Deletion of a Histidine-rich Loop of AtMTP1, a Vacular Zn\textsuperscript{2+}/H\textsuperscript{+} Antiporter of Arabidopsis thaliana, Stimulates the Transport Activity*

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Arabidopsis thaliana AtMTP1 belongs to the cation diffusion facilitator family and is localized on the vacuolar membrane. We investigated the enzymatic kinetics of AtMTP1 by a heterologous expression system in the yeast \textit{Saccharomyces cerevisiae}, which lacked genes for vacuolar membrane zinc transporters ZRC1 and COT1. The yeast mutant expressing AtMTP1 heterologously was tolerant to 10 mM ZnCl\textsubscript{2}. Active transport of zinc into vacuoles of living yeast cells expressing AtMTP1 was confirmed by the fluorescent zinc indicator FuraZin-1. Zinc transport was quantitatively analyzed by using vacuolar membrane vesicles prepared from AtMTP1-expressing yeast cells and radioisotope \textsuperscript{65}Zn\textsuperscript{2+}. Active zinc uptake depended on a pH gradient generated by endogenous vacuolar H\textsuperscript{+}-ATPase. The activity was inhibited by bafilomycin A\textsubscript{1}, an inhibitor of the H\textsuperscript{+}-ATPase. The $K_m$ for Zn\textsuperscript{2+} and $V_{\text{max}}$ of AtMTP1 were determined to be 0.30 mM and 1.22 nmol/min/mg, respectively. We prepared a mutant AtMTP1 that lacked the major part (32 residues from 185 to 216) of a long histidine-rich hydrophilic loop in the central part of AtMTP1. Yeast cells expressing the mutant became hyperresistant to high concentrations of Zn\textsuperscript{2+} and resistant to Co\textsuperscript{2+}. The $K_m$ and $V_{\text{max}}$ values were increased 2–11-fold. These results indicate that AtMTP1 functions as a Zn\textsuperscript{2+}/H\textsuperscript{+} antiporter in vacuoles and that a histidine-rich region is not essential for zinc transport. We propose that a histidine-rich loop functions as a buffering pocket of Zn\textsuperscript{2+} and a sensor of the zinc level at the cytoplasmic surface. This loop may be involved in the maintenance of the level of cytoplasmic Zn\textsuperscript{2+}.

Zinc is a trace element essential as a cofactor for many enzymes and a structural element for various regulatory proteins (1–3). These proteins and enzymes include zinc-finger proteins, RNA polymerases, superoxide dismutase, and alcohol dehydrogenase. Thus, zinc deficiency causes severe symptoms in all organisms including plants. However, when present in excess, zinc can become extremely toxic, causing symptoms such as chlorosis in plants. The essential but potentially toxic nature of zinc necessitates precise homeostatic control mechanisms to satisfy the requirements of cellular activity and to protect cells from toxic effects.

Plants have many kinds of zinc transporters and zinc channels (4–6). Typical zinc transporters include metal tolerance protein (MTP)\textsuperscript{3} (7, 8), ZIP (ZRT (zinc-regulated transporter)/IRT (iron-regulated transporter)-like protein) (4), and HMA (heavy metal ATPase) (9) families. The MTP family in \textit{Arabidopsis thaliana} consists of 12 members, and 4 members, MTP1-MTP4, form a subfamily. Both \textit{A. thaliana} AtMTP1 (10, 11) and AtMTP3 (12) are localized on the membrane of central vacuoles. A similar transporter in the zinc-hyperaccumulating plant species \textit{Arabidopsis halleri}, AhMTP1–3, has also been investigated (13). Zinc tolerance of \textit{A. halleri} has been suggested to be due to an increased copy number of the MTP1 gene and enhanced level of transcription (13, 14). AtMTP1 has high identity with \textit{A. halleri} AhMTP1–3 (13, 14). AtMTP1, AhMTP1–3, and AtMTP3 belong to a ubiquitous family of transition metal transport proteins called the cation diffusion facilitator protein family, which have been identified in bacteria, Archaea, and eukaryotes and have been demonstrated to transport zinc, cobalt, and cadmium (15).

AtMTP1 has been demonstrated to transport zinc from the cytosol into the vacuole in \textit{A. thaliana} (10–12) as well as AtMTP3 (12). AtMTP1 has been predicted from the hydropathy to have six transmembrane domains, long N- and C-terminal tails, and a long histidine-rich (His-rich) hydrophilic region between the fourth and fifth transmembrane domains (10, 16). A multiple histidine domain is also present in mammalian orthologues such as mouse ZnT-3 (17). The His-rich domain in these members has been estimated to serve as a zinc binding region (16, 17). However, the transport mechanism and structure-function relationship of these zinc transporters have not been clarified, although zinc transport activity of MTP1 was detected by using reconstituted proteoliposomes of the protein expressed in \textit{Escherichia coli} (18) and by a yeast complementation assay with a zinc-hypersensitive double mutant of \textit{ZRC1} and \textit{COT1} (\textit{zrc1 cot1}) (13, 19).

