A Combined Zinc/Cadmium Sensor and Zinc/Cadmium Export Regulator in a Heavy Metal Pump

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Heavy metal pumps (P1B-ATPases) are important for cellular heavy metal homeostasis. AtHMA4, an Arabidopsis thaliana heavy metal pump of importance for plant Zn\(^{2+}\) nutrition, has an extended C-terminal domain containing 13 cysteine pairs and a terminal stretch of 11 histidines. Using a novel size-exclusion chromatography, inductively coupled plasma mass spectrometry approach we report that the C-terminal domain of AtHMA4 is a high affinity Zn\(^{2+}\) and Cd\(^{2+}\) chelator with capacity to bind 10 Zn\(^{2+}\) ions per C terminus. When AtHMA4 is expressed in a Zn\(^{2+}\)-sensitive zrc1 cot1 yeast strain, sequential removal of the histidine stretch and the cysteine pairs confers a gradual increase in Zn\(^{2+}\) and Cd\(^{2+}\) tolerance and lowered Zn\(^{2+}\) and Cd\(^{2+}\) content of transformed yeast cells. We conclude that the C-terminal domain of AtHMA4 serves a dual role as Zn\(^{2+}\) and Cd\(^{2+}\) chelator (sensor) and as a regulator of the efficiency of Zn\(^{2+}\) and Cd\(^{2+}\) export. The identification of a post-translational handle on Zn\(^{2+}\) and Cd\(^{2+}\) transport efficiency opens new perspectives for regulation of Zn\(^{2+}\) nutrition and tolerance in eukaryotes.

P1B-type ATPases form a subfamily of P-type ATPases and pump metal ions across biological membranes (1–4). These pumps maintain metal homeostasis in all domains of life (5–8). Humans have only two P1B-ATPases, namely ATP7A and ATP7B, both of which transport Cu\(^{2+}\), and cause Menke and Wilson diseases, respectively, when mutated (8). In contrast, in the model plant Arabidopsis thaliana eight P1B-ATPase genes (heavy metal ATPases 1–8 (HMA1–HMA8)) are present (9, 10). Monovalent metal ions, such as Cu\(^{2+}\) and Ag\(^{+}\), are transported by HMA5–HMA8, which belong to the subclass P1B1, whereas divalent metal ions, such as Zn\(^{2+}\) and Cd\(^{2+}\), are transported by HMA2–HMA4 belonging to the P1B2 subgroup that is related to prokaryotic divalent heavy metal pumps (4, 11).

In A. thaliana, HMA2 and HMA4 are closely related in primary sequence and might have evolved as a result of gene duplication (10). In the physiology of A. thaliana, these two pumps are redundant in several functions (12, 13). Both genes are expressed in roots in the pericycle cells surrounding the xylem, a vascular tissue specialized in transport of inorganic nutrients and water to the shoot. Single knock-out mutants of AtHMA2 and AtHMA4 have weak phenotypes, whereas a double hma2 hma4 mutant accumulates zinc in root pericycle cells, which causes shoots to suffer from zinc deficiency. This strongly suggests that AtHMA2 and AtHMA4 are responsible for catalyzing zinc efflux from pericycle cells, thereby loading the xylem with zinc (12–13). In the zinc hyperaccumulator Arabidopsis halleri the gene encoding HMA4 has been copied three times (14). This, together with increased promoter strength, results in an increased capacity of this metallophyte to accumulate zinc in shoots (14).

P1B-ATPases have six to eight transmembrane segments responsible for metal ion coordination during transport, a large cytosolic portion divided into three catalytic domains (A, P, and N), and two terminal domains that contain metal-coordinating residues (the N terminus and the C terminus). The N-terminal domains of P1B-ATPases are not essential for the transport mechanism but play important roles in their post-translational regulation. In bacterial P1B-ATPases the N-terminal domains are characterized by Cys-X-X-Cys sequences (4, 7, 8, 15). The Cys residues are responsible for metal coordination and can be involved in binding both monovalent (Ag\(^{+}\) and Cu\(^{2+}\)) and divalent metal cations (Cu\(^{2+}\), Cd\(^{2+}\), and Zn\(^{2+}\)) (16–21). In plant Zn\(^{2+}\)-ATPases the Cys-X-X-Cys conserved sequence is replaced by a Cys-Cys-X-Glu motif (9, 12, 22, 23). Truncation of P1B-ATPase N-terminal metal binding domains or mutation in their metal binding sequences lead to reduced enzyme activity but does not affect the affinity of metal transport to membrane transport sites (24–29). The N-terminal metal binding domain of ATP7B has been studied in great detail. The Cu\(^{2+}\) bound by this domain is received from the...
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Intracellular Cu⁺ donor metallochaperone Atox1 (30). Following docking of Atox1 to the N-terminal domain and subsequent transfer of copper, the pump is activated. The N-terminal domain of ATP7B is furthermore required for Cu⁺-dependent intracellular targeting of the pump (31, 32) and is phosphorylated in a Cu⁺-stimulated manner (33). The N-terminal metal domain is essential for AthMA4 function in planta. Whereas expression of full-length AthMA2 can restore the zinc-deficiency phenotype of the hma2 hma4 mutant, removal of the entire N-terminal domain or mutation of the Cys residues within the conserved sequence CysX-X-Glu results in failure to complement (34).

