells were transformed with ZIP transporter cDNAs [(MtZIP1 (Genbank Acc. No. AY339054), MtZIP3 (AY339055), MtZIP4 (AY339056), MtZIP5 (AY339057), MtZIP6 (AY339058), and MtZIP7 (AY339059)] using the yeast expression vector pFL61 (Minet et al. 1992) as described previously (DiDonato et al. 2004; López-Millán et al. 2004). MtZIP2 (Burleigh et al. 2003) was not included in this study because we did not have the necessary clone. Yeast cells were transformed utilizing the lithium chloride method (Gietz and Schiestl 1991). Yeast cells were grown in a synthetic defined Dropout Base (DOB) culture media (MP Biomedicals, Solon, OH, USA) containing ammonium sulfate and 2% dextrose and the amino acid supplement (CSM) containing all auxotrophic amino acids required for growth, with the exception of uracil (MP Biomedicals). Yeast cells were incubated on a

rotary shaker at 30°C @ 275 RPM until they reached late log phase growth ($A_{600} = 0.8$).

Zn and Cd uptake studies

Yeast cells growing exponentially were centrifuged at 250 × *g* for 4 min at 4°C and washed three times with ice-cold assay Buffer A [10 mM MES (pH 6.1), 250 μM $CaCl_2$ and 2% glucose] before being resuspended in an equivalent volume of assay buffer relative to culture volume. Cells were kept on ice until just prior to use. Yeast cells were then transferred to a rotary shaker at 30°C @ 275 RPM for 15 min prior to beginning uptake studies. Zn assay solutions (Buffer B) for kinetic studies were made by the addition of 0.2 μCi ^{65}Zn as $ZnCl_2$; (Oak Ridge National Laboratory; Oak Ridge, TN) per milliliter of Buffer A and Zn concentrations for each assay buffer were adjusted by the addition of $ZnSO_4$ to Buffer B, yielding concentrations ranging from 0.5 to 10 μM. Complementation assays were performed in Buffer B with a Zn concentration of 10 μM. Inhibition studies were performed with Buffer B and a Zn concentration of 1 μM for MtZIP1 and 0.5 μM for MtZIP5 and MtZIP6, plus Cu at one of the following concentrations (1, 2, 4, 6, or 10 μM) or Cd at one of the following concentrations (0.5, 1, 2, or 3 μM). Cd uptake solutions (Buffer D) were made by the addition of 0.2 μCi ^{109}Cd (GE Healthcare; Piscataway, NJ) per milliliter of Buffer A. Cd kinetic analysis assays were performed in Buffer D with the following $CdSO_4$ concentrations (1, 2, 5, 10, 50, or 100 μM).

Uptake assays were performed by adding 500 μl of each assay buffer to 500 μl of cell suspensions and reactions were incubated at 30°C for 3 min. The reactions were terminated after 3 min by vacuum filtration through Whatman GF/C filters, and washed with 10 ml of ice-cold SSW buffer pH 4.1 (100 mM citrate, 25 mM $MgSO_4$, 5 mM EDTA, 5 mM KH_2PO_4, 5 mM $CaCl_2$, 1 mM $NaCl_2$) (Eide et al. 1992). Radioactivity associated with yeast cells and samples for determining specific activity were quantified via gamma detection using a PerkinElmer 1480 Wallac Wizard 3' automatic gamma counter (Waltham, MA). Specific activity was determined by quantifying the activity in a 500 μl aliquot of each assay buffer and determining the cpm (counts per minute) of ^{65}Zn or ^{109}Cd. The cpm to μmol ratio was subsequently used to convert cpm to μmol for rate determinations. Non-specific binding and transport was determined by transforming ZHY3 with pFL61 plasmid and performing uptake assays. The actual uptake rates of the ZIP transporters were determined by subtracting the uptake rates of yeast transformed with the control plasmid from yeast transformed with plasmids containing the ZIP genes.

