Metal Selectivity Determinants in a Family of Transition Metal Transporters

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Background: Metal selectivity is an important feature of the plant cation diffusion facilitator (CDF) transporter family.

Conclusion: Residues within the cytoplasmic histidine-rich loop and transmembrane domains define metal specificity.

Significance: This raises the possibility of engineering transporters for selective biofortification of cereal grains with nutritionally essential metals.

Metal tolerance proteins (MTPs) are plant members of the cation diffusion facilitator (CDF) transporter family involved in cellular metal homeostasis. Members of the CDF family are ubiquitously found in all living entities and show principal selectivity for Zn²⁺, Mn²⁺, and Fe³⁺. Little is known regarding metal selectivity determinants of CDFs. We identified a novel cereal member of CDFs in barley, termed HvMTP1, that localizes to the vacuolar membrane. Unlike its close relative AtMTP1, which is highly selective for Zn²⁺, HvMTP1 exhibits selectivity for both Zn²⁺ and Co²⁺ as assessed by its ability to suppress yeast mutant phenotypes for both metals. Expression of HvMTP1/AtMTP1 chimeras in yeast revealed a five-residue sequence within the AtMTP1 N-segment of the His-rich intracytoplasmic loop that confines specificity to Zn²⁺. Furthermore, mutants of AtMTP1 generated through random mutagenesis revealed residues embedded within transmembrane domain 3 that additionally specify the high degree of Zn²⁺ selectivity. We propose that the His-rich loop, which might play a role as a zinc chaperone, determines the identity of the metal ions that are transported. The residues within transmembrane domain 3 can also influence metal selectivity, possibly through conformational changes induced at the cation transport site located within the membrane or at the cytoplasmic C-terminal domain.

The transition metal zinc has a wide range of critical roles in biological systems. As a cofactor in a variety of enzyme classes, Zn²⁺ plays a central catalytic role in hydrolysis (e.g. zinc proteases) (1, 2) or in substrate coordination (e.g. alcohol dehydrogenase). Zinc is also involved in the stabilization of small protein structural motifs. The most widespread example is that of zinc finger proteins, in which zinc coordinates with cysteine residues, and often additionally with histidine, to generate a rigid local structure in transcription factors. These diverse roles of zinc notwithstanding, it is astonishing that bioinformatics approaches predict that no less than around 10% of the human proteome comprises zinc-binding proteins (3).

Despite the clear prevalence of zinc in the functioning of so many human proteins, the impact of dietary zinc deficiency was not even mooted until the 1960s (4). It has since become clear that extreme cases of zinc deficiency result in impaired infant growth and development (5), although dietary zinc supplementation reduces the prevalence of infectious disease in populations at risk of zinc deficiency, whether or not classified as undernourished (6). There is strong evidence that this more mild zinc deficiency contributes significantly to the 800,000 deaths annually worldwide from malaria, diarrhea, measles, and pneumonia that can be attributed to malnutrition (7).

One strategy to combat nutritional zinc deficiency is that of biofortification of cereals through enhancing zinc accumulation in the part of the grain, the endosperm, that is most commonly eaten (8). In principle, biofortification might be accomplished through expression of transporters that sequester the metal into an intracellular compartment. Therefore, insights into metal selectivity of such transporters are an essential first step in achieving this goal. Moreover, as most uptake and deposition transition metal transporters are not entirely specific for one metal, understanding their metal selectivity underpins approaches aimed at biofortification of beneficial metals.

Recent transcriptomics data suggest the presence of metal tolerance proteins (MTPs)² that might be responsible for transition metal transport within barley grain (9). MTPs are plant members of the large cation diffusion facilitator (CDF) family of transporters ubiquitous to all forms of life (archaea, bacteria, and eukaryotes) (10). Most members of the CDF family fall clearly into one of three clades that can be specified according to the principal metal transported: Zn²⁺, Mn²⁺, or Fe³⁺/Zn²⁺.

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² The abbreviations used are: MTP, metal tolerance protein; CDF, cation diffusion facilitator; TMD, transmembrane domain; SDM, site-directed mutagenesis; SC, synthetic complete.
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(11). Many CDFs will, however, transport more than one transition metal. CDFs function as homodimeric antiporters, transporting metal ions against concentration gradients using H\(^{+}\) or K\(^{+}\) to create an electrochemical gradient (12–16). In general, these possess six transmembrane domains (TMDs), cytosolic N- and C-terminals, and, in the case of Zn\(^{2+}\)-transporters only, a His-rich cytosolic loop (IL2) between TMD4 and TMD5 thought to be involved in metal binding (11, 15). Intriguingly, although a three-dimensional structure is available for a Fe\(^{2+}/\)Zn\(^{2+}\) CDF protein (the YiiP transporter from *Escherichia coli* (17, 18)), little is known concerning domains of CDFs that determine metal selectivity. Identifying the metal selectivity determinants of plant transporters, especially for edible crops, could make a major impact on tailoring the transporters for biofortification.