In addition to metal-binding N termini, plant Zn²⁺-ATPases have long C termini that can contain multiple Cys-Cys repeat sequences and stretches of consecutive His residues. In A. thaliana HMA2 the C-terminal domain includes 244 amino acid residues, and in A. thaliana HMA4 it contains ~470 residues. Because both the sulfhydryl and imidazole side chains of Cys and His, respectively, have metal-coordinating capabilities, it has been proposed that the C-terminal regions of plant Zn²⁺-ATPases constitute metal-binding domains (22). In accordance with this assumption, the isolated C-terminal domain of A. thaliana HMA2 binds three Zn²⁺ ions with very high affinity (Kd = 16 nM) (22). Removal of the C-terminal domain from HMA2 leads to a reduction in enzyme turnover of 43% but does not affect the Zn²⁺ dependence of ATP hydrolysis. This suggests that the C terminus of HMA2 is a regulatory domain involved in control of enzyme turnover rate. However, following introduction into an A. thaliana hma2 hma4 double mutant, a truncated version of AthMA2 encoding a pump lacking its C-terminal domain complemented most of the zinc deficiency phenotypes to the same degree as wild-type AthMA2 (34). Further, yeast complementation studies have produced conflicting results with respect to the role of the C-terminal domain of A. thaliana HMA4 in metal transport. A mutant AthMA4 gene encoding a truncated pump lacking the last 16 C-terminal amino acid residues (which include the C-terminal His-rich stretch) could not complement yeast ycf1 (Cd²⁺-sensitive) and zrc1 (Zn²⁺-sensitive) mutations (35). In contrast, truncation of the coding sequence for the whole C-terminal domain did not affect the activity of this gene to complement ycf1 yeast (36). Therefore, it is still not clear how the C-terminal extensions of plant Zn²⁺-ATPases influence their function and whether they play a common role in all of these pumps.

In this study, we describe the role of the C-terminal domain of A. thaliana HMA4. The Zn²⁺-binding capacity of this domain and the ATP phosphorylation kinetics of AthMA4 lacking this domain were investigated. In addition, the C-terminal domain and AthMA4 C-terminal deletion mutants were expressed in a Zn²⁺-sensitive yeast strain. Our results show that the C-terminal domain of AthMA4 contains 10 metal binding sites with the capacity to chelate zinc and cadmium. Removal of the C-terminal domain does not impact phosphorylation kinetics of the pump but influences its ability to export Zn²⁺ and Cd²⁺. We propose that the C-terminal domain of AthMA4 is a Zn²⁺ and Cd²⁺ sensor involved in regulation of Zn²⁺ and Cd²⁺ export efficiency.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Deletion mutants of AthMA4 (37) were generated by PCR using the primers given in supplemental Table I and inserted into the yeast expression vector p426. All mutations were verified by sequencing. In all constructs, AthMA4 was under control of a galactose inducible (GAL1) promoter. For localization purposes, a gene encoding green fluorescent protein (GFP) was fused to the 3’-ends of AthMA4, Athma4Δ18, and Athma4Δ59 in the p426 vector. The coding sequences of two C-terminal domains of AthMA4 (C₇; amino acid residue 716–1173 and C₅; A18, residues 716–1155) were amplified by PCR. C₇ and C₅ were inserted into p426 for expression in yeast and pET15b (Novagen) for expression in Escherichia coli (supplemental Table I). All mutations were verified by sequencing.

Yeast Complementation Experiments—Zn²⁺-hypersensitive Saccharomyces cerevisiae strain zrc1 cot1 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; zrc1::natMX cot1::kanMX4) (38) and the corresponding wild-type yeast strain BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) (Euroscarf, Frankfurt, Germany) were used for gene expression and for a positive control transformed with the empty vector (p426). Yeast cells were transformed as previously described (39). Transformed yeast cells (zrc1 cot1) were used for drop test experiments for measuring Zn²⁺ and Cd²⁺ tolerance of yeast expressing AthMA4 and mutants. Yeast cells were diluted in H₂O to A₆₀₀ = 0.5 and further diluted and spotted on minimal media containing 2% (w/v) galactose (Gal), 2% (w/v) bacto-agar, 0.7% (w/v) yeast nitrogen base, 20 μg/ml His, 30 μg/ml Met, 30 μg/ml Leu, and ZnCl₂ and CdCl₂ as indicated. Plates were incubated at 30 °C for 3–5 days.

Isolation of Spontaneous Mutants of AthMA4—Plasmids were recovered from yeast suppressor mutants by first turning them into spheroplasts by incubating them in 50 mM Tris-HCl (pH 7.5), 1.2 M sorbitol, 10 mM EDTA, 200 units of lyticase (Sigma) for 3–5 h at 37 °C, then collecting them in 50 mM Tris-HCl (pH 7.5), 1.2 M sorbitol, 20 μM ZnCl₂. Isolation of mutational sites of Athma4 by PCR using the primers given in supplemental Table I. All mutations were verified by sequencing. In all constructs, AthMA4 was under control of a galactose inducible (GAL1) promoter. For localization purposes, a gene encoding green fluorescent protein (GFP) was fused to the 3’-ends of AthMA4, Athma4Δ18, and Athma4Δ59 in the p426 vector. The coding sequences of two C-terminal domains of AthMA4 (C₇; amino acid residue 716–1173 and C₅; A18, residues 716–1155) were amplified by PCR. C₇ and C₅ were inserted into p426 for expression in yeast and pET15b (Novagen) for expression in Escherichia coli (supplemental Table I). All mutations were verified by sequencing.

