Functional and Biochemical Characterization of Cucumber Genes Encoding Two Copper ATPases CsHMA5.1 and CsHMA5.2*

Received for publication, October 19, 2014, and in revised form, April 29, 2015. Published, JBC Papers in Press, May 11, 2015, DOI 10.1074/jbc.M114.618355

Magdalena Migocka†‡, Ewelina Posyniak†, Ewa Maciaszczyk-Dziubinska‡, Anna Papierniak§, and Anna Kosieradzaka§

From the †Institute of Experimental Biology, Department of Plant Molecular Physiology, and §Institute of Experimental Biology, Department of Genetics and Cell Physiology, ‡University of Wroclaw, Kanonia 6/8, 50-328 Wroclaw, Poland

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s)KJ818254 and KJ818255.

†To whom correspondence should be addressed. Tel: 48-71-3754113, E-mail: mmigocka@biol.uni.wroc.pl.

‡This work was supported by Polish Ministry of Science and Higher Education Grant IP2011 035871 and Wroclaw University Grant 1227/M/IBR/11. The authors declare that they have no conflicts of interest with the contents of this article. The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s)KJ818254 and KJ818255.

§Institute of Experimental Biology, Department of Plant Molecular Physiology, and Institute of Experimental Biology, Department of Genetics and Cell Physiology, University of Wroclaw, Kanonia 6/8, 50-328 Wroclaw, Poland

Background: Plant copper HMA5-like ATPases have not been biochemically characterized yet.

Results: Cucumber copper ATPases CsHMA5.1 and CsHMA5.2 are tonoplast monovalent copper transporters differentially regulated by copper availability.

Conclusion: HMA5-like ATPases may contribute to vacuolar sequestration of copper excess in plant cells.

Significance: Orthologous transporters are differently displayed to achieve organismal copper homeostasis.

Cucumber copper ATPases CsHMA5.1 and CsHMA5.2, identified in the tonoplast of cucumber cells. Interestingly, the root-specific antibodies revealed the presence of CsHMA5.1 and CsHMA5.2 copper transporters, indicating that they contribute to copper homeostasis within plant cells, but until now other transporters that regulate copper transport in plants have not been identified. These data identify CsHMA5.1 and CsHMA5.2 as likely candidates for copper transport in plant cells, with CsHMA5.1 being responsible for the increased sequestration of copper in vacuoles of cucumber root cells under copper excess.

This article has been withdrawn by Magdalena Migocka, Ewelina Posyniak, Ewa Maciaszczyk-Dziubinska, and Anna Kosieradzaka. Anna Papierniak could not be reached. The CsCACS gels in Fig. 3 (A and B) were duplicated. The following were found in Fig 4. The CsHMA5.1 immunoblot was inappropriately manipulated. Lanes 6 and 7 of the CsHMA5.2 immunoblot were duplicated. Lanes 1 and 2 of the PM H-ATPase immunoblot and lanes 6-9 of the PPase immunoblot are the same. Additionally, lane 8 of the PM H-ATPase immunoblot was reused in lane 1 of the PPase immunoblot. Lanes 3-6 of the PM H-ATPase immunoblot were rotated 180° and reused as lanes 2-5 of the PPase immunoblot. Finally, the markers were reused between the PM H-ATPase and PPase immunoblots. Fig. 5 (A and B) contained many repeating features. In Fig. 8B, the first four colonies in the dilution series for the upper control CsHMA5.2 were reused in the lower control vector dilution series. Additionally, in the lower control panel, the rightmost in the dilution series was duplicated between vector and CsHMA5.2. Finally, some colonies shown in Fig. 8B and the V-PPase immunoblot in Fig. 9B were previously published in J. Exp. Bot. 2015 66:1001-1015, representing different experimental conditions.

Based on current knowledge, copper transport has been attributed to plant HMA1 (subgroup P_1B1) and HMA5–8 (subgroup P_1B4) P_1B-ATPases. Members of the P_1B4 subgroup include broad-spectrum transporters of metals. A. thaliana HMA1 was shown to be a Ca^{2+}/heavy metal pump localized in the plasma membrane, and nine CsHMA5.2 genes have been identified.

The abbreviations used are: HMA, heavy metal ATPase; BCS, bathocuproine disulfonate; sRT-PCR, semiquantitative RT-PCR; PPase, pyrophosphatase.

JUNE 19, 2015 • VOLUME 290 • NUMBER 25

WITHDRAWN

July 31, 2019
chloroplast envelope, which delivers copper from the cytosol into the chloroplast stroma and detoxifies chloroplasts from 
Zn$^{2+}$ under zinc excess (8, 9). A similar protein from barley (Hordeum vulgare), HvHMAl, was shown to be involved in mobilization of zinc and copper from plastids under zinc or copper deficiency or from the intracellular compartments of the mineral storage site of grains (aleurone cells) during grain filling and germination (10). In comparison, the P$_{1,3}$ subgroup of plant P$_{1,3}$-ATPases includes proteins that are highly specific for monovalent Cu$^+$ (11, 12). The first characterized plant HMA, AtHMA7 (RAN1), is a major component of the plant hormone ethylene signaling pathway because it delivers copper to the secretory pathway to supply the formation of functional copper-dependent ethylene receptors (13, 14). Chloroplast-localized HMA6 (PAA1) and HMA8 (PAA2) are responsible for the import of copper to the chloroplasts to provide the cofactor for copper-dependent activities, antioxidant enzyme CuZn-superoxide dismutase and plastocyanin, involved in photosynthesis (12, 15, 16). Contrary to AtHMA6–AtHMA8, which deliver copper to the target proteins, AtHMA5 appears to be involved in detoxification of Arabidopsis roots from copper excess (17). A similar function has been recently reported for the homologous protein OsHMA5 in rice (18). The athma5 knock-out mutant is hypersensitive to copper, whereas rice knock-out lines for OsHMA5 accumulated more copper in roots and shoots, when compared with wild type plants (18). In addition, OsHMA5 was shown to be localized in the plasma membrane (18). These observations suggest that HMA5 is involved in root-to-shoot transport of copper, thus protects plant roots from copper excess (17). In contrast to A. thaliana, rice possesses two duplicates of OsHMA5 and OsHMA5-like genes, one of which (OsHMA5-like P1B-ATPase) (20). Based on the homology