Here we report the identification of a barley CDF that transports zinc and localizes to the vacuolar membrane. Through construction of barley/*Arabidopsis* chimeric transporters, we show that a small region of five residues in the His-rich loop is responsible for selectivity for Zn\(^{2+}\) over Co\(^{2+}\). The importance of the histidine-rich loop in determining metal selectivity is underscored by the results of a yeast-based metal selectivity screen that additionally highlights the importance of TMD3 in the discrimination of Zn\(^{2+}\) and Co\(^{2+}\). The results are interpreted in terms of a structural model that might involve a metal chaperone role for the His-rich loop.

**EXPERIMENTAL PROCEDURES**

Amplification and Cloning of MTP1 from Barley and *Arabidopsis*—The full coding sequence of the putative *Hordeum vulgare* HvMTP1 is found in GenBank™ (accession no. AM286795). The published sequenced was from the Lofty Ninja cultivar. However, because we worked on the Golden Promise cultivar, we amplified and cloned the full coding sequence of the putative HvMTP1 from this particular cultivar. Bioinformatics databases revealed two barley Golden Promise cultivar expressed sequence tags (The Institute for Genomic Research TC/EST annotations Tentative Consensus 146169 and TC148957) corresponding to the beginning and end of the HvMTP1 coding sequence. No other barley ESTs corresponding to other putative Zn\(^{2+}\) CDFs were found. Seeds of *H. vulgare* cv. Golden Promise, provided by Preben Holm (Aarhus University, Flakkeberg, Denmark), were germinated on moist vermiculate. RNA was extracted from 10-day-old seedlings (Qiagen RNeasy mini kit). cDNA was obtained by reverse transcription from cDNA and genomic DNA with Phusion Hot Start high-fidelity DNA polymerase (Finnzymes) using primers designed against the 5' and 3' untranslated regions, predicted on the basis of the EST sequences: UTR-HvMTP1_F and UTR-HvMTP1_R (supplemental Table 1). The amplified HvMTP1 from roots was cloned, after A tailing, into pCR 2.1 (Invitrogen). Sequencing showed that the coding sequence of HvMTP1 from Golden Promise is identical with the one in the National Center for Biotechnology Information database.

*Arabidopsis thaliana* AtMTP1 was amplified from pFL61 donated by Ute Krämer (Ruhr University, Bochum, Germany) using primers AtMTP1-KpnI_F and AtMTP1-EcoRI-Stop_R (supplemental Table 1).

Chimeric Constructs—Chimeric mutants between AtMTP1 and HvMTP1, consisting of domain swaps or domain deletions, were achieved by a series of PCR amplifications and blunt end ligations. In brief, each of the two or three fragments to be assembled was amplified individually using specific primers (supplemental Table 1). The fragments were ligated, and the ligation product was further used for second-round PCR using primers specific for the desired assembled segment. A subsequent ligation step was performed in the case of addition of a third fragment. The ligation product was further used as a template for the final construct, using primers corresponding to the trails of the constructs.

Site-directed and Random Mutagenesis—Site-directed mutagenesis (SDM) and random mutagenesis were performed on *HvMTP1* and *AtMTP1* cloned in the pYES2 vector using QuickChange II XL site-directed mutagenesis and GeneMorph II random mutagenesis kits (Stratagene) according to the manufacturer’s instructions. Primers and mutations performed are listed in supplemental Table S2. All constructs in this work were confirmed by sequencing.

Expression in Yeast Expression Vectors—The binary vector pYES2 (Invitrogen) was used for expression of all constructs, including the WT MTP1, chimeric, and mutated proteins in yeast under the *GAL1* promoter. Primers designed against the start and trailer regions of WT MTP1s, including the restriction enzyme sites (KpnI/EcoRI) used for cloning, are shown in supplemental Table S1. All forward primers contained an additional AAA codon between the enzymatic restriction site and the ATG codon for placing the ATG in a Kozak consensus sequence.

*Yeast Strains and Growth Media*—Ionic selectivity of WT MTP1s, chimeras, and random and site-directed mutants was investigated by heterologous expression in budding yeast (*Saccharomyces cerevisiae*); BY4741 being the wild-type parental strain. Yeast mutants deficient in metal transporters were obtained from Euroscarf, and the Δzrc1Δcot1 mutant was obtained from Nathalie Verbruggen (Université Libre de Bruxelles, Brussels). Details of the yeast strains used are found in supplemental Table 3. Yeast cell transformation was performed by the lithium acetate method (20). Transformed yeasts were selected on synthetic defined medium without uracil (0.17% (w/v) yeast nitrogen base without amino acids and nitrogen (Formedium), 0.5% (w/v) ammonium sulfate, 2% (w/v) glucose, supplemented with the required amino acids histidine, leucine, and methionine (pH 6.0)).