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Purification of Yeast Membranes and Total Protein—Total protein and membrane purification from transgenic yeast cells was done as previously described (40) but without EDTA included. Protein concentrations were determined by the method of Bradford (41) using γ-globulin as a standard for membrane proteins and bovine serum albumin (BSA) as standard for purified C-terminal domain.

Western Blot Analysis—Proteins were separated by SDS-PAGE and immobilized on a polyvinylidene difluoride (PVDF) membrane (Millipore). Recombinant proteins were detected by immunostaining with either a specific antibody against the AthMA4 C terminus 883CASKNEKGEVKVAKS850 (Geno-sphere) or by Penta-His antibody (Qiagen).

Protein Purification—pET15b vector with AthMA4 C-terminal domain and pMAL-c2X vector (New England Biolabs) containing a maltose-binding protein were expressed in E. coli strain BL21(DE3)pLys (Novagen) by standard procedures.
Recombinant proteins were affinity-purified under native conditions according to the protocols provided by the manufacturers, either by Ni²⁺-NTA agarose (Qiagen) for purification of AtHMA4 C terminus by means of its endogenous His stretch or amylose resin (New England Biolabs) for purification of the maltose-binding protein.

**Zinc Quantification in Yeast**—Yeast pellets were lyophilized and digested using microwaves as previously described (42). The digests were diluted and measured with either ICP-Optical Emission Spectroscopy (Optima 5300 DV, PerkinElmer Life Sciences) using standard settings or with ICP-MS equipped with an octopole reaction cell system (7500c, Agilent Technologies, UK). The analytical accuracy was validated using a certified reference material (1515 apple leaf, National Institute of Technology). Only elements determined with accuracy better than 90% were accepted.

**Localization of GFP Fusion Proteins in Yeast**—Transgenic yeast cells (zrc1 cot1) expressing C-terminal GFP fusion proteins of AtHMA4, Athma4Δ18, and Athma4Δ459 were localized in yeast as described previously (43) with minor modifications.

**Dithizone Staining**—Zn²⁺ bound to protein immobilized on a PVDF membrane was following renaturation detected with dithizone (0.5 mg/ml) as described (44).

**Phosphorylated Intermediate Detection**—Phosphorylation status of AtHMA4 polypeptide expressed in yeast microsomal membranes was measured at 0 °C (45, 46). Microsomes of yeast expressing AtHMA4, Athma4Δ18, and Athma4Δ268 were diluted in 20% (v/v) glycerol, 50 mM Tris-HCl, pH 7.5, and 1 mM DTT to a concentration of 10 µg/µl. 20 µg of total protein was added to 48 µl of standard reaction buffer (50 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, 5 mM Na₃P₂O₇, 50 mM KNO₃, 0.25 mM Na₂MoO₄, 1 mM DTT and varying concentrations of ZnCl₂ and EGTA). The assay was started by addition of 2.5 µCi of [γ-P³²]ATP/0.73 µM ATP and was left on ice for 30 s or as indicated before quenching by addition of 500 µl stop buffer (8% trichloroacetic acid, 1 mM phosphoric acid). For dephosphorylation experiments chase solutions of EGTA/ATP or EGTA/ADP were added to a final concentration of 5 mM EGTA and 0.5 mM ATP/ADP at t₀ before quenching at the indicated times. The samples were incubated on ice for 20 min followed by centrifugation at 16,000 × g for at least 15 min. The pellet was washed once in 500 µl of stop buffer and dissolved in loading buffer (50 mM NaH₂PO₄, pH 6.0, 0.01% SDS, 20 mM mercaptoethanol, 0.014% bromphenol blue, and 0.1 g/ml Li-SDS). An equal amount of protein (10–20 µg) was loaded onto 8% acid SDS-PAGE gels. After running, the radioactivity was visualized in a PhosphorImager scanner (Storm 860, Molecular dynamics or Cyclone Phosphoimage Storage System, Packard), and individual bands corresponding to AtHMA4 protein were quantified. Graphs were fitted to Michaelis-Menten or exponential decay kinetics using GraphPad Prism 5.0, and rate constants were estimated from the program.