\textit{A. thaliana} vacuolar zinc transporter AtMTP1 plays a key role in zinc tolerance and has a His-rich loop. The importance

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\textsuperscript{3} The abbreviations used are: MTP, metal tolerance protein; CCCP, carbonyl cyanide m-chlorophenylhydrazone; V-ATPase, vacuolar H\textsuperscript{+}-ATPase; MES, 4-morpholineethanesulfonic acid.
of its physiological function was revealed by the gene disruption mutant of AtMTP1 (10). The His-rich loop consists of 81 residues including 25 histidine residues. Several cation diffusion facilitator family members such as ShMTP1 of hyperaccumulator plant *Stylosanthes hamata* lack a His-rich region and have different ion selectivity (20). In the present study we expressed AtMTP1 in *Saccharomyces cerevisiae* to determine the kinetic properties; namely, affinity for zinc, ion selectivity, and dependence of the transport activity on a pH gradient. A zinc fluorescent probe enabled us to monitor zinc accumulation into vacuoles in living yeast cells. We also carried out quantitative analysis of zinc transport using radioactive $^{65}$Zn$^{2+}$ and a vacuolar-membrane-enriched fraction from AtMTP1-expressing yeast. Furthermore, expression of a mutant AtMTP1, which lacked a large part of the His-rich loop, revealed the critical role of this motif. We report the experimental results and discuss the biochemical meaning of the His-rich loop of AtMTP1, especially the ion selectivity, affinity to Zn$^{2+}$, and transport efficiency.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Media**—The zinc-sensitive mutant *zrc1 cot1* (*zrc1::LEU2 cot1::TRP1*) of yeast (*S. cerevisiae*) was generated from BJ5458 (ABC710) (MATa, ura3-52, leu2Δ1, lys2-801, his3Δ200, pep4::HIS3, prb1Δ1.6R, can1, trp1, GAL) (21) by the PCR-mediated gene disruption method (22). The ZRC1 locus was replaced with LEU2 gene, and the COT1 locus was replaced with the TRP1 gene. The oligonucleotides used were (5’-ZRC1) 5’-ATGATCACGGTAAAGAATGAGAAATCAGGGGATCC-3’ and (3’-ZRC1) 5’-TTACAGGGAAATGAGGATGGAGCGTCTCTTCTC-3’. These primers were used to amplify the corresponding genomic DNA region. The obtained plasmid was introduced into the EcoRI-SalI site of URA3-marked, high copy yeast strain, which lacks endogenous zinc transporters, by the lithium acetate/single-strand DNA/polyethylene glycol transformation method. Positive *Ura*+ colonies were selected, and the expression of AtMTP1 was confirmed by immunoblotting with the anti-AtMTP1 antibodies prepared previously (10).

**Construction of DNA for AtMTP1 Mutant**—The DNA of AtMTP1 mutant that lacks the major part (Gly-185 to His-216) of histidine-rich loop (His-rich loop) (shown in Fig. 6A) was constructed by a PCR and type IIS restriction enzyme-based method (24). A type IIIS Esp3I restriction site (underlined) was present in each primer. We used the primer pair 5’-TAGATG-CGTCTCTTCTCATGAAATGCGTTCAAGC-3’ (forward) and 5’-TTAGACGTTCATGATGATGCTTAC-3’ (reverse). The obtained clone was used as a Δ185–216 mutant AtMTP1. The mutant *AtMTP1* DNA was introduced into a *zrc1 cot1* strain of yeast by the same method for the wild-type *AtMTP1* described above.

**Vacuolar Membrane Vesicle Preparation from Yeast**—Yeast cells were grown to the exponential phase in SC-U medium that contained 2% glucose, 0.145% yeast nitrogen base without amino acids and ammonium sulfate, 0.002% adenine, 0.008% inositol, 0.008% p-aminobenzoic acid, and 0.008% amino acids, except for 0.04% leucine and no cysteine. Cells were harvested and treated with zymolase, and spheroplasts were obtained. The spheroplast suspension was homogenized using a Teflon homogenizer. Vacuolar membranes were isolated from the spheroplast homogenate by a sucrose discontinuous gradient (15 and 35% (w/w) sucrose) centrifugation (25). The obtained membranes were suspended in 5 mM Tris-HCl, pH 6.9, 300 mM sorbitol, 1 mM dithiothreitol, 0.5 mM MgCl$_2$, 100 μM p-(amidinophenyl) methanesulfonyl fluoride hydrochloride, and 1 μg/ml leupeptin and stored at −80 °C until used.