Results

The Zn transport capacity of the ZIP family members was determined by expressing the transporters in the yeast mutant ZHY3 that lacks both high and low affinity Zn transport systems (Zhao and Eide 1996b). These transporters were examined by following the uptake of ^{65}Zn in a liquid assay culture system at a Zn concentration maintained at 10 μM. MtZIP1, MtZIP5 and MtZIP6 were the only transporters from the six ZIP family members studied that had the capacity to transport Zn, relative to the pFL61 vector control (Fig. 1), with MtZIP1 showing the highest transport rate. Therefore, these three transporters were chosen for further analysis to examine their biochemical characteristics in metal transport.

The three MtZIP transporters showed saturating kinetics for Zn, with MTZIP1 (Fig. 2a) requiring higher Zn concentrations to reach saturation than either MtZIP5 or MtZIP6 (Fig. 2b). Because the transporters showed saturating kinetics, the kinetic

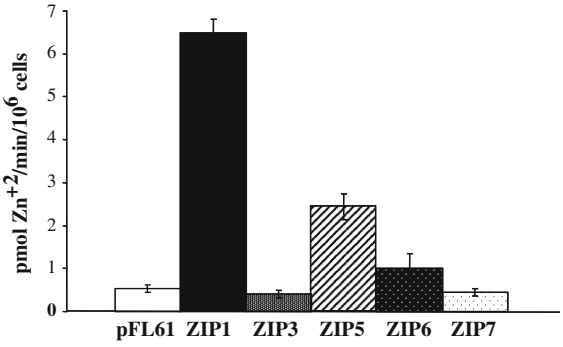

Fig. 1 Zn uptake capacity of the ZIP transporters from *Medicago truncatula*. Transporters were cloned into the expression vector pFL61 and transformed into the yeast strain ScZHY3 that lacks both the high and low affinity Zn transporters ZRT1 and ZRT2, respectively. Zn transport was measured at 10 μM Zn

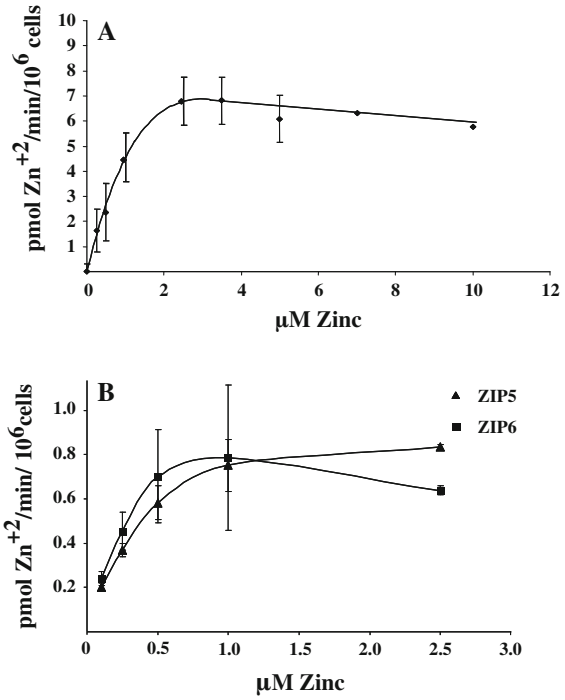

Fig. 2 Zn transport rates for MtZIP1, MtZIP5, and MtZIP6 were determined by examining the uptake rates of Zn at varying Zn concentrations. The Zn levels were varied between 0.25 and 10 µM resulting in nonlinear kinetics for **a** MtZIP1, and **b** MtZIP5 and MtZIP6