**SEC-ICP-MS**—AtHMA4 C-terminal domain was reconstituted with Zn²⁺, and the ⁴⁸SO⁴⁻ and ⁶⁶Zn²⁺ ratio was subsequently quantified (42, 47). AtHMA4 C-terminal domain (400 µl; 0.6–1.0 µg protein/µl) was dissolved in 20 mM Tris(2-carboxyethyl)phosphine/1% trifluoroacetic acid and immediately transferred to a 15-ml ultrafiltration device with a 3 kDa cut-off (Amicon Ultra, Millipore). The ultrafiltration device was filled with H₂O (Ultrapure, Millipore) and centrifuged at 4000 × g (2 °C) to 0.5 ml. The sample was washed with degassed 20 mM Hepes/150 mM KCl (pH 6.8) and incubated for 30 min on ice with 15 ml of 2 µM Zn²⁺ to exchange Ni²⁺, which originated from the Ni²⁺-NTA column, with Zn²⁺. The sample was spun down on the ultrafiltration device, and the incubation procedure was repeated, and then the sample was washed to remove desorbed Ni²⁺ and residual Zn²⁺. The reconstituted Zn-AtHMA4 complex was analyzed by SEC using two sequentially connected HiTrap columns (Amersham Biosciences) hyphenated to an ICP-MS system equipped with an octopole reaction cell (7500ce, Agilent Technologies, UK). The ratio between ⁴⁸SO⁴⁻ and ⁶⁶Zn²⁺ was quantified in triplicate, using Cu/Zn superoxide dismutase (Sigma-Aldrich) as a calibrator with a well defined 4 S/Zn stoichiometry. The stoichiometry obtained for the AtHMA4 C-terminal domain was validated using three zinc-containing metalloproteins with known S/Zn ratios, as described (47). The accuracy of Zn/S ratios was always higher than 95%.

**RESULTS**

**The AtHMA4 C-terminal Domain Has a High Zn²⁺-binding Capacity**—The presence of an 11-residue His stretch and 13 Cys pairs in the C-terminal region of AtHMA4 (Fig. 1), points to the presence of metal binding sites in this region. To test whether the AtHMA4 C-terminal domain binds Zn²⁺ and Cd²⁺, we expressed the C terminus in E. coli cells and purified the recombinant protein by Ni-NTA chromatography using the endogenous His tag in the C terminus. The isolated domain readily bound Zn²⁺ and Cd²⁺ as evidenced by the color reaction with dithizone, which turns red following Zn²⁺/Cd²⁺ binding (Fig. 2A). To confirm that Zn²⁺ binds to AtHMA4, the purified C terminus was analyzed for metal-binding affinity by SEC-ICP-MS. The C terminus was initially incubated in a zinc solution to remove residual Ni²⁺ from the purification. No traces of Ni²⁺ (⁶⁶Ni²⁺), or any other element, were observed together with protein at the elution time of the sulfur signal (⁴⁸SO⁴⁻). In contrast, a clear peak of Zn²⁺ (⁶⁶Zn²⁺) eluted exactly together with the protein peak, corresponding to the Zn-AtHMA4 C terminus complex (Fig. 2B). The Zn²⁺-binding capacity of the C terminus determined by analyzing the S/Zn ratio in the peak containing the Zn-AtHMA4 C terminus complex was 5.2 ± 0.2. Because the expressed C-terminal domain contains 50 sulfur groups (43 cysteines and 7 methionines), this corresponds to ~10 Zn²⁺ ions per C terminus.

**The C Terminus of AtHMA4 Serves as a Zn²⁺ and Cd²⁺ Chelator in Vivo**—To test whether the C-terminal domain can serve as a Zn²⁺ and Cd²⁺ chelator in living cells, we expressed the 458 C-terminal residues of AtHMA4 (residues 716–1173) along in the yeast zrc1 cot1 strain; this lacks some of the main transporters controlling cellular Zn²⁺ homeostasis and, as a consequence, is sensitive to elevated Zn²⁺ in the growth medium. Yeast cells expressing AtHMA4 C terminus to high levels were able to grow on 500 µM Zn²⁺ and furthermore accumulated Zn²⁺ in contrast to yeast cells transformed with an
empty vector (Figs. 2C and 5C). When grown on Cd\(^{2+}\)/H\(^{+}\), C-terminal-expressing yeast also accumulated Cd\(^{2+}\)/H\(^{11001}\) to a higher level than the control (see Fig. 6B). These results are consistent with a supposed role of the C-terminal domain as a Zn\(^{2+}\)/H\(^{11001}\) and Cd\(^{2+}\)/H\(^{11001}\) metal chelator. Compared with the full-length C-terminal domain, truncating the terminal 18 residues (Ct\(^{18}\)) only slightly decreased the ability to induce yeast Zn\(^{2+}\)/H\(^{11001}\) tolerance (Fig. 2C).

Yeast Expressing AtHMA4 Has Increased Zn\(^{2+}\)/H\(^{11001}\) and Cd\(^{2+}\)/H\(^{11001}\) Tolerance—Depending on the growth conditions, full-length AtHMA4 conferred Zn\(^{2+}\)/H\(^{11001}\) tolerance to zrc1 cot1 yeast cells. Complementation showed a strong pH dependence, and the rescue effect was most pronounced when the growth medium was at pH 4–5 (supplemental Fig. 1). In the conditions generally used in the experiments in this study, pH 5.5–6, the efficiency of AtHMA4 in suppressing the Zn\(^{2+}\)/H\(^{11001}\) sensitivity of zrc1 cot1 was markedly reduced (supplemental Fig. 1). This phenomenon is supportive of a mechanism of Zn\(^{2+}\)/H\(^{+}\) countertransport for AtHMA4.