**Measurements of Zinc Content in Vacuoles with FuraZin-1**—Zinc content in yeast vacuoles was measured with a zinc fluorescent probe (26). Yeast cultures (5 ml) were grown to the log phase in Chelex-treated synthetic defined medium supplemented with 10 μM ZnCl$_2$. Chelex-treated synthetic defined medium was prepared by treating the medium, which contained 2% glucose, 0.56% yeast nitrogen base without divalent cations or potassium phosphate (Bio101 Systems, Vista, CA) and 0.002% adenine, 0.008% inositol, 0.008% p-aminobenzoic acid, and 0.008% amino acids except for 0.04% leucine and no cysteine medium (1 liter), with 25 g of Chelex 100 resin (Sigma-Aldrich) for 12 h at 4 °C. The resin was removed, and the medium was supplemented with 7.3 mM KH$_2$PO$_4$, 10 μM FeCl$_3$, 2 μM CuSO$_4$, and 10 μM ZnCl$_2$. The solution was adjusted to pH 4.2 with HCl and then filter-sterilized into polycarbonate flasks. Cells were harvested, washed twice with phosphate-buffered saline, and resuspended in the same saline to make a final density of 3 × 10$^9$ cells ml$^{-1}$. A zinc fluorescent probe, FuraZin-1 acetoxymethyl ester (Molecular Probes, Eugene, OR; 50 μg) was dissolved in 16.6 μl of 20% pluronic solution (Molecular Probes), and the solution was diluted 4-fold with Me$_2$SO to give a stock solution of 1.25 mM fluorophore and 5% pluronic solution. Fluorophore was added to a 250-μl aliquot of the cell suspension to give a final concentration of 25 μM. The cell suspensions were incubated at 30 °C in the dark for 1 h with agitation. The cells recovered by centrifugation were washed 3 times with 0.01% aspartic acid, 0.01% glutamic acid, 0.002% histidine, nophenyl) methanesulfonyl fluoride hydrochloride, and 1 μg/ml leupeptin and stored at −80 °C until used.

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Function of His-rich Loop of Zinc Transporter AtMTP1

1 ml of chilled solution of 10 mM MES-Tris, pH 6.5, 4 mM MgCl₂, 2% glucose (for use as zinc uptake buffer), and 1 mM EDTA and then incubated at 30°C for 1 h. The cells were then chilled and washed twice with the uptake buffer. Cell pellets were resuspended in 1 ml of uptake buffer and maintained on ice before zinc uptake assays. Fluorometric assay of fluorophore speciation was performed using a RF5300PC fluorospectrophotometer (Shimadzu, Kyoto, Japan). The assay was started by adding an aliquot of yeast cells (10 μl) to 2 ml of MES-Tris uptake buffer containing 100 μM ZnCl₂. With the instrument set at maximum scan speed, the excitation wavelength was scanned from 250 to 450 nm, and the intensity of emission at the fixed wavelength of 500 nm was recorded. The corrected traces were scanned to obtain emission intensities at the excitation wavelengths of 325 and 380 nm. The zinc content is referred to as the ratio of excitation fluorescence at 325 nm to that at 380 nm (F₃₂₅/F₃₈₀ ratio).

Zn²⁺ Transport Assay—Zn²⁺ uptake into vacuolar membrane vesicles was measured using the filtration method (27). Assays were performed at 25°C in aliquots of medium containing of 300 mM sorbitol, 5 mM MES-Tris, pH 6.9, 25 mM KCl, 1 mM dithiothreitol, 5 mM MgCl₂, 200 μM NaN₃, 100 μM vanadate, 3 mM ATP-Tris, and 5 μM ⁶⁵ZnCl₂. Radioisotope ⁶⁵ZnCl₂ was purchased from Japan Radioisotope Association (Tokyo, Japan) and RIKEN (Wako, Japan). The specific radioactivity of ⁶⁵ZnCl₂ used for experiments was at a range of 0.3 to 925 TBq/mol. For the assay of the Zn²⁺/H⁺ antiporter, membrane vesicles were preincubated with 3 mM ATP-Tris for 5 or 10 min, and then Zn²⁺ transport was started by adding ⁶⁵ZnCl₂. After the reaction, the mixture was filtered through a presoaked 0.45-μm nitrocellulose filter (13 mm in diameter, Advantech, Tokyo, Japan). The filter was washed twice with 750 μl of cold wash buffer (300 mM sorbitol, 5 mM MES-Tris, pH 6.9, 25 mM KCl, and 100 μM ZnCl₂). The radioactivity associated with the filters was measured by a liquid scintillation counter. Background values resulting from incubation with 0.2 μM bafilomycin A₁ (Wako Pure Chemicals, Osaka, Japan), a potent inhibitor of vacuolar H⁺-ATPase (V-ATPase), were subtracted from corresponding values in the absence of bafilomycin A₁ and calculated as V-ATPase-dependent Zn²⁺/H⁺ exchanger activity. The proton pumping activity of endogenous V-ATPase in yeast vacuolar membranes was measured as the initial rate of fluorescence quenching of quinacrine at 25°C with a Shimadzu (Kyoto, Japan) RF5300PC fluorescence spectrophotometer set at 425 nm for excitation and 498 nm for emission as described previously (28).