### Experimental Procedures

#### Plant Material and Growth Conditions—Cucumber (Cucumis sativus var. Krak) plants were grown in hydroponics as described earlier (21). To induce copper deficiency, following germination the seedlings were grown for 2 weeks in nutrient solutions completely deprived of copper. To impose copper toxicity, 2-week-old cucumber seedlings were transferred to the fresh nutrition media supplemented with 20 μM CuCl$_2$ and grown for the next 72 h.

#### Yeast Expression, Metal Tolerance, and Accumulation—Saccharomyces cerevisiae (ACE1::kanMX4) and Δyef1 (Δyef1::kanMX4) BY4742 (MATa his3Δ1 leu2Δ0 rec1Δ0 ura3Δ0) were purchased from Euroscarf. CsHMA5.2 sequences from contigs of cucumber were retrieved from the GenBankTM. For heterologous expression, full-length CsHMA5.1 and CsHMA5.2 were amplified by PCR using primers introducing restriction sites (forward 5'-AAA TCT AGA GTT ATG CTG GTC GAC CTC AAC TCT AAT GCC TTG TAT CTC A-3' and reverse 5'-TTT TTT TTT TTT TTT TTG AAG TTA CCG CGG TG-3') and SpeI (reverse 5'-TTT TTT TTT TTT TTT TTG AAG TTA CCG CGG TG-3') and SalI (forward 5'-AAA TCT AGA GTT ATG CTG GTC GAC CTC AAC CAC TAT TCC ATT CAT TGG AAT-3') and XbaI (forward 5'-AAA TCT AGA GTT ATG CTG GTC GAC CTC AAC CAC TAT TCC ATT CAT TGG AAT-3') and XbaI/SpeI or XbaI/SalI

#### Isolation of Vacuolar Membranes from Yeast Cells and Plant Roots—Exponentially growing yeast cellsexpressing CsHMA5.1 or CsHMA5.2 using the yeast transformation kit (Sigma). For the drop test experiments, yeast cell cultures at an initial $A_{600}$ of 0.3 were serially diluted and spotted on standard complete minimal media without uracil, containing 2% (w/v) glucose (Gal), 2% (w/v) bacto-agar, 0.7% (w/v) yeast nitrogen base (YNB), and amino acids without methionine (SC-U/Glu), supplemented or not (control) with 50 μM CuCl$_2$ or 15 μM AgNO$_3$. Plates were incubated at 30 °C for 3 to 5 days. For copper uptake, the liquid SC-U/Glu yeast culture (OD$_{600}$ ~ 0.5) was supplied with 100 μM CuCl$_2$ and subsampled after 1, 2, 4, and 8 h of growth in the presence of metal excess. The intracellular copper content was determined essentially as described earlier (24, 25) using the Atomic Absorption Spectrophotometer 3300 (PerkinElmer Life Sciences).

### Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2

Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2 includes proteins that are highly specific to HMA5-like P1B-ATPase (20). Based on the homology

### Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2

Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2 includes proteins that are highly specific to HMA5-like P1B-ATPase (20). Based on the homology

### Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2

Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2 includes proteins that are highly specific to HMA5-like P1B-ATPase (20). Based on the homology

### Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2

Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2 includes proteins that are highly specific to HMA5-like P1B-ATPase (20). Based on the homology

### Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2

Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2 includes proteins that are highly specific to HMA5-like P1B-ATPase (20). Based on the homology

### Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2

Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2 includes proteins that are highly specific to HMA5-like P1B-ATPase (20). Based on the homology
Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2

Immunolocalization of CsHMA5.1 and CsHMA5.2 in Yeast and Cucumber Cells—Polyclonal anti-CsHMA5.1 and anti-CsHMA5.2 antibodies were raised in rabbit against the keyhole limpet hemocyanin-conjugated peptides ISKDGT-DHRSREVC, corresponding to N-terminal amino acids 101–114 of CsHMA5.1 protein and TGSGRYKATIFPEGC, corresponding to N-terminal amino acids 202–215 of CsHMA5.2 protein (GenScript). The position of peptides is marked in Fig. 2A. In addition, commercially available antibodies (Agrisera) against marker enzymes for plasma membrane (H^+-ATPase, AS09 260, diluted 1:10,000 in PBS-T) and tonoplast (vacuolar pyrophosphatase Pase, AS12 1849, diluted 1:2000 in PBS-T) were used to determine membrane purity. Western blot analysis was performed essentially as described earlier (20), using 1:1000 diluted antibodies for CsHMA5.1 or CsHMA5.2. Following incubation with the primary antibodies, the binding of anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (diluted 1:2000 in PBS-T; AS09 602, Agrisera) was visualized using the enhanced chemiluminescence system (ECL; Amersham Biosciences). Total RNA were extracted from CsHMA5.2 transformed with empty vector were subtracted from the results obtained in membranes containing cucumber CsHMA5.1 or CsHMA5.2.