Full-length AtHMA4 complemented zrc1 cot1 cells to a much weaker degree than the C-terminal domain expressed alone on both Zn\(^{2+}\)/H\(^{11001}\) and Cd\(^{2+}\)/H\(^{11001}\) (see Figs. 4C and 6A). AtHMA4 was expressed in yeast to a much lower degree than the soluble C-terminal domain alone (Fig. 2D), which may suggest less chelation. However, the zinc tolerance conferred to zrc1 cot1 cells by AtHMA4 was due to a transport function rather than chelation as the D401A mutant, in which the essential Asp residue required for pump activity is mutated, no longer confers resistance (supplemental Fig. 1). In addition, we investigated the effect of gradually removing parts of the C terminus from AtHMA4 to determine how this affected complementation by the pump.

We used stringent conditions where the wild-type pump could not suppress the zrc1 cot1 mutant phenotype (high ZnCl\(_2\), pH 6.0). Initially we tested a truncated version of the pump that lacks the 18 C-terminal residues (AtHMA4\(^{\Delta18}\)), because this region had previously been shown to be essential.
for catalytic activity (35). Surprisingly this showed significantly improved Zn^{2+} tolerance (Fig. 3A). One explanation could be that the modified C terminus in some way chelates excess cytoplasmic Zn^{2+} more efficiently than the wild-type pump. However, introducing the D401A active site mutant into the Athma4\Delta18 background completely abolished the positive effect on growth (supplemental Fig. 2). This demonstrates that genetic complementation of the zrc1 cot1 mutant by Athma4\Delta18 was linked to the catalytic activity of the pump and not to its Zn^{2+}-chelating property.

Spontaneous Suppressor Mutations in Athma\Delta18 Result in C-terminal Deletions of the Pump—When zrc1 cot1 cells expressing Athma4\Delta18 were plated on Zn^{2+}-containing media, rapidly growing colonies occasionally appeared on the plates. Suppressor mutants of zrc1 cot1 were never observed with yeast cells expressing wild-type Athma4. All expression plasmids rescued from suppressor mutants and reintroduced into zrc1 cot1 yeast cells conferred Zn^{2+} tolerance (Fig. 3B), and, following sequencing, spontaneous mutations could be observed in the Athma4\Delta18 DNA sequences of the plasmids. When 15 such mutations were compared they formed three mutant groups; these contained stop codons due to frameshift mutations that resulted in C-terminal deletions of 204, 359, or 369 residues, respectively (supplemental Table II).

Sequential Deletion of C-terminal CC Pairs of Athma4 Results in a Gradual Increase in Zn^{2+} Tolerance of Transformed zrc1 cot1 Cells—To study the role of C-terminal CC pairs in more detail, 13 deletion mutants, lacking between 32 to 459 of the last amino acid residues, and from 1 to 13 CC pairs, respectively (Fig. 1), were generated by introducing stop codons in the sequence by site-directed mutagenesis. Expression in yeast demonstrated that a gradual increase in Zn^{2+} tolerance of the mutant yeast strain was correlated with the number of amino acid residues removed from the C terminus of Athma4 (Fig. 4). Removal of 157 residues (Athma4\Delta157) produced a maximal effect (Fig. 4). None of the C-terminally deleted pumps containing the D401A mutation supported yeast growth on Zn^{2+}-containing media (supplemental Fig. 2).

When GFP was fused to the C termini of Athma4, Athma4\Delta18, and Athma4\Delta459, all constructs showed the same intracellular localization in transgenic yeast as visualized by confocal laser scanning microscopy. GFP fluorescence was intense in the periphery of the cells indicating plasma membrane localization and was also seen around the nucleus indicating localization to the endoplasmic reticulum, as often seen when plasma membrane proteins are overexpressed in yeast cells (43, 48) (Fig. 5B). A similar trend in zinc tolerance as observed for the non-tagged pumps was observed for the GFP-tagged pumps (supplemental Fig. 3) confirming that the GFP-tagged pumps were functional. Hence, the differences in mediating yeast Zn^{2+} tolerance were not caused by differential localization of wild-type and mutant pumps.

Cytoplasmic Zn^{2+} Is Reduced in Yeast Expressing Truncated Athma4—When metal concentrations in transgenic yeast cells were analyzed by ICP-Optical Emission Spectroscopy or ICP-MS we found that cells expressing either Athma4\Delta18 or Athma4\Delta459 had significantly lower Zn^{2+} concentrations than yeast cells expressing Athma4 and control cells transformed with empty vector (Fig. 5B and Table 1). This result is consistent with Athma4\Delta18 and Athma4\Delta459 functioning at the plasma membrane to pump Zn^{2+} out of the cell and hence confer tolerance to high Zn^{2+}. Although cells expressing Athma4\Delta18 and Athma4\Delta459 had comparable total Zn^{2+} contents, cells expressing Athma4\Delta459 grew better than those expressing Athma4\Delta18 on high Zn^{2+}.