SDS-PAGE and Immunoblotting—Proteins were separated by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). After blocking with de-fatting milk, the membrane filter was incubated with the primary antibody and then with horseradish peroxidase-conjugated protein A (1:2000 dilution) (GE Healthcare). Chemiluminescent reagents of ECL (GE Healthcare) were used for detection of antigens. A specific antibody to the N-terminal region of AtMTP1 (At2g46800, positions 37–50, Cys-GFSKASNAGDAHE) was prepared by injecting a synthetic peptide into rabbits and used as anti-AtMTP1 antibody (1:2000 dilution) (10). An antibody to the subunit A of V-ATPase was prepared previously (29) and used as anti-VHA-A antibody (1:2000 dilution).

**RESULTS**

Functional Expression of AtMTP1 in Yeast—To determine the functionality of AtMTP1, we expressed AtMTP1 in *S. cerevisiae* that contained neither Zrc1 nor Cot1 (zrc1 cot1). AtMTP1 protein was detected at 43 kDa in an immunoblot of the vacuolar membrane fraction from yeast cells transformed with AtMTP1 and was immunooblotted with the anti-AtMTP1 antibody. The arrow indicates the position of AtMTP1 (43 kDa). B, zinc tolerance assay of original yeast strain cells (BJ5458) expressing empty vector and mutant zrc1 cot1 yeast cells expressing empty vector (zrc1 cot1) or AtMTP1 (AtMTP1). Yeast cultures were adjusted to 1.0 optical density at 600 nm (A₆₀₀) and 5-μl aliquots were applied to HC-U plates supplemented with indicated concentrations of ZnCl₂. Colony growth was recorded 2 days after incubation at 30°C. C, zinc tolerance of yeast cells in liquid culture. Yeast strains transformed with AtMTP1 (open circles) or empty vector (closed squares) were incubated in liquid HC-U medium supplemented with indicated concentrations of ZnCl₂, and A₆₀₀ of culture was recorded 21 h after incubation at 30°C. The initial density of cell cultures was 0.01 A₆₀₀.
To monitor the active incorporation of zinc into vacuoles in living yeast cells, we used a ratiometric fluorescent zinc indicator FuraZin-1 according to the method reported previously (26). Yeast cells were incubated with the acetoxymethyl ester of FuraZin-1, washed with EDTA, and then treated with glucose to start the zinc uptake. The fluorophore was accumulated predominantly in vacuoles of \( \text{AtMTP1} \)-expressing cells (Fig. 2A).

In assay medium containing 100 \( \mu \text{M} \) \( \text{ZnCl}_2 \), vacuoles of \( \text{AtMTP1} \)-expressing yeast showed a rapid increase in the \( F_{325}/F_{380} \) ratio in a time-dependent manner (Fig. 2B). These observations indicate a marked accumulation of \( \text{Zn}^{2+} \) into vacuoles of living yeast cells with \( \text{AtMTP1} \). Thus, we concluded that the yeast cells are useful for kinetic analysis of \( \text{AtMTP1} \).

**Zn\(^{2+}/H^+\) Antiport Activity of \( \text{AtMTP1} \)—Vacuolar membranes from yeast with \( \text{AtMTP1} \), but not from yeast with an empty vector, showed high activity of zinc uptake in an ATP-dependent manner (Fig. 3A).**

The membrane vesicles were acidified by V-ATPase and then the \( \Delta \text{pH} \)-dependent \( \text{Zn}^{2+} \) uptake activity was determined. Preincubation of membrane vesicles with 3 mM ATP for 5 min resulted in maximal activity of the \( \text{Zn}^{2+}/H^+ \) antiport (data not shown). Zinc accumulation into vesicles was inhibited to the control level by 0.2 \( \mu \text{M} \) bafilomycin, a specific inhibitor of V-ATPase (30). Next, the \( \text{Zn}^{2+} \) accumulation was measured in the presence of a protonophore, carbonyl cyanide \( m \)-chlorophenylhydrazone (CCCP) (Fig. 3B). \( \text{Zn}^{2+} \) accumulation was inhibited by 10 \( \mu \text{M} \) CCCP to less than 30%, indicating that the zinc accumulation is tightly associated with a proton gradient generated by V-ATPase.