Growth Assays—Yeast were grown to an A600 of 1 in liquid non-inducing synthetic complete medium (SC) (as SD but supplemented with yeast synthetic dropout mix without uracil (Formedium) (pH 6.0)), and 10 μl were spotted on agar SC galactose 2% (Formedium)-inducing medium. At least three independent transformants for each construct were tested.

Intracellular Localization Studies in Plant Cells—Cellular localization of HvMTP1 in plant cells was assessed by transient expression in barley leaf protoplasts using in-frame C-terminal fusion with mGFP5 in XmaI site of pART7. Cells were visualized by inverted confocal laser scanning microscopy (21).
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In Yeast—Cellular localization of WT, chimeric, and mutated proteins in yeast was assessed using in-frame C-terminal fusions with GFP, the marker being previously cloned in Xhol/ XbaI (22). Yeast vacuolar membranes were selectively stained with the red lipophilic styryl dye FM4–64 (Invitrogen) (23). The GFP fluorescence of yeast was observed by confocal laser scanning microscopy with a Zeiss LSM510 Meta head based on an Axiovert 200 m microscope (Zeiss, Jena, Germany).

Zn Transport Assay—Yeasts microsomes were isolated from log-phase cultures of Δzrc1/Δcot1 transformed with either the empty vector (pYES2) or the vector containing HvMTP1 according to Nakanishi et al. (24). Vesicles were stored at –80 °C in resuspension buffer containing 5 mM Bis-Tris propane/Mes (pH 7.5), 300 mM sorbitol, 5 mM MgCl₂, 1 mM DTT, 10 μM PMSF, 1 mg/L leupeptin, and 2 mg/L pepstatin (21). The uptake experiments were carried out by the filtration method as described (21), with minor modifications. Filtration was performed using 120 μg microsomes that were preincubated at 25 °C in 600 μL of uptake buffer (5 mM BTP (pH 7.2), 300 mM sorbitol, 25 mM KCl, 1 mM DTT, 1 mM ATP, 5 mM MgCl₂). Reactions were performed at several time intervals upon addition of 65ZnCl₂. Net Zn²⁺ uptake was obtained upon subtraction of nonspecific adsorption, determined in the uptake buffer in the absence of MgCl₂, from the corresponding values in the uptake buffer in the presence of MgCl₂.

Metal Analysis—Concentration of Zn²⁺ and Co²⁺ in yeast cells was determined by atomic absorption spectrometry (Hitachi Z-5300 Polarized Zeeman atomic absorption spectrophotometer). Yeast cell cultures, at a density of 10⁷ cells/mL, were supplemented with sulfate salts with 200 μM Zn²⁺, 250 μM Co²⁺, or no added metal as control. After 24 h growth at 30 °C with shaking (200 rpm), cells were collected by centrifugation and washed three times with ice-cold 50 mM Tris-HCl (pH 6.5) and 10 mM EDTA and once with water (25). Pellets were dried for 24 h at 60 °C and digested in 3 mL HNO₃ at 80 °C using an open-vessel microwave system (MARS 5, CEM, Mathews, NC).

Protein Extraction and Gel-Blot Analysis—Total cell lysate from yeast was prepared after 24 h induction in SC medium without uracil and supplemented with galactose medium by disruption with glass beads in ice-cold lysis buffer containing 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10% (v/v) glycerol, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 10 μg/mL pepstatin A (Sigma). Protein extracts (20 μg) were loaded on 10% SDS-PAGE gel and transferred to nitrocellulose membrane (Protran Whatman). Immuno blot analyses were probed with rabbit anti-His, antibodies (1:1000) (Abcam, catalog no. 9108) and goat anti-rabbit HRP-conjugated antibodies (1:8000) (Sigma, catalog no. A-9169). Proteins were revealed by enhanced chemiluminescence using the Western pico revelation kit (Perbio Science).