Isoelectric focusing of Cu-ATPases CsHMA5.1 and CsHMA5.2 isolated from yeast vacuole confirmed the presence of Cu-ATPase isoforms CsHMA5.1 and CsHMA5.2 in yeast vacuole. 300 pmol of CuSO_4 or 1 mM BCS and 300 nmol of bafilomycin to avoid the background resulting from the activity of vacuolar V-ATPase. 300 pmol of CuSO_4 or 1 mM BCS (complex specific chelator) was included in the assay containing copper. Following a 10-min-long incubation at room temperature, the reactions were initiated by 30 mM Mg-ATP and carried out for 30 min at 30 °C. The rate of Pi release due to HMA activity was estimated according to Ames (27). The presence of Cu-ATPase was followed by a melt cycle that consisted of a stepwise increase in temperature from 72 to 99 °C. The PCR products were sequenced to confirm primer specificity. The relative quantification analysis (ΔΔCt method) was performed using the LightCycler® 480 software, Version 1.5.

Vacuolar Membranes Isolated from Yeast Cells or Plant Roots—Polyclonal anti-CsHMA5.1 and anti-CsHMA5.2 antibodies were raised in rabbit against the keyhole limpet hemocyanin-conjugated peptides ISKDGT-DHRSREVC, corresponding to N-terminal amino acids 101–114 of CsHMA5.1 protein and TGSGRYKATIFPEGC, corresponding to N-terminal amino acids 202–215 of CsHMA5.2 protein (GenScript). The position of peptides is marked in Fig. 2A. In addition, commercially available antibodies (Agrisera) against marker enzymes for plasma membrane (H^+-ATPase, AS09 260, diluted 1:10,000 in PBS-T) and tonoplast (vacuolar pyrophosphatase Pase, AS12 1849, diluted 1:2000 in PBS-T) were used to determine membrane purity. Western blot analysis was performed essentially as described earlier (20), using 1:1000 diluted antibodies for CsHMA5.1 or CsHMA5.2. Following incubation with the primary antibodies, the binding of anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (diluted 1:2000 in PBS-T; AS09 602, Agrisera) was visualized using the enhanced chemiluminescence system (ECL; Amersham Biosciences). Total RNA were extracted from CsHMA5.2 transformed with empty vector were subtracted from the results obtained in membranes containing cucumber CsHMA5.1 or CsHMA5.2.

Isoelectric focusing of Cu-ATPases CsHMA5.1 and CsHMA5.2 isolated from yeast vacuole confirmed the presence of Cu-ATPase isoforms CsHMA5.1 and CsHMA5.2 in yeast vacuole. 300 pmol of CuSO_4 or 1 mM BCS and 300 nmol of bafilomycin to avoid the background resulting from the activity of vacuolar V-ATPase. 300 pmol of CuSO_4 or 1 mM BCS (complex specific chelator) was included in the assay containing copper. Following a 10-min-long incubation at room temperature, the reactions were initiated by 30 mM Mg-ATP and carried out for 30 min at 30 °C. The rate of Pi release due to HMA activity was estimated according to Ames (27). The presence of Cu-ATPase was followed by a melt cycle that consisted of a stepwise increase in temperature from 72 to 99 °C. The PCR products were sequenced to confirm primer specificity. The relative quantification analysis (ΔΔCt method) was performed using the LightCycler® 480 software, Version 1.5.

JU NE 19, 2015 • VOLUME 290 • NUMBER 25

A S B M B • J O U R N A L O F B I O L O G I C A L C H E M I S T R Y 15719

WITHDRAWN
Sequence Analyses—The hydropathy profile and location of putative transmembrane helices was determined by TMHMM Server2.0 and visualized using TMRPres2D software. Multi-alignment and homology estimation between cucumber HMA sequences was performed using ClustalW. The phylogenetic tree of plant HMA5–8 proteins was constructed using MEGA6.0 software (32) and the Maximum Likelihood method with the bootstrap (1000 replicates). The sequences from A. thaliana and O. sativa were retrieved from TAIR and TIGR databases, respectively. The previously annotated (19) sequences from Chlamydomonas reinhardtii, Physcomitrella patens, P. trichocarpa, S. bicolor, S. moellendorffii, and V. vinifera were retrieved from customized databases (GenBankTM, TAIR, Phytozome Version 9.1). The PCR-amplified CsHMA5.1 and CsHMA5.2 sequences have been submitted to the GenBankTM data library and are available under accession numbers KJ818254 and KJ818255.

Statistical Analysis—Each experiment was repeated three to six times using material prepared from different plants or yeast cultures. Values are expressed as means ± S.D. Data were analyzed using one-way analysis of variance followed by Tukey’s test. Statistical analysis of the data were performed using paired or unpaired Student’s t-test; significance was accepted when p < 0.05. The data from ATPase activity assay and metal transport assay were fitted to the Michaelis-Menten equation using GraphPad Software (GraphPad Software, Inc.).