The \( \text{V-ATPase-dependent (bafilomycin-sensitive) Zn}^{2+} \) accumulation was measured with the passage of time (Fig. 4A). The amount of \( \text{Zn}^{2+} \) in membrane vesicles increased linearly up to 20 min and quickly decreased after the addition of the zinc ionophore pyrithione. Membrane vesicles of the vector control...
did not accumulate Zn\(^{2+}\). These results indicate that AtMTP1 functions as an active transporter of Zn\(^{2+}\) using a pH gradient. The dependence of the activity on the substrate concentration was determined (Fig. 4B). The zinc uptake activity of AtMTP1 was saturated above 2 \(\mu\)M Zn\(^{2+}\). The curve fitted the Michaelis-Menten equation. The \(K_m\) value was obtained at a Zn\(^{2+}\) concentration of 0.30 \(\mu\)M, and the \(V_{max}\) value was 1.22 nmol/min/mg of membrane protein by the Hanes plot analysis (see Fig. 7C).

The Zn\(^{2+}\) uptake activity of membrane vesicles from the AtMTP1/zrc1 cot1 strain was measured in the presence of other metal ions in addition to 5 \(\mu\)M \(^{65}\)ZnCl\(_2\) (Fig. 5). Mn\(^{2+}\) at 50 \(\mu\)M had no effect on the transport activity. Cd\(^{2+}\) suppressed the activity to 40%, and Fe\(^{2+}\) and Ca\(^{2+}\) suppressed to less than 70% of the control activity. Zinc accumulation was completely inhibited by Ni\(^{2+}\) and Co\(^{2+}\). The activity was thoroughly suppressed in the presence of 50 \(\mu\)M cold Zn\(^{2+}\). We could not test the effect of Mg\(^{2+}\) because the reaction medium contained 5 mM MgCl\(_2\) as a cofactor of V-ATPase. Magnesium ion is the most abundant metal in the cytoplasm. However, Mg\(^{2+}\) has no negative effect on AtMTP1 since the activity was detected in the presence of the ion. The negative effect of these ions on the AtMTP1 was either because the metal ions compete with Zn\(^{2+}\) as actual substrates or the metal ions inhibit AtMTP1 through binding to a sensitive site.

**Functional Properties of Mutant AtMTP1 Lacking a Histidine-rich Region**—AtMTP1 has a log His-rich loop with 81 residues as a characteristic structure (Fig. 6A). We examined its biochemical role by heterologous expression of a mutant AtMTP1, which lacked a major part (32 amino acid residues; positions 185–216) of the loop, in S. cerevisiae zrc1 cot1 mutant strain. Both the wild-type and His-loop mutant (Δ185–216) AtMTP1 proteins in the vacuolar membrane vesicles were detected at similar levels by immunoblotting (Fig. 6B). The mutant AtMTP1 had a slightly smaller molecular size (40 kDa) than the 43-kDa wild type because the mutant lacked 32 residues. The immunostained intensity of the subunit A of V-ATPase (VHA-A), an internal marker protein, was not
Deletion of the His-rich loop changed the enzymatic properties. Membrane vesicles from yeast cells expressing Δ185−216 AtMTP1 gave extremely high activity of Zn$^{2+}$ uptake compared with the wild type when assayed at 5 μM $^{65}$Zn$^{2+}$ (Fig. 7A). The wild-type and Δ185−216 AtMTP1 showed a linear increase in the activity for 20 and 10 min, respectively. Thus, we determined the substrate concentration dependence using a reaction time of 2 min (Fig. 7B) and determined kinetic parameters from the Hanes plots (Fig. 7C). $K_m$ values for Zn$^{2+}$ of the wild-type and Δ185−216 AtMTP1 were calculated to be 0.30 and 0.64 μM, respectively. $V_{max}$ values of the wild-type and mutant AtMTP1 were 1.22 and 13.6 nmol/min/mg of protein, respectively. The protein amount of wild-type and mutant AtMTP1 on the basis of vacuolar membrane protein was not changed (Fig. 6B). The protein amount of the subunit A of V-ATPase and the activity in yeast cells expressing the mutant were also similar to those of cells expressing wild-type AtMTP1. These results indicate that enhancement of the transport activity was not due to the increase in the amount of the AtMTP1 protein or proton pump activity. Thus, deletion of a His-rich region from AtMTP1 caused a decrease in affinity for Zn$^{2+}$ and an 11-fold increase in the maximal velocity of Zn$^{2+}$ transport.

We prepared another His-loop-deletion mutant, which lacked a part (51 residues; positions 182–232) of the loop. The yeast cells expressing the mutant became tolerant to 0.2 mM Co$^{2+}$ and an 11-fold increase in the maximal velocity of Zn$^{2+}$ transport.

**DISCUSSION**

The aim of this study was to clarify the biochemical mechanism of zinc transport by AtMTP1 and the biochemical roles of its His-rich loop. Heterologous expression of A. thaliana vacuolar membrane AtMTP1 in S. cerevisiae cells provided a good experimental system for evaluation of AtMTP1. Plants have two zinc transporters in vacuoles, AtMTP1 (10, 11) and AtMTP3 (12). The vacuolar localization of AtMTP1 and AtMTP3 was demonstrated by expression of their fusion protein with green fluorescent protein in A. thaliana suspension-cultured cells and subcellular fractionation of membranes prepared from plants (10–12). The physiological roles of zinc decreased or increased in yeast cells expressing Δ185−216 AtMTP1.