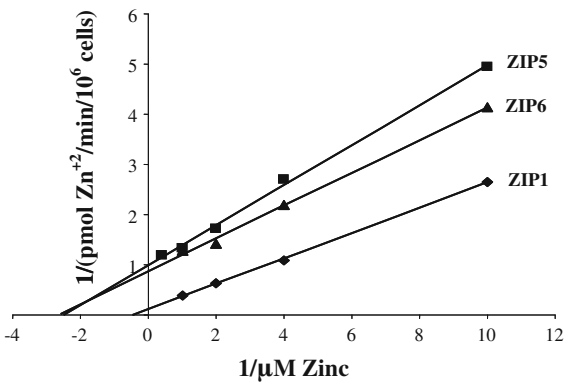

Fig. 3 Transport kinetic constants for MtZIP1, MtZIP5, and MtZIP6 were determined by linearization of Zn transport data using the double reciprocal plot

parameters were determined for each transporter by transforming the data using double reciprocal plots (Fig. 3). The kinetic constants calculated for MtZIP1 were a K_m of 1 µM and a V_{max} of 8 pmol $min^{-1}10^6$ $cells^{-1}$, while MtZIP5 and MtZIP6 had K_m's of 0.4 µM and 0.3 µM, respectively, with similar values for V_{max} of approximately 1 pmol $min^{-1}10^6$ $cells^{-1}$. Zn concentrations in subsequent competition studies were maintained near the K_m concentration for each transporter.

The Zn transport rates of MtZIP1, MtZIP5, and MtZIP6 were studied in the presence of the divalent metal Cu. It inhibited the transport of Zn via MtZIP1, MtZIP5, and MtZIP6 by 25–30% when Cu was added to the uptake solution at a concentration of 0.5 µM (Fig. 4). Cu further inhibited Zn transport in MtZIP1, MtZIP6, and MtZIP5 to 44, 47, and 56% of control rates, respectively, as the Cu concentration was increased to 1 µM. At Cu concentrations of 3 µM and higher, the Zn transport of MtZIP1, MtZIP5, and MtZIP6 were maximally inhibited, reaching approximately 80–85 percent inhibition.

Zn transport via MtZIP1 was inhibited 20% by 1 µM Cd, with the effect increasing to 55% as the Cd concentration was increased to 3 µM (Fig. 5). However, MtZIP5 and MtZIP6 appeared to be less sensitive to the effect of Cd, because Zn transport via MtZIP5 or MtZIP6 in the presence of 3 µM Cd was only inhibited 20 and 30%, respectively.

The capacity for Cd transport was also examined for MtZIP1, MtZIP5, and MtZIP6. MtZIP6 was the only one of these transporters that demonstrated measureable Cd transport capacity (Fig. 6). For yeast cells transformed with MtZIP1 and MtZIP5, Cd transport was not significantly different from yeast cells transformed with the vector control plasmid (pFL61) (Fig. 6a). The transport of Cd by MtZIP6 showed saturating kinetics that conformed to Michaelis–Menten kinetics. The kinetic parameters were

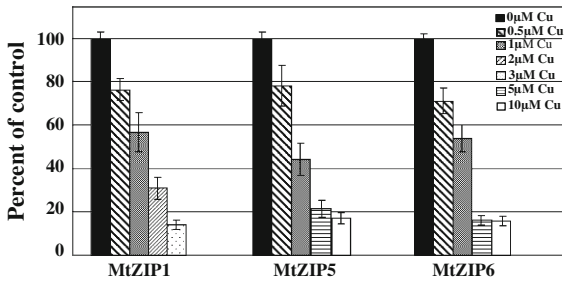

Fig. 4 The effect of Cu on the Zn transport capacity of MtZIP1, MtZIP5, and MtZIP6 was determined in the presence of Cu at concentrations between 0.5 and 10 µM. Transport reactions were performed at 1 µM Zn for MtZIP1 and 0.5 µM for MtZIP5 and MtZIP6