The C Terminus of Athma4 Is Involved in Handling of Cadmium in zrc1 cot1—Athma4 has been shown to increase cadmium tolerance in wild-type yeast and in the
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Cd\(^{2+}\)-sensitive yeast strain ycf1 (35, 36). To compare the effect of AtHMA4 C terminus in handling Cd\(^{2+}\) in addition to Zn\(^{2+}\), we analyzed AtHMA4, Athma4\(\Delta 18\), Athma4\(\Delta 459\), and the C terminus alone by expressing them in the same yeast strain, zrc1 cot1, but grown with increasing amounts of Cd\(^{2+}\) (Fig. 6A). Expression of AtHMA4 resulted in toxicity when grown on Cd\(^{2+}\) compared with the vector control. The toxicity was not evident with the pump lacking the His stretch, Athma4\(\Delta 18\), whereas removal of the whole C terminus resulted in a pump that was able to increase the Cd\(^{2+}\) tolerance of zrc1 cot1 (Fig. 6A). The toxic effect of AtHMA4 in zrc1 cot1 was supported by ICP-MS data, where AtHMA4 accumulated Cd\(^{2+}\) in contrast to Athma4\(\Delta 18\) and Athma4\(\Delta 459\), which had a significantly lower level of Cd\(^{2+}\) (Fig. 6B). In contrast to the growth experiment with zrc1 cot1 cells expressing Athma4\(\Delta 18\) and Athma4\(\Delta 459\), no effect could be seen with these two mutant pumps in the ICP-MS analysis compared with the vector control. This is likely due to the much lower concentration of CdCl\(_2\) (1 \(\mu\)M) used for the ICP-MS analysis enabling all transformants to grow in contrast to the growth analysis.

These results indicate that expression of full-length AtHMA4 causes mis-handling of Cd\(^{2+}\) in zrc1 cot1 cells. Partial or total removal of the C terminus results in a better handling of Cd\(^{2+}\) by zrc1 cot1 cells, which causes an increase in Cd\(^{2+}\) tolerance in a similar manner as seen for the Zn\(^{2+}\) tolerance mediated by Athma4\(\Delta 459\) and Athma4\(\Delta 18\) (Fig. 4). In both circumstances removal of the whole C terminus resulted in the best handling of Zn\(^{2+}\) and Cd\(^{2+}\).

In zrc1 cot1 cells, expression of the C terminus alone results in an improved Cd\(^{2+}\) tolerance on as high a concentration as 160 \(\mu\)M CdCl\(_2\) (Fig. 6A). This is most likely due to chelation of free Cd\(^{2+}\) by binding to the C terminus as mentioned above (Fig. 2A). This is in agreement with an increased total amount of Cd\(^{2+}\) in the yeast cells expressing the C terminus, as shown by ICP-MS (Fig. 6B).
Phosphorylation Kinetics Are Not Influenced by the C-terminal Domain of AtHMA4—Both wild-type and truncated pumps (here exemplified by Athma4Δ18 and Athma4Δ268) were readily expressed to the same degree in yeast (Fig. 7A). However, it was not possible to observe Zn\textsuperscript{2+}-dependent ATPase activity in membranes from transformed yeast cells. Therefore, as an alternative approach to biochemically characterize the expressed proteins, we measured the steady-state phosphorylation levels of AtHMA4 following incorporation of \textsuperscript{32}P from \textsuperscript{32}P-labeled ATP. This analysis took advantage of the fact that, in all P-type ATPases, an obligatory step of the reaction cycle is phosphorylation of a conserved aspartate residue by ATP-Mg (1, 2).

AtHMA4 and truncated mutants were all able to generate a phosphorylated intermediate even in the absence of added Zn\textsuperscript{2+} expected to be essential for the reaction (Fig. 7, B and C).

This was probably due to trace amounts of Zn\textsuperscript{2+} present in the preparation as phosphorylation levels could be diminished by adding EGTA (0.5 mM) (Fig. 7C). As expected, ATP did not phosphorylate the Athma4D401A mutant (Fig. 7B). When titrating pump proteins with Zn\textsuperscript{2+} in the presence of EGTA (0.5 mM) the steady-state phosphorylation level of Athma4Δ268 was slightly but significantly higher than that of the wild-type protein (Fig. 8A). Similar amounts of protein were used in each experiment; this indicates that a somewhat larger pool of protein was active in the Athma4Δ268 preparation than in that of the wild type or, alternatively, that truncation had displaced the E1–E2 conformational equilibrium toward a phosphorylated state.