The zrc1 cot1 mutant did not grow in the presence of Zn$^{2+}$ at more than 0.2 mM, although the original strain BJ5458 was tolerant to Zn$^{2+}$ (Fig. 6C, left panel). The yeast cells, into which the wild-type AtMTP1 was introduced, were tolerant to zinc even at 10 mM. The cells expressing a Δ185−216 mutant also were tolerant to zinc and showed a slightly high tolerance to zinc at more than 10 mM compared with the control cells. Thus, the His-rich region is not essential for zinc transport. Sensitivity to other metal ions was examined on an agar plate supplemented with high concentrations of metals. There was no marked difference in tolerance to Ni$^{2+}$ and Cd$^{2+}$ between yeast cells with an empty vector, the wild-type AtMTP1, and Δ185−216 AtMTP1 (Fig. 6C, right panel). This suggests that neither the wild-type nor Δ185−216 AtMTP1 preferentially transport Ni$^{2+}$ or Cd$^{2+}$ into vacuoles. It should be noted that the yeast cells expressing the mutant became tolerant to 0.2 mM Co$^{2+}$. This means that the His-rich region is not essential for zinc transport and ion selectivity for Ni$^{2+}$ and Cd$^{2+}$ of AtMTP1.

**FIGURE 6. Ion selectivity of AtMTP1 and a His-rich-loop-modified variant.** A, schematic model of the His-rich loop between the transmembrane domain 4 and 5. Histidine residues in the loop are highlighted. The His-rich loop mutant lacked the main part of the loop (Δ185−216). B, immunoblots of membranes from yeast cells expressing the wild-type (WT, lane 1) and His-loop mutant (Δ185−216, lane 2) AtMTP1 with anti-AtMTP1 and anti-VHA-A (subunit A of V-ATPase) antibodies. C, yeast mutant strain of zrc1 cot1 was transformed with an empty vector (zrc1 cot1), AtMTP1 (WT AtMTP1), or Δ185−216 mutant (Δ185−216). Original yeast strain BJ5458 expressing empty vector (BJ5458) was also examined. Concentration of cultured cells was adjusted to 1.0 $A_{600}$, and then 5-μl aliquots of serial dilutions (from top to bottom in each panel) were spotted on HC-U plates supplemented with ZnCl$_2$ at the indicated concentrations (left panel). The plates were supplemented with 0.5 mM NiCl$_2$, 0.20 mM CoCl$_2$, or 0.12 mM CdCl$_2$ (right panel). All plates were incubated for 2 days at 30 °C.
detoxification under zinc oversupply were demonstrated by phenotypic analysis of AtMTP1 (10) and by overexpression analysis of AtMTP3 (12). AtMTP1 complements the function of yeast vacuolar Zrc1 and Cot1 (Fig. 1). The zrc1 cot1 double mutant strain is highly sensitive to zinc and unable to grow in the presence of low concentrations of ZnCl₂. Zrc1 and Cot1 are known to contribute to zinc tolerance by sequestration of excess zinc into vacuoles in yeast (26, 31, 32), and Zrc1 is considered the major transporter for zinc tolerance (26). Thus, we concluded that AtMTP1 was correctly localized in the vacuoles and kept its functionality in yeast cells.

**Functionality of AtMTP1 as a Zn²⁺/H⁺ Antiporter in the Vacuolar Membrane**—The present study revealed that AtMTP1 actively transports Zn²⁺ into vacuoles by using a pH gradient formed by V-ATPase. The heterologously expressed AtMTP1 in yeast cells has been demonstrated to function on the vacuolar membranes by using a fluorescent probe of Zn²⁺ (Fig. 2). The transport of Zn²⁺ was supported by a pH gradient across the membrane. In yeast cells, V-ATPase acidifies vacuoles, and AtMTP1 uses the pH gradient as an energy source. Zn²⁺ uptake was completely inhibited by a potent V-ATPase inhibitor bafilomycin A₁ (30) and was strongly suppressed by CCCP a protonophore (Fig. 3). The membrane vesicles accumulated zinc in a time- and dose-dependent manner (Fig. 4). In addition to V-ATPase, H⁺-translocating pyrophosphatase works as a proton pump in vacuoles of plants (33–35), although the enzyme is absent in yeast. In growing young tissues of plants, the proton pump activity of H⁺-pyrophosphatase is comparable or higher than that of V-ATPase (36). Thus, AtMTP1 utilizes a pH gradient generated by two vacuolar proton pumps, V-ATPase and H⁺-pyrophosphatase.