Results

Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2

The two genes coding for HMA5-like proteins in cucumber are located on different chromosomes of the cucumber Chinese long genome. CsHMA5.1, which is more closely related to AtHMA5, was found on chromosome 5 (ACHR02000005.1), whereas CsHMA5.2 is located on chromosome 4 (ACHR02000004.1). HMA5s are not the only plant Cu⁺-ATPases that have been multiplied. The genes encoding HMA6s and HMA7s are also present in multiple copies in some plants, but the number of their copies is lower when compared with HMA5s (Fig. 1). Phylogenetic analysis of plant monovalent copper ATPases revealed that they form two clearly distinct phylogenetic clusters, one including HMA6s and HMA8s and the other containing HMA7s and HMA5s, which originated from a common ancestral protein related to the present HMA7 proteins from the microalga C. reinhardtii (CrHMA7.1 and CrHMA7.2) (Fig. 1). Interestingly, HMA5 genes are absent in green alga C. reinhardtii, suggesting...
that they originated from the HMA7-like ancestor after the emergence of vascular land plants. Hence, HMA5s are most closely related to HMA7-like pumps. Indeed, when compared with other cucumber Cu\textsuperscript{2+}-transporting ATPases, CsHMA5.1 and CsHMA5.2 are the most similar to CsHMA7 (40 and 44%, respectively) and show low sequence similarity to CsHMA6 (16 and 17%, respectively) and CsHMA8 (21 and 26%, respectively). In addition, the in silico predictions (PlantLoc, TargetP servers) of subcellular localization based on sequence comparisons to the already fixed locations suggest that different cucumber Cu\textsuperscript{2+}-ATPases might be targeted to different cellular membranes. CsHMA6 and CsHMA8 contain chloroplast-targeting peptides and localize with the highest probability to chloroplasts (data not shown), consistent with the chloroplast localization of their Arabidopsis homologs AtHMA6 and AtHMA8 (15, 16). A similar chloroplast-targeting sequence was identified in CsHMA5.2; however, this protein is predicted to localize with much higher probability in Golgi and vacuolar membranes (data not shown). In comparison, CsHMA5.1 was strongly predicted to localize mainly in the tonoplast or endoplasmic reticulum (with lower probability), whereas the closest homolog of CsHMA5s, CsHMA7, might be targeted with the same probability to different intracellular membranes, including endoplasmic reticulum, Golgi, and tonoplast (data not shown). These results are consistent with the phylogenetic analysis and the level of sequence homology between cucumber Cu\textsuperscript{2+}-ATPases and suggest that CsHMA5.1 and CsHMA5.2 localize to intracellular membranes rather than to the plasma membrane.

Maintenance and the expansion of HMA5s play important functions in different plants. However, only AtHMA5 and OsHMA5 have been functionally characterized to date, so the function of additional HMA5 isoforms in plants is not known. Using cDNA of cucumber roots (cv. Krak), the full-length coding regions of CsHMA5.1 and CsHMA5.2 were amplified by PCR, sequenced, and deposited in the GenBank\textsuperscript{TM} database. cDNA sequences of CsHMA5.1 and CsHMA5.2 isolated from cucumber Krak were identical to the relative sequences generated from genomic contigs of cucumbers Chinese long and Borszczagowski (data not shown). They encode two putative proteins of 973 and 926 amino acids, respectively (20). Protein sequence comparisons revealed that CsHMA5.1 shows 58% similarity to CsHMA5.2 and that both proteins show a comparable level of similarity (67 and 65%, respectively) to the homologous protein from A. thaliana (AtHMA5). Nevertheless, the phylogenetic analysis suggests that CsHMA5.1 and AtHMA5 are more closely related (Fig. 1). Both orthologs, CsHMA5.1 and CsHMA5.2, possess the features typical for P-ATPases (conservative motif DKTGTLT with an Asp residue that is phosphorylated and dephosphorylated during the catalytic cycle) and P\textsubscript{1B}-ATPases (eight transmembrane-spanning domains, CPC(X)\textsubscript{6}P motif, and locus HP) (Fig. 2, A and B). In addition, the putative copper-binding motifs CXXC (two motifs in the N termini), YN (TMDVII), and MXXS (TMDVIII), which are characteristic for copper ATPases (33, 34), were identified in CsHMA5.1 and CsHMA5.2 (Fig. 2A), suggesting that cucumber pumps are putative copper transporters.

**FIGURE 2. Comparative sequence analysis of cucumber HMA5.1 and HMA5.2 proteins.** A, ClustalW alignment of deduced amino acid sequences of CsHMA5.1 and CsHMA5.2. Consensus amino acid residues are marked with asterisks. Boxes indicate the position of motifs CXXC, YN, and MXXS, which are specific for copper ATPases, the motif DKTGTLT common for all P-ATPases, as well as the motifs CPC and locus HP, both characteristic for the subfamily of P\textsubscript{1B}-ATPases. The sequences of peptides used for preparation of two antibodies specific for CsHMA5.1 and CsHMA5.2 are underlined. The putative transmembrane domains (TMDs) are shaded in gray and numbered. B, membrane topology of CsHMA5.1 and CsHMA5.2 proteins predicted with TMHMM server 2.0 and visualized in TMRpres2d.

**Organ and Subcellular Localization of CsHMA5.1 and CsHMA5.2**—The function of heavy metal transporters is strongly determined by their tissue and subcellular localization.
Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2