The half-maximal concentration at which Zn\textsuperscript{2+} induced phosphorylation was the same for both proteins (~5 \(\mu\text{M}\) Zn\textsuperscript{2+}) indicating that they have comparable Zn\textsuperscript{2+} affinities (Figs. 8A and Table 2). This supports the notion that the C terminus does not have an effect on the Zn\textsuperscript{2+} binding to the translocation site. In steady-state experiments, the ATP affinity of both pumps was ~2 \(\mu\text{M}\) (Fig. 8B). Measured under the same conditions (0 °C), the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase has a significantly higher ATP affinity of 0.1 \(\mu\text{M}\) (46). Maximal phosphorylation did not occur before 20 s with half-maximal phosphorylation obtained after ~8 s (Fig. 8C). For comparison, the Ca\textsuperscript{2+}-ATPase is under the same conditions (2 \(\mu\text{M}\) ATP and 0 °C) fully phosphorylated in ~1 s (46). When the decay of the phosphoenzymes was studied as a function of time, both could be fitted with a monoexponential curve with half-maximal dephosphorylation observed after ~5 s (Fig. 8D). The rate of the dephosphorylation reaction could not be increased by ADP addition and again could be fitted by a monophasic curve similar for both wild-type AtHMA4 and truncated enzyme (Fig. 8E). This is compatible with a model in which AtHMA4 rapidly undergoes the E1P to E2P conformational transition associated with Zn\textsuperscript{2+} transport and subsequently accumulates in the E2P state. From the data it appears that the C terminus does not have an effect on the E1P to E2P transition. The relatively low ATP affinity and the slow kinetics of phosphorylation and dephosphorylation of AtHMA4 expressed in yeast indicate a very low turnover rate and readily explain why significant ATPase activity of the enzymes could not be detected.

**DISCUSSION**

Plants are the only eukaryotic organisms with Zn\textsuperscript{2+}-ATPases, and the extended C termini of these pumps are unique for P1B-ATPases. Taking into consideration the important physiological roles of plant Zn\textsuperscript{2+} ATPases, it is to be expected that mechanisms have evolved to fine-tune their activity at the posttranslational level. In Cu\textsuperscript{2+}-ATPases, it has been demonstrated that N-terminal metal binding domains play regulatory roles. In this light, the long C-terminal extensions of plant Zn\textsuperscript{2+}-ATPases being rich in Cys and His residues are interesting, but their role in metal binding and pump regulation remains unclear. To understand the function of these C-terminal domains uniquely associated with eukaryotic Zn\textsuperscript{2+}-ATPases we used the AtHMA4 C-terminal domain as a model. We investigated whether it is able to bind metals and whether it plays a role in the pump efficiency of AtHMA4. The results presented...
support a function of this domain as a dual metal sensor and pump regulator.

The Metal-binding Capability of the C-terminal Domain of AtHMA4—In this work we found that the C-terminal domain of AtHMA4 contains 10 metal binding sites, which is significantly more than the three zinc sites identified in the related AtHMA2 pump (22, 47). However, because these termini have various lengths, the shortest being that of Arabidopsis halleri HMA3 with 61 amino acid residues and the longest that of Thlaspi caerulescens HMA4 with 479 residues, and because they lack overall homology, it is not to be expected that they share identical properties and functions.

The C terminus of AtHMA4 contains multiple Cys-Cys sequence motifs and a long terminal stretch of 11 consecutive His residues not found in AtHMA2. In AtHMA2, the C terminus contains a number of HXH repeats not found in AtHMA4 and several Cys residues. Analysis by zinc K-edge x-ray absorption spectroscopy and chemical modification has shown that in AtHMA2 His residues are most important for zinc coordination, whereas Cys residues play a less prominent role (22). Further experiments are needed to test how Cys and His residues in AtHMA4 communicate to generate the metal binding sites.

The Functional Role of the C-terminal Domain of AtHMA4—Deletions in the C-terminal domain of AtHMA4 resulted in a pump protein that was superior compared with the wild-type pump in rescuing Zn$^{2+}$/H_11001 and Cd$^{2+}$/H_11001 sensitivity of the S. cerevisiae zrc1 cot1 strain. We suggest that this is due to a better handling of the metals, possibly through extrusion from the cytoplasm and that the C-terminal domain reduces pump efficiency. A recurring theme in P-type ATPases is that extended terminal domains play negative regulatory roles. For example, P2B Ca$^{2+}$/H_11001-ATPases and P3A H$^+$-ATPases are equipped with a regulatory domain (R-domain) situated in either an extended N or C terminus. In both examples, the R-domains function as autoinhibitors. Autoinhibition is relieved following binding of regulatory proteins with pump activation as the result (49–51).

| Protein expressed | $K_m$ for Zn$^{2+}$ (µM) | Steady-state EP$^{2+}$ level$^a$ (pmol EP$^{2+}$/min/total µg protein) |
|-------------------|--------------------------|-------------------------------------------------|
| ATHMA4            | 3.7 ± 0.8                | 9.41 ± 1.03                                     |
| Athma4Δ268        | 4.8 ± 0.7                | 13.74 ± 1.17                                    |

$^a$ Numbers refer to picomoles of EP$^{2+}$ formed per minute per total micrograms of protein compared to the activity in the absence of added Zn$^{2+}$.