We examined the metal selectivity of AtMTP1 by two methods. From the competition assay, Ni²⁺ and Co²⁺ strongly inhibited the Zn²⁺ uptake activity, and Cd²⁺, Fe²⁺, and Ca²⁺ also partially inhibited the transport activity (Fig. 5). The metals other than Fe²⁺ did not inhibit V-ATPase in yeast vacuolar membrane at the assay concentrations (data not shown). Fe²⁺ inhibited V-ATPase to 73% of the control activity. The results suggest that these metals, such as Ni²⁺ and Cd²⁺, competitively inhibit AtMTP1 as additional transport substrates. However, the possibility was not confirmed by further experiments. AtMTP1 expressed in yeast cells showed no capacity to transport Ni²⁺, Co²⁺, or Cd²⁺ in the yeast growth test (Fig. 6C). The atmtp1 mutant plants of A. thaliana were hypersensitive to Zn²⁺ but not to Ni²⁺, Co²⁺, Mn²⁺, and Cd²⁺ (10). Therefore, AtMTP1 might function via a Zn²⁺/H⁺ antiporter mechanism.
with a relatively high selectivity to zinc both \textit{in vitro} and \textit{in vivo}. Metal specificity varied with the transporters. Yeast vacuolar \textit{Zrc1} has a high specificity to zinc, and yeast vacuolar \textit{Cot1} has the capacity to transport zinc and cobalt (26). \textit{Thlaspi goesingense} vacuolar membrane \textit{TgMTP1} has been estimated to have a broad specificity for zinc, cadmium, nickel, and cobalt (37). \textit{A. thaliana} vacuolar membrane \textit{AtMTP3} that contains a short His-rich loop has also been reported to be specific to zinc (12).

\textbf{Role of a His-rich Loop in \textit{AtMTP1}}—As mentioned above, the His-rich region of \textit{TgMTP1} was tightly related to the metal selectivity (37). In \textit{hZIP4}, a human plasma membrane zinc importer, a His-rich cluster has been reported to mediate the ubiquitination and degradation of \textit{hZIP4} at the upper tier of physiological zinc concentrations more than 20 $\mu$M (38). In the presence of excessive zinc, the zinc-saturated \textit{hZIP4} becomes a target molecule of ubiquitination. This mechanism is useful for protection against zinc cytotoxicity because the decrease in \textit{hZIP4} suppresses the import of excess zinc into the cells. In contrast to the present study, histidine residues in the His-rich loop have been reported to be essential for zinc transport in PC-3 cells (39). The differences in roles of His-rich regions may be due to their amino acid sequences. His-rich loop of \textit{AtMTP1} is richest in histidine residues among zinc transporters.

We examined the contribution of the region to the metal selectivity. The His-rich loop is a candidate for the metal selectivity determinant. In \textit{T. goesingense}, two \textit{TgMTP1} mRNA are generated from a single gene; a spliced (\textit{TgMTP1t1}) and a unspliced (\textit{TgMTP1t2}) transcript (37). The sequence of \textit{TgMTP1} is similar to that of \textit{AtMTP1}. \textit{TgMTP1t2} lacks a His-rich region (Fig. 8). This region contains 13 histidine residues and has an identity of 58\% with \textit{AtMTP1}. \textit{TgMTP1t1} has been demonstrated to transport cadmium, cobalt, and zinc, and \textit{TgMTP1t2} conferred the high tolerance to nickel (37). Analysis of the \textit{T. goesingense} transporter suggested that the His-rich loop might be involved in the substrate specificity of \textit{TgMTP1}. The present results showed that the His-rich region is involved in the substrate specificity because the tolerance to Co$^{2+}$ was increased in the yeast strain expressing a 185–216 mutant (Fig. 6). However, the contribution of the region to ion selectivity was not critical, since the tolerance to Ni$^{2+}$ and Cd$^{2+}$ was apparently not changed. Ni$^{2+}$ and Co$^{2+}$ may compete with Zn$^{2+}$ for binding to the His-rich region because the His-rich region of dehydrin from citrus has been reported to have a high affinity for Cu$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, and Co$^{2+}$ (40). Capacity of the mutant \textit{AtMTP1} to transport Co$^{2+}$ and Ni$^{2+}$ remains to be examined by the direct assay using radioisotopes of the metals. The yeast strain expressing a 185–216 mutant did not become tolerant to Ni$^{2+}$ like \textit{TgMTP1t2}. Thus, an ion selectivity filter may exist in the other domains including transmembrane domains. The filter might recognize the ion radius of zinc. Zn$^{2+}$ and Co$^{2+}$ have similar ion radii of 75.0 and 74.5 pm, respectively, and are different in size from Ni$^{2+}$ (69 pm) and Cd$^{2+}$ (95 pm).
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pm) (41). We estimate that the deletion of the His-rich region slightly changes the configuration of ion selectivity filter and that the mutant AtMTP1 transports Zn\(^{2+}\) and Co\(^{2+}\) with a similar ion radius.