RESULTS

HvMTP1 Characterization and Metal Selectivity—We cloned from barley a CDF family member that we termed HvMTP1. Phylogenetic analysis (not shown) of HvMTP1 positions it in the Zn²⁺ clade of the CDF family, alongside the Arabidopsis thaliana MTPs 1 through 4. The HvMTP1 transcript is ubiquitously present in barley organs including roots, shoots, and seeds, and, in common with homologous MTP1s from dicotyledonous species, the coding sequence consists of one exon (Fig. 1A). Cellular localization of HvMTP1 is apparent at the vacuolar membrane both when it is expressed heterologously in yeast (Fig. 1B) or transiently in protoplasts from barley mesophyll (C). Ionic selectivity of HvMTP1 was assessed by heterologous expression in a number of yeast mutants deficient in various metal transporters. Among the yeast mutants selected (Δcot1 (Co²⁺/Zn²⁺-sensitive), Δzrc1 (Zn²⁺), Δzrc1Δcot1 (Zn²⁺/Co²⁺), Δcup2 (Cu²⁺), Δsmf1 (Ni²⁺), Δpmr1 (Mn²⁺), and Δycf1 (Cd²⁺)), only those sensitive to high concentrations of Zn²⁺ or Co²⁺ were complemented by expression of HvMTP1 (Fig. 1D and data not shown). Thus, in contrast to its counterpart AtMTP1, which is highly selective for Zn²⁺, HvMTP1 appears to transport both Zn²⁺ and Co²⁺. Direct Zn²⁺ uptake measurements in yeast microsomes showed that HvMTP1 can facilitate transport of the metal, and therefore, the yeast growth phenotype observed on medium supplemented with metals cannot be due just to binding (Fig. 1E).

Metal Selectivity of Histidine-rich Loop Chimeric Constructs between HvMTP1 and AtMTP1—Previous experiments with plant Zn²⁺ MTPs have shown that removing the entire intracytoplasmic His-rich loop results in a non-functional protein (TcMTP1, Ref. 26). However, deletion of only 32 amino acid residues from the N-segment of the His-rich loop of AtMTP1 confers on the transporter the ability to complement yeast mutants defective not only in Zn²⁺ transport but also in Co²⁺ (15). Nicotiana tabacum and Nicotiana glauca MTP1s have also been shown to complement the growth of yeast mutants on media supplemented with either Zn²⁺ or Co²⁺ (27). The authors suggested that the presence of more His residues in the His-rich loop of Nicotiana species in comparison to AtMTP1 might be responsible for additional Co²⁺ transport capability. Therefore, we hypothesized that if the residues in the His-rich loop are involved in conferring the metal selectivity, then swapping the loop between AtMTP1 and HvMTP1 (Fig. 2A) should lead to inverted metal selectivity.

Metal selectivity assessed in yeast indeed showed that interchanging the His-rich loops of AtMTP1 and HvMTP1 resulted in exchanged metal specificities (Fig. 2B). To further investigate the role of the His-rich loop in conferring metal specificity, we divided it into an N-segment comprising 36 residues (181–216) for AtMTP1 and 38 residues (180–217) for HvMTP1 and a C-segment consisting of 45 residues (217–263) for AtMTP1 and 66 residues (218–283) for HvMTP1 (Fig. 2A). Exchange of the N- and C-segments of the loop between the two proteins showed that the restriction to Zn specificity resides with the N- and C-segments of the loop between the two proteins were changed with the equivalent ones in the homologous protein, either as single, double, or triple mutations (data not shown). Results showed that a single five-residue sequence within the His-rich loop of AtMTP1 is responsible for restricting its metal selectivity to Zn²⁺. Deletion of the 5 residue sequence in a mutated protein able to complement yeast growth both on Zn²⁺ and Co²⁺. Mutations of each of the five residues from AtMTP1 to the corresponding ones in HvMTP1 showed that single mutations of four of the
residues result in the ability to also complement Co\(^{2+}\) (Fig. 2C). Furthermore, yeast expressing the wild-type AtMTP1 and HvMTP1 and the chimeric constructs between the two also showed that interchanging the His-loop or the five residue sequence resulted in a marked elevation of Co\(^{2+}\) concentration in strains expressing AtMTP1 containing the HvMTP1 His loop variants (Fig. 3A) without significantly affecting the concentration of Zn\(^{2+}\) (Fig. 3B). This finding mirrors that of the respective growth phenotypes. Cellular localization of the At/Hv chimeras within yeast cells showed a vacuolar localization similar to the wild-type proteins (supplemental Fig. 1), confirming that the chimeric proteins are expressed and functional.

It is possible in principle that some chimeric proteins complement the growth of yeast on Co\(^{2+}\) because they are expressed more highly than those that fail to complement. To check this, C-terminal His\(_6\)-tagged constructs were created for wild-type AtMTP1, AtMTP1/Hv-His loop, and AtMTP1-NSEDD. The metal selectivity of the His\(_6\) constructs was checked by expression in the S. cerevisiae double mutant \(\Delta\text{zrc1}/\Delta\text{cot1}\) (supplemental Fig. 2A). The results showed a similar pattern of behavior as the non-His\(_6\) tagged proteins (Fig. 2B). The level of expression of the His\(_6\)-tagged proteins in the \(\Delta\text{zrc1}/\Delta\text{cot1}\) mutant was no higher in the Co\(^{2+}\)-complementing variants than in the wild-type AtMTP1 that complements the Zn\(^{2+}\) phenotype alone (supplemental Fig. 2B). Therefore the Co\(^{2+}\) phenotype observed for some of the AtMTP1 chimeric proteins is indeed due to a shift in metal specificity that cannot be attributed to an increase in their level of expression.