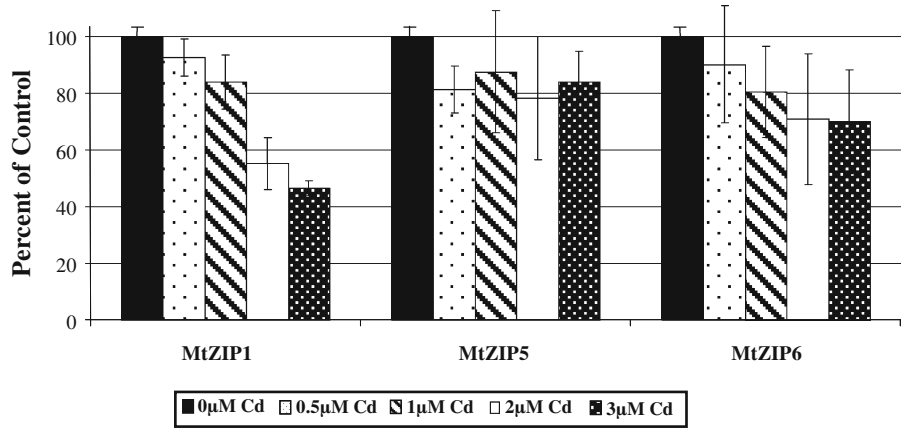

Fig. 5 The effect of Cd on the Zn transport capacity of MtZIP1, MtZIP5, and MtZIP6 was determined in the presence of Cd at concentrations up to 3 μM. Transport reactions were performed at 1 μM Zn for MtZIP1 and 0.5 μM for MtZIP5, and MtZIP6

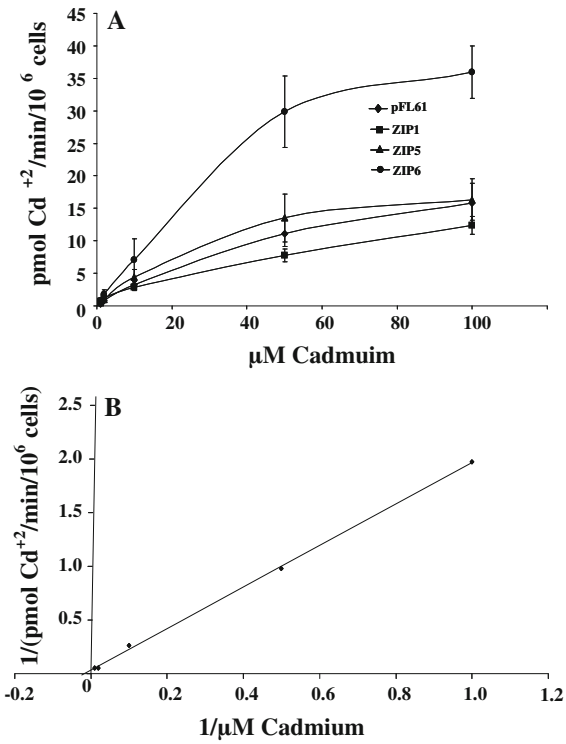

Fig. 6 Cd transport rates of the control vector, MtZIP1, MtZIP5, and MtZIP6 were determined by examining the uptake rates of Cd at varying concentrations. **a** The Cd concentrations were varied between 5 and 100 μM. **b** The Cd transport kinetic constants were determined for MtZIP6 after subtracting nonspecific transport (control vector) and using the double reciprocal plot

determined by double reciprocal plot to be a K_m of 57 μM and V_{max} of 29.4 pmol min^{-1} 10^6 $cells^{-1}$ (Fig. 6b).

Discussion

Zinc transport

The current study examines the Zn transport capacity of several members of the ZIP family of divalent metal transporters to better understand their function in Zn nutrition in the model legume *Medicago truncatula*. Three transporters from this family (MtZIP1, MtZIP5, and MtZIP6) demonstrated Zn transport capabilities when expressed in the ScZHY3 yeast mutant (Fig. 1), consistent with previous qualitative complementation assays using the same yeast strain (López-Millán et al. 2004). Because detailed kinetic analyses had not been performed previously, we examined the ability of these three transporters to transport Zn and determined their kinetic parameters and the effect of other divalent metals on their capacity to transport Zn.