The present study revealed that the His-rich region is not essential to zinc transport (Fig. 6). A key question to be addressed is what role the His-rich region plays in the AtMTP1. The kinetic analysis of the Δ185–216 mutant revealed that the removal of the His-rich region markedly increased the apparent maximal velocity of zinc transport and lowered the affinity to Zn\(^{2+}\) (Fig. 7). This multiple histidine region of AtMTP1 is composed of 32 residues and contains 16 histidine residues and only two acidic residues (Fig. 6). This region has a high affinity to Ni\(^{2+}\), Co\(^{2+}\), and Zn\(^{2+}\) because the histidine residue has a high affinity to these metal ions (42). The present results suggest that the His-rich region binds Zn\(^{2+}\) ions in the cytosol and that the ions are transferred from the His-rich region to the pore region consisting of transmembrane domains. A high affinity for Zn\(^{2+}\) means stable binding of ions, and this becomes an energy barrier for transfer of ions. Removal of Zn\(^{2+}\) from histidine residues might require relatively high energy. When AtMTP1 lacked the His-rich region, Zn\(^{2+}\) ion can be transported without being trapped at the His-rich region in the cytoplasmic space. In this case the energy required for ion transport may be low, and consequently, the transport velocity might increase. In other words, the His-rich region functions as a concentration sensor and buffering pocket of cytoplasmic Zn\(^{2+}\) to collect the excess ions in the vacuole. Thus, this structure has a physiological merit to plant cells. This also keeps the cytoplasmic concentration of Zn\(^{2+}\) at a low level and protects the cells from zinc toxicity.

The characteristic of the Δ185–216 AtMTP1 was a slight decrease in the affinity for Zn\(^{2+}\) (Fig. 7). The relatively high affinity of wild-type AtMTP1 may be due to the Zn\(^{2+}\) concentration effect at the His-rich loop. In total, the His-rich loop collects Zn\(^{2+}\) ions in the cytoplasm and may function as a sensor of zinc concentration in the cytosol for zinc deposition into the vacuole. At low Zn\(^{2+}\) concentrations, the ions are trapped by the loop and not transported into vacuoles. This is important to keep Zn\(^{2+}\) in the cytoplasm, where many zinc-proteins and zinc-enzymes utilize the ion. When zinc is oversupplied, the His-rich loop may be saturated with Zn\(^{2+}\), and the ions are easily transferred to the zinc entrance pocket of AtMTP1 and then transported into vacuoles. The \(K_m\) value of AtMTP1 for Zn\(^{2+}\) was 0.30 \(\mu M\) in yeast membrane vesicles (Fig. 7). This value was comparable to the \(S. cerevisiae\) endogenous zinc transporter ZRC1 (0.16 \(\mu M\)) (43), \(E. coli\) ZitB (1.4 \(\mu M\)) (44), and human hZIP4 (2.5 \(\mu M\)) (38). The obtained \(K_m\) of AtMTP1 is of importance physiologically, since most zinc-proteins have high affinity to zinc (3), and the zinc toxicity was observed above 200 \(\mu M\) in the AtMTP1 knockout mutant atmtp1 plants (10). The \(K_m\) value is one of the determinant factors of the concentration of free Zn\(^{2+}\) remained in the cytosol.

In conclusion, we propose that the His-rich region of AtMTP1 has unique functions, namely, serving as a buffering pocket to catch and stock zinc in the vacuole and as a sensor of the cytoplasmic free zinc ions. Excess Zn\(^{2+}\) in the cytosol may be trapped by the His-rich region and then actively transported into vacuole through AtMTP1. On the other hand, the trapped Zn\(^{2+}\) may be released to the cytosol when the zinc concentration is decreased. The His-rich loop has been reported to function as a sensor for high periplasmic levels of zinc has been discussed for the Synechocystis zinc transporter that belongs to the ABC transporter family (45). Furthermore, Lu and Fu (46) recently reported the x-ray structure of a \(E. coli\) Zn\(^{2+}\)/H\(^{+}\) exchanger Yiip in complex with zinc. Yiip, consisting of 298 residues and 6 transmembrane domains, exists as a Y-shaped structure of homodimer. The C-terminal part of Yiip forms a zinc binding portion. At present, it is impossible to adopt this structural model to AtMTP1 because Yiip is a member of the zinc transporter group lacking the His-rich region. Identification of the tertiary structure and kinetic properties of the His-rich loop, especially the His-rich region from positions 185 to 216, and further investigations of the phenotypic properties of the plants overexpressing the His-rich region and Δ185–216 mutant AtMTP1 should provide insight into how the loop associates with the physiological roles of the transporter.

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