It has been proposed that the cytoplasmic histidine-rich loop might act as a sensor and, therefore, a binding site for cytoplasmic Zn\(^{2+}\) (15). The possibility therefore arises that the suppres-
sion of Zn\(^{2+}\) - and Co\(^{2+}\)-sensitive phenotypes in yeast arises through chelation of metals in the loop region rather than through transport \textit{per se}. We therefore expressed the AtMTP1 histidine-rich loop (as a C-terminal His\(_6\)-tagged variant) on its own in the \(\Delta\text{zrc1}/\Delta\text{cot1}\) yeast mutant. No complementation of yeast growth on any metals was observed (supplemental Fig. 3A). To check the possibility that the histidine-rich loop was not expressed, a C-terminal GFP fusion construct was made. Confocal microscopy showed that this protein is expressed and is located, as expected for a soluble protein, within the cytoplasm (supplemental Fig. 3B). Moreover, immunoblotting showed that the His\(_6\) C-terminal fusion of the loop is also expressed within yeast (Fig. 3C). Thus, even if the histidine-rich loop is involved in chelation of the metal ions, the amount bound is too small to account for suppression of metal-sensitive phenotypes in yeast. Metal transport into the vacuole is required.

Identification of Additional Metal Selectivity Determinants within AtMTP1 with Random and Site-directed Mutagenesis—

To investigate further the nature of Zn\(^{2+}\) versus Zn\(^{2+}\)/Co\(^{2+}\) selectivity, the Zn\(^{2+}\)-selective transporter gene AtMTP1 was subjected to random mutagenesis followed by expression in \(\Delta\text{cot1}\) yeast and screened for the ability to grow on 1.6 mM Co\(^{2+}\). From about 42,000 \(\Delta\text{cot1}\) transformants, 15 Co\(^{2+}\)-tolerant yeast colonies were selected for further analysis.

**FIGURE 2. A five-residue sequence within the histidine-rich loop of AtMTP1 restricts transporter selectivity to Zn\(^{2+}\).** A, sequence alignment of the His-rich intracytoplasmic loops of \(H.\) \textit{vulgare} and \(A.\) \textit{thaliana} MTP1. The His-rich loops of AtMTP1 G181-G261 and HvMTP1 G180-S283 are underlined in black. The N- and C-segments of the His-rich loops are delimited by arrows (top panel). B and C, metal selectivity of HvMTP1/AtMTP1 His-rich loop chimeras by heterologous expression in the yeast \(\Delta\text{zrc1}/\Delta\text{cot1}\) mutant strain transformed with empty vector (pYES2) (B), chimeric constructs of the two plant transporters (C), and SDMs of AtMTP1 and HvMTP1 consisting of interchange of corresponding single residues in the five-amino acid sequence within the His-rich loop. Yeast cells were grown on inducing agar SC medium without uracil with no added metals (Control) or supplemented with metals. Plates were incubated for 4 days at 30 °C.
identified. Plasmids were extracted and sequenced. Fig. 4 highlights residues that, when mutated, confer the ability to suppress the yeast Co\textsuperscript{2+}/H\textsubscript{11001}-sensitive mutant phenotype.

The mutation G140A arose twice independently as a single mutant. Two of the AtMTP1 random mutants in the C-segment of the His-loop (E217K and E226D) conferred a very subtle phenotype on Co\textsuperscript{2+}/H\textsubscript{11001} (data not shown), and, therefore, they were not considered any further. Three of the 15 mutants carried double substitutions. However, mutations at positions His-201 and His-212 were also identified as single substitutions. Therefore, we further focused on the single substitutions and confirmed their phenotype by generating the same residue substitutions by SDM. The ability of these mutants to confer Co\textsuperscript{2+}/H\textsubscript{11001} tolerance was verified by transformation of \textsl{/H9004}zrc1/\textsl{/H9004}cot1 cells (Fig. 5).