FIGURE 8. Kinetics of phosphorylation and dephosphorylation of AtHMA4 and the C-terminal mutant Athma4Δ268. A, Zn$^{2+}$-dependent phosphorylation. Phosphorylation was initiated in the presence of 500 µM EGTA with addition of Zn$^{2+}$ as indicated. B, ATP-Mg-dependent phosphorylation. Phosphorylation was initiated at conditions giving a maximal steady-state phosphorylation level above, with addition of ATP as indicated so that the ratio of hot to cold ATP remained the same for all measurements. C, time course of the phosphorylation reaction. D, dephosphorylation in the presence of ATP. Dephosphorylation was initiated after phosphorylation for 30 s by addition of a chase solution producing final concentrations of 5 mM EGTA and 0.5 mM unlabeled ATP. E, dephosphorylation in the presence of ADP. Dephosphorylation was initiated after phosphorylation for 30 s by addition of a chase solution producing final concentrations of 5 mM EGTA and 0.5 mM unlabeled ADP. Values reported are the mean ± S.E. for at least three independent measurements.
Expression of the 458 C-terminal residues of AtHMA4 in zrc1 cot1 similarly rendered this mutant able to grow on high levels of zinc and cadmium. How can complementation of zrc1 cot1 yeast cells be achieved by expression of a pump lacking the C-terminal domain when the C-terminal domain alone produces the same effect? The very different expression levels for the two polypeptides in yeast provide a simple explanation for this apparent contradiction. The C-terminal domain alone is expressed to very high levels in its heterologous host, and we propose that it lowers cytoplasmic zinc and cadmium by acting as a passive Zn\(^{2+}/\text{Cd}^{2+}\) chelator. As wild-type and truncated AtHMA4 are expressed at much lower levels their passive contribution to lowering cytoplasmic zinc and cadmium is negligible. However, the truncated pump is contributing to example zinc tolerance by actively extruding Zn\(^{2+}\). In yeast, wild-type AtHMA4 is not as efficient as the C-terminally truncated pump. Full activation of AtHMA4 by elevated cytoplasmic zinc might require additional proteins or factors not present in the heterologous system.

To study the impact of the C-terminal domain on enzyme catalysis we studied partial enzyme reactions by measuring formation and degradation of the phosphorylated reaction cycle intermediate. Both in the presence and in the absence of the C-terminal domain the phosphorylation and dephosphorylation rates were very slow compared with other P-type ATPases. The very low rate of enzyme turnover readily explains why it has not been possible so far to assay the ATP hydrolytic activity of AtHMA4 using traditional ATPase assays. It requires further studies to ascertain whether the low turnover number of AtHMA4 is an intrinsic property of the pump or reflects the lack of subsidiary proteins or ligands in the heterologous system. A surprising finding was that the phosphorylation kinetics of AtHMA4 were not affected by deletion of the C-terminal domain. The rate of protein phosphorylation as well as the rate of dephosphorylation remained the same with and without the C-terminal domain, which indicates that this region does not influence enzyme turnover. In contrast, deletion of the C-terminal domain of AtHMA2 leads to a 43% reduction in enzyme turnover (22).

An intriguing question arises as to how the C-terminally truncated AtHMA4 can have a strong positive impact on the ability of transformed yeast cells to handle Zn\(^{2+}\), when the kinetics, localization, and expression levels of the truncated pump remain essentially the same as the wild-type protein. The simplest explanation is that deletion of the C-terminal domain results in an improved Zn\(^{2+}\) pumping efficiency, i.e. the number of zinc ions transported per ATP in the wild-type pump is lower compared with that of the truncated pump. The ability to alternate between tightly coupled and non-tightly coupled (slipping) states is common among biological pumps, including P-type ATPases (52). Plasma membrane H\(^{+}\)-ATPase (53–55) and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (56) are examples of P-type ATPases in which pumping efficiency can be regulated by slipping.

An alternative, but more speculative, hypothesis is that the C-terminal sensor could have alternative or additional functions that cannot be detected in the heterologous expression system employed in this work, because they require accessory partners only found in the homologous system. The Menke disease protein ATP7A normally resides in Golgi membranes but at elevated Cu\(^{2+}\) levels escapes the Golgi to be delivered to the plasma membrane by a mechanism involving metal binding domains in its N terminus (6, 57). The yeast Cd\(^{2+}\) pump Pca1p has a degradation signal for ubiquitin-mediated proteolytic degradation in its metal binding N-terminal domain making the protein extremely unstable under normal conditions (58, 59). Increased Cd\(^{2+}\) in some way masks this signal and allows the pump to escape degradation and enter the secretory pathway for surface expression (58). In both these examples, elevation of heavy metals is transduced into a trafficking response of P1B-ATPases. It remains to be tested whether the intracellular localization of AtHMA4 in planta is dependent on Zn\(^{2+}\) or Cd\(^{2+}\) binding to its C terminus.

In this study we have analyzed the role of the C-terminal domain of AtHMA4 in more detail. Based on our results we suggest a model in which the metal binding domain serves a dual role as Zn\(^{2+}/\text{Cd}^{2+}\) sensor and negative regulator of heavy metal ion export activity. AtHMA2 and AtHMA4 are closely related pumps, but their C-terminal extensions are very different in length and show no sequence homology to each other. Deleting the C terminus of AtHMA2 causes reduced ATP hydrolytic activity (22), whereas deletion of the same region in AtHMA4 has no apparent effect on enzyme turnover but results in more efficient zinc or cadmium pumping. At present the physiological relevance of this diversity remains unclear. Because zinc is an essential nutrient that at high concentrations becomes toxic, differential regulation of related isoforms could be a means to cope with multiple challenges resulting from too low and too high zinc or cadmium toxicity. This might require diversified Zn\(^{2+}/\text{Cd}^{2+}\) sensors that through different regulatory mechanisms evoke different responses.

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