The organ expression pattern of CsHMA5.1 and CsHMA5.2 was determined in vegetative organs including roots, hypocotyls, cotyledons, and leaves, using RT-PCR and semiquantitative reverse transcription-polymerase chain reaction analysis of CsHMA5.1 and CsHMA5.2 expression in roots, hypocotyls, cotyledons, and leaves, respectively. The detection was performed using the primary antibodies for CsHMA5-GFP with anti-CsHMA5.1 or anti-CsHMA5.2 antibodies and confirmed that both cucumber proteins were present in vacuolar membranes of yeast cells (Fig. 5, A and B). The strain lacking the ATP-dependent ABC transporter YCF1 was used to avoid background activity resulting from YCF1-mediated ATP hydrolysis. The immunostaining of vacuolar membranes isolated from yeast expressing CsHMA5.1-GFP or CsHMA5-GFP with antibodies raised against the peptides from the deduced amino acid sequences of the N-terminal domains of CsHMA5s (Fig. 2A). Based on the amino acid composition, the molecular masses of CsHMA5.1 and CsHMA5.2 were expected to be close to 105 and 98 kDa, respectively. Western blot analysis of different cucumber membranes fractionated on a sucrose gradient showed that CsHMA5.1 and CsHMA5.2 colocalize with the marker protein for vacuolar membrane (vacuolar PPase) and not with the marker protein for plasma membrane (H^+-ATPase). The antibodies reacted with the ∼100-kDa proteins and did not cross-react with other proteins (Fig. 4). Subcellular localization of CsHMA5.1 and CsHMA5.2 was determined in vegetative organs of 2-week-old cucumbers, including roots, hypocotyls, cotyledons, and leaves, using sRT-PCR. sRT-PCR was also used to establish the CsHMA5s expression level in roots, hypocotyls, cotyledons, and leaves, using sRT-PCR and semiquantitative real time PCR. sRT-PCR was also used to establish the CsHMA5s transcription-polymerase chain reaction analysis of CsHMA5s in different cucumber organs. Inset, the semiquantitative reverse transcription-polymerase chain reaction analysis of CsHMA5.1 and CsHMA5.2 in the same organs. B, semiquantitative reverse transcription-polymerase chain reaction analysis of CsHMA5.1 and CsHMA5.2 expression in cucumber flowers and fruits. mp, male perianth; pist, pistil; fp, female perianth; pet, petiole; stam, stamen.

FIGURE 3. Organ expression profile of CsHMA5.1 and CsHMA5.2 in cucumber. A, quantitative real time PCR analysis of CsHMA5.1 and CsHMA5.2 expression in roots, hypocotyls (hyp), cotyledons (cot), petioles (pet), and leaves. The letters indicate significant differences (p < 0.05) between CsHMA5.1 and CsHMA5.2 expression level in roots (a) or between the amount of CsHMA5.1 transcript in different cucumber organs. Inset, the semiquantitative reverse transcription-polymerase chain reaction analysis of CsHMA5.1 and CsHMA5.2 in the same organs. B, semiquantitative reverse transcription-polymerase chain reaction analysis of CsHMA5.1 and CsHMA5.2 expression in cucumber flowers and fruits. mp, male perianth; pist, pistil; fp, female perianth; petiole; stam, stamen.

FIGURE 4. Subcellular localization of CsHMA5.1 and CsHMA5.2 in cucumber. A, subcellular localization of CsHMA5.1 and CsHMA5.2 was determined in vegetative organs of 2-week-old cucumbers, including roots, hypocotyls, cotyledons, and leaves, using sRT-PCR and semiquantitative reverse transcription-polymerase chain reaction analysis of CsHMA5.1 and CsHMA5.2 expression in roots, hypocotyls, cotyledons, and leaves, respectively. The detection was performed using the primary antibodies for CsHMA5-GFP with anti-CsHMA5.1 or anti-CsHMA5.2 antibodies and confirmed that both cucumber proteins were present in vacuolar membranes of yeast cells (Fig. 5, A and B). The strain lacking the ATP-dependent ABC transporter YCF1 was used to avoid background activity resulting from YCF1-mediated ATP hydrolysis. The immunostaining of vacuolar membranes isolated from yeast expressing CsHMA5.1-GFP or CsHMA5-GFP with antibodies raised against the peptides from the deduced amino acid sequences of the N-terminal domains of CsHMA5s (Fig. 2A). Based on the amino acid composition, the molecular masses of CsHMA5.1 and CsHMA5.2 were expected to be close to 105 and 98 kDa, respectively. Western blot analysis of different cucumber membranes fractionated on a sucrose gradient showed that CsHMA5.1 and CsHMA5.2 colocalize with the marker protein for vacuolar membrane (vacuolar PPase) and not with the marker protein for plasma membrane (H^+-ATPase). The antibodies reacted with the ∼100-kDa proteins and did not cross-react with other proteins (Fig. 4). Subcellular localization of CsHMA5.1 and CsHMA5.2 was similar to OsHMA5, a plasma membrane-localized homologous transporter in rice (18).

ATPase and Transport Activity of CsHMA5.1 and CsHMA5.2—Copper ATPases characterized so far, CopA, ATP7A, ATP7B, and PAA1/HMA6, required monovalent copper for maximal activation of their ATPase activity (12, 35–38). To establish whether copper or other metals are essential for CsHMA5.1-mediated and CsHMA5.2-mediated ATP hydrolysis, we studied the ATPase activity in vacuolar membranes isolated from the Δycf1 strain expressing CsHMA5.1-GFP or CsHMA5-GFP. The strain lacking the ATP-dependent ABC transporter YCF1 was used to avoid background activity resulting from YCF1-mediated ATP hydrolysis. The immunostaining of vacuolar membranes isolated from yeast expressing CsHMA5.1-GFP or CsHMA5-GFP with antibodies raised against the peptides from the deduced amino acid sequences of the N-terminal domains of CsHMA5s (Fig. 2A). Based on the amino acid composition, the molecular masses of CsHMA5.1 and CsHMA5.2 were expected to be close to 105 and 98 kDa, respectively. Western blot analysis of different cucumber membranes fractionated on a sucrose gradient showed that CsHMA5.1 and CsHMA5.2 colocalize with the marker protein for vacuolar membrane (vacuolar PPase) and not with the marker protein for plasma membrane (H^+-ATPase). The antibodies reacted with the ∼100-kDa proteins and did not cross-react with other proteins (Fig. 4). Subcellular localization of CsHMA5.1 and CsHMA5.2 was similar to OsHMA5, a plasma membrane-localized homologous transporter in rice (18).
Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2