Random mutagenesis showed that in addition to residues within the His-rich loop, including two identified previously in the five residue sequence (VTVTT), TMDs 2 and 3 arose as possible regions for conferring the metal specificity (Fig. 4). A previous study demonstrated that other mutations in TMD2 of the \textsl{S. cerevisiae} ZRC1 transporter modulate the metal selectivity of this Zn\textsuperscript{2+} transporter (28). Therefore, site-directed mutagenesis was also performed for the equivalent residue Ser-93 (TMD2) of AtMTP1. Metal selectivity of the resulting mutant transporters (AtMTP1 S93A and S93T) was comparable with that of wild-type AtMTP1 when investigated in yeast deficient in Zn\textsuperscript{2+}, Co\textsuperscript{2+}, and Mn\textsuperscript{2+} transport (Fig. 5).

One of the randomly generated mutants of AtMTP1, I135F, located within TMD3, exhibited reduced growth on Zn\textsuperscript{2+} but resulted in the gain of complementation for Co\textsuperscript{2+} and even partially for Mn\textsuperscript{2+}. Therefore, we further investigated whether the restriction of the transporter selectivity for Zn\textsuperscript{2+} could be due to interactions between Ile-135 and the VTVTT sequence. Corresponding mutations were therefore created in the wild-type HvMTP1 (without VTVTT) and in the chimera HvMTP1-VTVTT that displayed a similar metal complementation phenotype in yeast to AtMTP1. Mutation of I134F in the HvMTP1-VTVTT resulted, as for AtMTP1, in growth complementation on Co\textsuperscript{2+} and impaired growth on Zn\textsuperscript{2+}. However, reduced yeast growth on Zn\textsuperscript{2+} was also observed for HvMTP1 I134F (data not shown), thus suggesting that Ile-135 and...
VTVTT sequence act independently in confining transport to Zn\(^{2+}\)/H\((+)\). We further investigated whether charge, size, or a specific side chain at position 135 has a key role in defining the metal specificity in AtMTP1. Hence, Ile-135 was extensively and variably substituted, and the metal selectivity of the mutated transporters was assessed in yeast (Fig. 6). Among the variants tested, only I135V and I135G displayed a similar behavior to the wild-type. Interestingly, substitution of Ile-135 with Leu, Tyr, Gln, Asn, and Glu resulted, as with the I135F mutant, in strong complementation not only of the Co\(^{2+}\)/H\((+)\)-sensitive phenotype but also that of Mn\(^{2+}\)/H\((+)\) (Fig. 6). Taken together, these results indicate a key role for Ile-135 in determining metal selectivity.

**DISCUSSION**

HvMTP1 is a Zn\(^{2+}\)/Co\(^{2+}\)-specific transporter. Cation diffusion facilitators play widespread roles in transition metal detoxification. Their principal affinities are for Zn\(^{2+}\), Fe\(^{3+}\), and Mn\(^{2+}\). Several studies have reported characterization of MTPs from dicotyledonous plants (Arabidopsis (19, 21, 29), Thlaspi (26), Brassica (30), Nicotiana (27), and poplar (22)) and, recently, from a monocot (rice (31)). Here, we report on a barley MTP named HvMTP1 as a member isolated from cereals.

Our results confirm and extend previous analyses showing that HvMTP1 transcripts are present in cereal seeds (9) by demonstrating protein expression of HvMTP1 at the vacuolar membrane. Therefore, the HvMTP1 transporter could be involved in the deposition of metals in the endosperm during grain filling. Because HvMTP1 could significantly affect the metals accumulated in the edible parts of cereals, its metal selectivity was assessed. Heterologous expression of the transporter in yeast reveals that it is specific for Zn\(^{2+}\) and Co\(^{2+}\). A number of other plant Zn\(^{2+}\) CDFs have also been shown to select for Co\(^{2+}\) and/or other non-essential or even toxic heavy metals. Such transporters include AtMTP3 (29), and MTP1s from Thlaspi caerulescens and Thlaspi geosingense (26, 32), N. tabaccum and N. glauca (27), and Brassica juncea (30).

**The Histidine-rich Loop (IL2) Plays a Role in Zn\(^{2+}\)/Co\(^{2+}\) Discrimination**—Metal specificity is an important feature of all transporters. Recognition, binding, and transport are crucial in uptake and distribution of essential metals for cellular homeostasis. Few data are available regarding the metal specificity determinants of transition metal transporters, including CDFs. Recent bioinformatics analysis suggests that metal specificity of CDFs might reside at the cytosolic sites (33). Additional experimental work (15) has highlighted the potential role of the N-segment of the His-rich loop of AtMTP1 in encoding metal selectivity. Focusing on the plant Zn\(^{2+}\) CDF subgroup, we identified amino acid residues in the His-rich loop and in additional domains that play a key role in selecting and differentiating between metal ions.