The transporters were classified as either high or low affinity based on their affinity (K_m) for Zn; both classes of transporters were found amongst the *M. truncatula* ZIP family. MtZIP1, with a K_m of 1 μM was assigned as a low affinity transporter, and both MtZIP5 and MtZIP6, with K_m's of 0.4 and 0.3 μM, respectively, were assigned as high affinity transporters. The K_m of MtZIP1 aligns with reported K_m values from other plant species, including *Arabidopsis thaliana* (AtZIP1, 13 μM; AtZIP2, 2 μM; AtZIP3, 14 μM) (Grotz et al. 1998), *Thlaspi caerulescens* (TcZNT1, 7.5 μM) (Pence et al. 2000), and *Pisum sativum* (PsRIT1, 4 μM) (Cohen et al. 2004). However, the kinetic analyses of MtZIP5 and MtZIP6

reveal a 5-fold lower K_m than that of MtZIP1, or those of ZIPs from other plant species. Although high and low affinity assignments are relative, it would appear that *M. truncatula* has both types, similar to the high affinity (ScZRT1; apparent $K_m = 0.6$ μM Zn) (Zhao and Eide 1996a) and low affinity (ScZRT2; apparent $K_m = 3.6$–8.0 μM Zn) (Zhao and Eide 1996b) Zn transporter systems in yeast.

The high affinity characteristics of MtZIP5 and MtZIP6 for Zn suggest a role for these transporters in the uptake of Zn from the rhizosphere, because the concentration of available Zn in the soil ranges between 0.01–1 μM in calcareous soils (Carroll and Loneragan 1968; Kochian 1991). In contrast, MtZIP1's lower affinity for Zn suggests that this transporter has a role in movement of Zn within the plant, because the concentration of Zn in plant compartments has been shown to be much higher than in the soil. The Zn concentration of xylem sap can range from 4 to 22 μM (Clark et al. 1986; Hocking et al. 1978; Hocking 1980; White et al. 1981) and phloem concentrations of ~240 μM (Hocking 1980) have been measured. The wide variation in Zn concentrations between different cellular and developmental compartments suggests that each of the transporters may provide distinct roles in maintaining Zn homeostasis.

The maximum transport rates (V_{max}) are estimates, because the actual protein expression of each transporter was not determined; however, V_{max}'s for MtZIP1, MtZIP5 and MtZIP6 were determined to be 8, 1, and 1 pmol min^{-1} 10^6 $cells^{-1}$, respectively. Therefore, the ability of MtZIP1 to transport Zn at a higher capacity than MtZIP5 and MtZIP6 is speculative until confirmed with quantitative analysis of each transporter.

Competition studies

The effect that other divalent metals have on the ability of MtZIP1, MtZIP5, or MtZIP6 to transport Zn was examined using Cu^{2+} and Cd^{2+} as competing ions. Cu inhibited Zn transport by MtZIP1, MtZIP5, and MtZIP6, with the inhibition more pronounced as the Cu concentration was increased. Inhibition of Zn transport rose to greater than 80 percent in MtZIP1, MtZIP5, and MtZIP6 at Cu concentrations of 3, 10, and 5 μM, respectively (Fig. 5). The inhibition of Zn transport by Cu could be due to an interaction with the transporter's active site, which is possible because of the size and charge similarities of these two ions. On the other hand, because MtZIP5 and MtZIP6 have been shown to transport Fe in addition to Zn (López-Millán et al. 2004), these transporters and MtZIP1 may also have the capacity to transport Cu. Zn and Cu have similar physical characteristics that include charge and ionic radii of 72 and 74 pm, respectively (Weast 1976). The possibility that Cu could utilize the same transporter as Zn is suggested from several studies in which Cu and Zn have been shown to inhibit the transport of other metals (Bowen 1969; Giordano et al. 1974; Kausar et al. 1976; Hawf and Schmid 1967; Chaudhry and Loneragan 1972). These studies suggest that there is a direct interaction of the two metals at the plasma membrane. Although Cu uptake studies could be performed to examine the ability of MtZIP1, MtZIP5, and MtZIP6 to transport this metal, these experiments are problematic due to the short half-life of ^{64}Cu and ^{67}Cu (12 h and 2.5 days, respectively). Therefore, without Cu uptake studies, it cannot be ruled out that the Zn transport inhibition may be caused by the ability of Cu to bind to amino acids in the transporter protein, including arginine, lysine, and histidine (Bal et al. 1993; Freedman et al. 1982; López-Millán et al. 2004; Orfei et al. 2003). The binding of Cu could cause a conformational change in the protein leading to a decrease in Zn transport, perhaps by causing an increased transporter turnover such as occurs with the yeast Cu transporter ScCtr1 (Ooi et al. 1996; Petris et al. 2003).