The ATPase activity of CsHMA5.1 and CsHMA5.2 in vacuolar membranes isolated from yeast strain Δycf1. A and B, immunolocalization of the CsHMA5.1-GFP (A) or CsHMA5.2-GFP (B) fusion proteins in fractions enriched in vacuolar membranes isolated from Δycf1 strain. The membranes prepared from yeast transformed with empty plasmid or plasmids carrying CsHMA5.1 or CsHMA5.2 were analyzed by immunoblotting with specific antibodies generated against CsHMA5.1 or CsHMA5.2. C, effect of monovalent copper chelator BCS, reducing agents, and cysteine on Cu-ATPase activities of CsHMA5.1 and CsHMA5.2. The ATPase activities were determined in the presence of 5 μM CuCl₂ (saturating copper concentration). Data represent means ± S.D. Different letters indicate the significant differences between CsHMA5.1 or CsHMA5.2 activities determined with or without BCS, reducing agents and cysteine.

Instead of Na₂SO₃, cysteine was previously shown to positively affect P₁β-ATPase activity, probably through the binding of metal and forming a chelated complex interacting with the binding domains of a heavy metal transporter (35, 39, 40). The assay confirmed that CsHMA5.1 and CsHMA5.2 are copper ATPases, as expected from their homology to other characterized copper P₁β-ATPases. Both pumps were activated by copper, and the effect of metal was markedly increased by the reducing agent DTT or Na₂SO₃ (Fig. 5C). In contrast, BCS almost completely abolished the stimulatory effect of copper on ATP hydrolysis by CsHMA5.1 and CsHMA5.2, indicating that the monovalent Cu⁺ rather than divalent Cu²⁺ is the putative substrate for cucumber proteins (Fig. 5C). The maximal activation of CsHMA5.1 and CsHMA5.2 was observed upon addition of cysteine to the media containing both the reducing agent and copper, implicating the importance of copper thiolate formation for the interaction of the metal with both transporters (Fig. 5C). Further ATPase activities, including other metal ions, revealed that CsHMA5.1 and CsHMA5.2 are also activated by Ag⁺, although Cu⁺ was 2- or 3-fold less when compared with Na₂SO₃. ATPase activities of CsHMA5.1 and CsHMA5.2 were activated by other metals, including Zn²⁺, Cd²⁺, and Co²⁺, but to a markedly lower extent than for CsHMA5.2 was also slightly activated by Cd²⁺ (Fig. 5E).

Cysteine was pre-
ed in the reaction media (a), or the activities measured in the presence of Na₂SO₃ with or without cysteine (b). C, effect of monovalent copper chelator BCS, reducing agents, and cysteine on Cu-ATPase activities of CsHMA5.1 and CsHMA5.2. The ATPase activities were determined in the presence of 5 μM CuCl₂ (saturating copper concentration). Data represent means ± S.D. Different letters indicate the significant differences between CsHMA5.1 or CsHMA5.2 activities determined with or without BCS, reducing agents and cysteine.
Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2

**FIGURE 6.** Cu \(^+\) and Ag \(^+\) dependence of ATPase activities in vacuolar membranes isolated from yeast expressing fusion proteins CsHMA5.1-GFP and CsHMA5.2-GFP. The rate of Cu-dependent ATPase activity was measured in the presence of different concentrations of metal ions, determined at different concentrations of chelating molecules supply copper to CsHMA5.1 and CsHMA5.2 in cucumber root cells. Some chelating molecules supply copper to CsHMA5.1 and CsHMA5.2 in the intracellular environment, so it is likely that some chelating molecules supply copper to CsHMA5.1 and CsHMA5.2 in cucumber root cells.

To confirm that the Cu\(^+\)-dependent activation of CsHMA5.1 and CsHMA5.2 is related to the ATP-dependent transport of copper across the membranes, copper uptake was measured in vacuolar membrane vesicles isolated from the Δycf1 strain expressing CsHMA5.1-GFP and CsHMA5.2-GFP. Similarly to ATPase activities, copper transport by CsHMA5.1 or CsHMA5.2 was also dependent on metal concentration and increased with the increase of copper in reaction media up to 5 μM (Fig. 7). Higher copper concentrations (10 μM, 50 μM) had an inhibitory effect on CsHMA5.1 and CsHMA5.2 transport activities (Fig. 7). When compared with CsHMA5.1, the copper transport of copper across the membranes, copper uptake was determined on the control and metal-supplemented media. The growth of mutants transformed with the empty vector pUG35 or vectors carrying CsHMA5.1 or CsHMA5.2 was monitored on the control and metal-supplemented media. The presence of CsHMA5.1-GFP and CsHMA5.2-GFP fusions in the vacuolar membranes of the mutant strain was confirmed by fluorescence microscopy (Fig. 8A). The copper-sensitive mutant phenotype was fully complemented by CsHMA5.1 or CsHMA5.2 (Fig. 8B). Both proteins also conferred increased resistance of the same yeast to silver (Fig. 8B). These data suggest that cucumber CsHMA5s contribute to copper and silver detoxification in yeast cells. To investigate further the mecha-
Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2

nism of CsHMA5.1-mediated and CsHMA5.2-mediated tolerance of Δace1 to copper, the accumulation of copper was compared in mutant strains transformed with an empty vector or with vectors carrying cucumber genes. Analysis of metal content in cells growing in liquid medium supplemented with 100 μM CuCl2 showed that yeast expressing CsHMA5.1 or CsHMA5.2 accumulated a higher amount of copper during 8 h of exposure to metal than the strain transformed with the empty vector (Fig. 8C), suggesting that CsHMA5-mediated copper tolerance is related to the enhanced intracellular sequestration of copper rather than the increased copper efflux out of the yeast cells. This finding was in agreement with the vacuolar localization of CsHMA5s in yeast.