In contrast to other CDF metal subgroups, members from the Zn\(^{2+}\) clade possess a long His-rich intracytoplasmic loop (IL2) linking TMDs 4 and 5. Although all plant CDF members in the Zn\(^{2+}\) clade possess the His-rich loop, some are highly
FIGURE 5. Metal selectivity determinants within TMDs of AtMTP1 identified with random and site-directed mutagenesis. Complementation of S. cerevisiae mutants Δzrc1/Δcot1 (A) and Δpmr1 (B) grown on selective media supplemented with Zn$^{2+}$, Co$^{2+}$, and Mn$^{2+}$, respectively. Yeast wild-type strain BY4741(WT) and mutant strains were transformed with the empty vector (pYES2), AtMTP1(wt), or AtMTP1 SDMs identified by random mutagenesis to confer complementation of growth on Co$^{2+}$. Mutations marked by a were identified by initial random mutagenesis as a double mutant. Mutations marked by b were selected on the basis of their equivalency to residues on ScZRC1 that altered the WT metal specificity (28). B, growth of Δpmr1 on medium supplemented with Mn$^{2+}$ is shown only for those AtMTP1 SDMs that were able to partially rescue yeast sensitivity and compared with AtMTP11, a Mn transporter, used as a positive control (21). Yeast cells were grown on inducing agar SC without uracil medium with no added metals (Control) or supplemented with metals. Plates were incubated for 4 (Δzrc1/Δcot1) or 5 (Δpmr1) days at 30 °C.
specific for Zn$^{2+}$, such as PtdMTP1 (22), AtMTP1 (19, 34), and AhMTP1 (35), whereas others are able to transport a broader spectrum of divalent ions such as TcMTP1 (Zn$^{2+}$, Cd$^{2+}$, Ni$^{2+}$, Co$^{2+}$ (26)), NtMTP1, NgMTP1 (Zn$^{2+}$ and Co$^{2+}$ (27)), and HvMTP1 (Zn$^{2+}$ and Co$^{2+}$ (this study)). The possibility that the variation in the number of His residues within the His-rich loop might be responsible for confining the metal selectivity was suggested (27).

By creating chimeric constructs between plant Zn$^{2+}$ CDFs with solely Zn$^{2+}$ specificity (AtMTP1) and with Zn$^{2+}$/Co$^{2+}$ selectivity (HvMTP1) we were able to show that the N-segment of the His-rich loop plays a role in Zn$^{2+}$/Co$^{2+}$ selectivity in accordance with previous findings (15). Moreover, we narrowed the critical region to a five-residue stretch (VTVT) that, when present, restricts the transporter selectivity to solely Zn$^{2+}$. Transferring this short sequence from AtMTP1 to HvMTP1 hampers the ability of the barley transporter to complement the growth of yeast on Co$^{2+}$. Furthermore, mutations of single amino acids within this sequence also expanded the AtMTP1 complementation ability to Co$^{2+}$. It therefore appears that this five-residue sequence is critical in determining metal specificity for Zn$^{2+}$. This conclusion is supported by the fact that mutations of single residues from the corresponding sequence in barley (NSEDD) do not impair the complementation for Co$^{2+}$ (Fig. 2C). Thus, we show that the His-rich loop serves not only as a sensor and a binding site for the metal ions (15) but also has a fundamental role in the CDF metal selectivity.

An Additional Role of TM3 in Determining the Metal Selectivity—Mutant screening for gain of function for transport of metals could help identify key residues involved in metal selectivity. Random mutagenesis of AtMTP1 resulted in identification of residues within TMDs 2 and 3 as well as the His-rich loop that led to complementation not only for Zn$^{2+}$ but also for Co$^{2+}$. Two of the mutations, T206A and T208A, reside within the previously identified five-residue sequence from the N-segment of IL2. Residues in TMD2 are located in the vicinity of the likely transport site represented by the highly conserved residues HXXHD within TMDs 2 and 5 (17). Possibly, substitution of the two relevant residues (Ala-97 and Ala-99) by more bulky residues forces a reorientation of the helix and disruption of the Zn$^{2+}$ coordination site. In contrast, residues in TMD3 are more likely to affect the release of the metal on the other side of the membrane, whereas with TMD6, this domain forms a separate cluster from that of TMDs 1, 2, and 5 (18).

Intriguingly, two of the residues in TMD3 (I135F and E145G) are in equivalent relative positions to those identified in a study of ScZRC1 (L87H and E97G) that were reported to change the metal selectivity completely from Zn$^{2+}$ to Fe$^{2+}$ and Mn$^{2+}$ (28). In contrast to yeast, the corresponding Arabidopsis mutants were still able to confer partial or full complementation for Zn$^{2+}$ (Fig. 5A). Moreover, they also extended the range of metal specificity to Co$^{2+}$ and Mn$^{2+}$. Mutation of Ile-135 revealed no correlation between the polarity or charge of the residue and the metal selectivity. Importantly, minor structural modification of the side chain (I135L) can have a profound effect on widening metal selectivity.