Cadmium is a contaminating divalent metal present in many soils and has been shown in previous studies to inhibit the transport capacity of the Zn transporters AtZIP1, AtZIP2, and AtZIP3 (Grotz et al. 1998) and AtIRT1 (Eide et al. 1996). Cd reduced MtZIP1 Zn transport by 45% as compared to the control plasmid, while Zn transport by MtZIP5 and MtZIP6 was less affected (Fig. 5). The mechanism of inhibition by Cd could be similar to that of Cu. Cd could directly compete with Zn for the active sites of MtZIP1, MtZIP5, and MtZIP6. The binding of Cd to the transport site would either compete with Zn for transport, or block Zn binding to the transporter without Cd being transported into the cell; Cd has a larger ionic radii (109 pm) relative to Zn (74 pm) (Weast 1976).

Cadmium transport

Cadmium is a common contaminant of phosphate fertilizers and a major problem for crops grown in amended soils (Brown et al. 1995; Sukkariyah et al. 2005). The uptake and accumulation of Cd is detrimental to many plant processes due to its interference with other essential divalent metals including Fe and Zn (Das et al. 1997). Furthermore, the increase of Cd in plants creates a human health issue because the uptake of Cd by the plant will lead to an increase of Cd in the edible portions of the plant (Wagner 1993). Therefore, knowledge about the selectivity of various divalent metal transporters for Cd is relevant, especially as Cd transport has previously been demonstrated by plant metal transporters, including PsRIT1 (Cohen et al. 2004), TcZNT1 (Pence et al. 2000), and PtdMTP1 (Blaudez et al. 2003, Grotz et al. 1998; Korenkov et al. 2007; Korshunova et al. 1999; Mills et al. 2003).

Amongst the Zn transporting members of the MtZIP family, only MtZIP6 transports Cd (Fig. 6a). MtZIP6 has amino acid sequence similarity to the transporters in a clade of ZIP transporters that contains IRTs from several species including *Arabidopsis thaliana, Cucumis sativus, Lycopersicon esculentum, Oryza sativa*, and *Pisum sativum*. There are few reports of functional complementation and kinetic studies for Cd transport by plant Zn transporters in yeast. However, the Zn transporter TcZNT1 (Pence et al. 2000) was shown to exhibit saturating kinetics for Zn transport, while also showing linear kinetics with respect to Cd transport up to 80 μM Cd. Therefore, kinetic constants could not be determined for Cd. In contrast, both PsRIT1 (Cohen et al. 2004) and MtZIP6 transport Cd, Fe, and Zn and show saturating kinetics with respect to Cd transport and Zn transport. Similarly high K_m values for Cd between MtZIP6 and PsRIT1 (57 and 110 μM), respectively, suggest that both transporters have low affinity for Cd.

Acknowledgments This research was supported in part by HarvestPlus under Agreement number 58-6250-4-F029 and by the USDA-ARS under Agreement number 58-6250-6-003 to MAG. The contents of this publication do not necessarily reflect the views or policies of the US Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

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