CsHMA5.1 and CsHMA5.2 Are Differentially Regulated by Copper Stress—Both CsHMA5.1 and CsHMA5.2 are expressed in cucumber roots. To determine how copper stress affects the level of CsHMA5.1 and CsHMA5.2 transcripts, quantitative PCR was performed using the bulk of roots from plants grown upon copper excess or copper deficiency. As shown in Fig. 9A, the expression of CsHMA5.1 was not significantly affected by any of the treatments. Similarly, the level of CsHMA5.1 protein in tonoplast membranes isolated from the same roots was not significantly changed upon copper stress (Fig. 9B). In contrast, CsHMA5.2 transcript and protein levels were markedly reduced or elevated upon copper deficiency or copper excess, respectively. These findings suggest that CsHMA5.1 is a constitutive root-specific copper transporter unaffected during copper stress, whereas CsHMA5.2 functions in copper detoxification and excretion.

ATP-Dependent Copper Transport Operates in Tonoplast Membranes—Immunolocalization of CsHMA5s in yeast. This finding was in agreement with the vacuolar localization of CsHMA5s in cucumber cells. Indeed, ATP-dependent copper transport activity was detected in tonoplast vesicles prepared from the same roots (Fig. 9C). Moreover, the rate of active copper accumulation within vacuolar membranes was significantly dependent on the availability of copper in the nutrient solution. Specifically, the ATP-dependent copper transport in vesicles prepared from plants grown under copper toxicity or copper deficiency was over 4-fold higher or almost 5-fold lower, respectively, when compared with the control (Fig. 9C). These results were consistent with the changes in CsHMA5.2 transcript and protein levels under copper stress, suggesting that CsHMA5.2 plays a role in the increased vacuolar copper sequestration in cucumber roots in conditions of copper excess.

Discussion

So far, only two genes encoding plant HMA5-like P1B-ATPases have been functionally characterized as follows: OsHMA5 from rice and AtHMA5 from Arabidopsis, respectively (17, 18, 45). They are expressed predominantly in pericycle cells of roots and are involved in Cu⁺ compartmentalization and detoxification, probably through active copper loading to the xylem of roots and other organs (17, 18, 45). Moreover, rice OsHMA5 has been localized in the plasma membrane, confirming the predicted role of plant HMA5s in copper export from root cells (18). Although AtHMA5 has not been localized yet, the knock-out of the gene encoding the Arabidopsis transporter results in increased hypersensitivity of plants to copper and enhanced accumulation of the metal in roots under copper excess, suggesting that AtHMA5 may be involved in copper efflux from root cells, and therefore it could also localize to the plasma membrane (17). However, neither OsHMA5 nor AtHMA5 has...
Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2

been subjected to biochemical and kinetic studies confirming their roles in copper distribution in plant cells. Although Arabidopsis possesses only one HMA5 pump, multiple genes encoding homologous proteins have been identified in other plants, including rice; however, the relevance of these extra Cu-ATPases is not known (19).

This work provides the first functional and biochemical characterization of plant HMA5-like P1B-ATPases, CsHMA5.1 and CsHMA5.2 from cucumber, and it reveals that orthologous transporters in plants are differently displayed to achieve organismal copper homeostasis.

Contrary to plasma membrane-localized OsHMA5, CsHMA5.1 and CsHMA5.2 are associated with the vacuolar membrane, indicating that they function in vacuolar metal sequestration rather than in metal efflux out of the cells. However, different expression of CsHMA5.1 and CsHMA5.2 in cucumber organs suggests different biological roles for the two cucumber HMA5s. When studied in yeast vacuolar membranes, CsHMA5.1 and CsHMA5.2 were activated in the presence of copper and silver, similarly to homologous proteins in bacteria (CopA), humans (ATP7A and ATP7B), and A. thaliana (PAA1) (12, 35–38), confirming that they are putative copper ATPases. Interestingly, the maximal copper-dependent ATPase activity of CsHMA5.1 and CsHMA5.2 required the presence of Cys and reducing agents in the assay medium. Similar Cys and reducing agent-dependence have previously reported for the Cu-ATPases ZntA and ZntB from A. thaliana, respectively (39–47). This work demonstrated that metal-thiolate complexes rather than metal ions were the substrates for heavy metal transport. Using a yeast two-hybrid assay, this work demonstrated that the metal-binding domains of CsHMA5.1 and CsHMA5.2 interact with Arabidopsis ATX1-like cop-trains, confirming that chelated copper rather than metal ions is a substrate for the copper pump. Here, we show that copper thiolate is also a putative substrate for the cucumber homolog of HMA5 pumps. Contrary to Cys, the positive effect of reducing agents on copper ATPase activity probably results from their action on the copper redox state (47, 48). Reducing agents appear to be necessary to keep copper in a reduced state, a form activating phosphorylation of copper ATPases. Hence, both DTT and Na₂SO₃ had a similar positive effect on CsHMA5.1 and CsHMA5.2 activities, probably by maintaining copper in a monovalent form (12). A strong negative effect of the Cu⁺ chelator BCS on CsHMA5.1 and CsHMA5.2 activities confirmed that Cu⁺ is required for cucumber protein activity. Interestingly, in the absence of reducing agents the copper-mediated activation of CsHMA5.1 and CsHMA5.2 was still significant when compared with the copper-deprived control assay, suggesting that in the conditions of our experiment copper was predominantly present in the Cu⁺⁺ form. Indeed, recent studies on AthmA6/PAA1 in membranes isolated from Lactococcus lactis demonstrated that a majority (70%) of copper in the reaction medium was rapidly reduced (in less than 5 min) by the buffer and isolated membranes (12).