Thus, in contrast to ScZRC1, we did not identify any single mutation able to completely shift the metal selectivity of AtMTP1 from Zn$^{2+}$ to other metals, although several Ile-135 mutations led to a decrease in Zn$^{2+}$ and enhancement of Co$^{2+}$ and Mn$^{2+}$ complementation. It is possible that in higher organisms the metal selectivity of the CDFs is more tightly regulated (33), with more than one residue being involved in confining the metal specificity. This difference between plant and fungal representatives of the Zn$^{2+}$-group of CDFs is not entirely sur-
prising, as phylogenetic analysis showed that they cluster in distinct subgroups (11).

Structural Interpretation of Mutant-induced Specificity Changes in Relation to Metal Transport—To understand at a structural level the impact of mutations of those residues involved in determination of metal selectivity, we constructed a model of AtMTP1 structure using the protein structure prediction server I-TASSER (36). Briefly, I-TASSER threads the target sequence over the library of representative structures to identify cases of partial sequence-structure complementarity and then uses a complex algorithm to generate and optimize the plausible models. In our case, the final models turned out to be strongly biased toward the *E. coli* *YiiP* (3H90, Ref. 18), the only complete structure of a CDF available to date. Because in the metal-bound state the bacterial transporter assembles as a homodimer, the plausible dimeric structure of AtMTP1 was created by superimposing two AtMTP1 models over the *YiiP* dimer, placing missing Zn$^{2+}$/H11001 ions, and optimizing the final model by constrained energy minimization (Fig. 7A). In the Zn$^{2+}$/H11001-bound state, Ec*YiiP* adopts a Y-shape, with the TMDs of the two monomers clearly separated and the C-terminal cytoplasmic domains tightly associated, constituting the dimerization region (17, 18). Four Zn$^{2+}$ ions are present on each monomer, associated with one transport site (site A) within the membrane, a second (site B) at the membrane-cytoplasm interface, and a third (site C) at the cytoplasmic domains, binding the remaining two ions from each monomer in a complex cluster. At sites A and C, there are a number of highly conserved
residues implicated in metal binding, including the HXXXD sequence (site A) and a number of acidic and His residues at site C. In contrast, residues at site B are neither well conserved nor, in the case of AtMTP1, indicative of favoring metal binding, whereas in YiiP, Asp-68 and His-75 are implicated in Zn$^{2+}$ binding. There are no acidic or His residues in the equivalent domain of AtMTP1. There is also some uncertainty regarding the position of the His-rich loop, which is not present in YiiP and whose position in the final I-TASSER models varies. Fig. 7A shows one output from I-TASSER that envisages the His-rich loop as largely cytoplasmic and unstructured, unlabeled with three regions of α helix.

The five-residue stretch that we have identified in the His-rich loop as critical for selectivity of Zn$^{2+}$ over Co$^{2+}$ in AtMTP1 might reside in the first of the three α helices and exhibit proximity to binding site B. Cherezov et al. (37) proposed a crucial role for an unidentified Zn$^{2+}$ chaperone in delivering the metal to site B to facilitate transfer to the intramembrane transport site A. The absence of readily identifiable candidate residues for Zn$^{2+}$ binding at the AtMTP1 site B leads us to hypothesize, for AtMTP1, that the His-rich loop, which is essential for Zn$^{2+}$ binding (15) and for transport activity (15, 26), might perform the chaperone role by delivering Zn$^{2+}$ to a region of the protein occupied by site B in other CDF members.

Random mutagenesis resulted in identification of clusters of residues involved in metal selectivity not only, as expected, on the His-rich loop, but also focused on TMDs 2 and 3. Residues on TMD2 are directly involved in metal binding, and it is therefore not surprising that mutations in Ala-97 and Ala-99, both of which reside near the transport site (Fig. 7B), can change metal specificity. Although TMD3 is not directly implicated in coordinating Zn$^{2+}$, Fig. 7B shows that a number of residues controlling metal selectivity reside close to the transport site, with Ile-135 especially close to the conserved Zn$^{2+}$-coordinating His residue in TMD5. It is possible that single residue mutations within TMD3 not only influence the release of the ions at site A, but also through their connection to the flexible loop in the C-terminal domain that serves as a pivotal axis (18, 38) could modify the cytosolic cavity of the His-rich loop and thus broaden the metal specificity of the transporter. Thus, our genetic studies align with structural insights to suggest fundamental ways in which CDF transporters within the Zn$^{2+}$ clade can encode metal selectivity.

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