The apparent Kₘ values of CsHMA5.1 and CsHMA5.2 for Cu⁺, estimated using ATPase and a transport assay, were slightly different (close to 1 and 0.5 μM, respectively) and suggest that CsHMA5.2 binds copper more efficiently under copper-limiting conditions. Detailed studies on human ATP7B revealed that copper-mediated activation of ATPase is a result...
Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2

of copper binding to autoinhibitory CXXC motifs, inducing conformational changes within the protein that enable ATP hydrolysis (49, 50). The copper ATPases from other organisms, including plants, are probably regulated by the same autoinhibitory mechanism. As shown in Fig. 2, two CXXC motifs are present in CsHMA5.1 (CSAC and CNSC) and CsHMA5.2 (CSAC and CTSC). The presence of different amino acids in one of the two motifs (asparagine in CsHMA5.1 instead of threonine in CsHMA2) could affect copper binding and thus Cu-ATPase activity of both pumps. Nevertheless, the apparent $K_m$ values of CsHMA5s for copper were very similar to those previously reported for human ATPases PTP7B and ATP7A (1 and 2.5 $\mu M$, respectively) (37), for CopA from E. coli and from A. fulgidus (5 and 3.9 $\mu M$, respectively) (35, 36), and for plant AtHMA6/PAA1 (0.5 $\mu M$) (12).

In comparison with Cu$^+$, the affinities of both cucumber pumps for Ag$^+$ were very similar and lower ($K_m$ $\sim 2.5$ $\mu M$), suggesting that the mechanism of Cu$^+$ and Ag$^+$ binding by these proteins might be different. Ag$^+$ is not a physiological substrate for Cu-ATPases; hence, its stimulatory effect on activity of copper pumps probably results from the chemical (electropositive, singly charged cations) similarity between Ag$^+$ and Cu$^+$ ions (12). However, the difference in the Ag$^+$ (0.126 nm) and Cu$^+$ (0.096 nm) ionic radii could affect metal binding and thus ATPase activity of CsHMA5s. Differences in affinity of Cu$^+$ and Ag$^+$ were previously shown for CopA from B. globus fulgidus (35) and AthMA6/PAA1 from A. thaliana, whereas CopA from Bacillus subtilis showed higher affinity for both ions (51). In addition, the rate of ATP hydrolysis and CsHMA5.2-mediated ATP hydrolysis increased only in the presence of Cu$^+$ than upon addition of Ag$^+$ to the reaction media. The phosphorylation of AthMA6/PAA1 was also much higher in the presence of copper than Ag$^+$ (12). In contrast, CopA from A. fulgidus was four times faster by Ag$^+$ than by Cu$^+$. The pump from B. subtilis was activated by both ions with similar efficiency (51). It has already been evidenced that Cu-ATPases from various organisms might behave similarly or differently with copper and silver. Moreover, CopA from A. fulgidus is mainly present in the ion-binding phosphorylated form in the presence of copper or in a metal-free phosphorylated form in the presence of silver (35). In contrast, the ion-binding phosphorylated form of AthMA6/PAA1 is prevalent in the presence of either copper or silver (12).

Although the hydrolytic activities of CsHMA5.1 and CsHMA5.2 were copper- or silver-dependent, the highest concentration of metals resulted in the inhibition of ATP hydrolysis. A similar inhibitory effect of higher copper concentrations was observed when the ATP-dependent copper transport was assayed. Excessive copper also inhibited the ATPase activity of prokaryotic copper ATPases (CopB from Enterococcus hirae and A. fulgidus) as well as human ATP7A and plant AthMA6/PAA1 (11, 12, 38, 52, 53). High concentrations of metal ions could prevent rapid dissociation of the transported ions from the metal-binding site of P$_{1B}$-type ATPase and thus reduce the enzyme turnover rate (12). However, copper-mediated inhibition of P$_{1B}$-ATPase may result from the negative effect of metal excess on the protein structure, on the active sites of the enzyme, or on the membrane lipids. Excessive copper was found to inhibit protein activity by inducing protein precipitation or by inducing conformational changes at several key residues of proteins (54, 55).

The ability of CsHMA5.1 and CsHMA5.2 to transport silver and copper was confirmed by studying copper and silver tolerance of yeast expressing cucumber proteins. Both CsHMA5s increased yeast tolerance to copper and silver through intracellular copper sequestration, indicating that they actually transport Cu$^+$ and Ag$^+$ in vivo. Similarly, AthMA5, AthMA6/PAA1, and OsHMA5 have been shown to participate in intracellular copper sequestration when expressed in the Δccc2 yeast strain, lacking the Golgi-resident endogenous copper Cu$^+/Ag^+$-ATPase Ccc2p (12, 18, 45). According to available data, P$_{1B}$-ATPases participate in the maintenance of copper homeostasis either through the import of copper into the cell (PCC7942 and PCC6803 in cyanobacteria Synechococcus and Synechocystis, respectively) and intracellular compartments to supply copper to copper-dependent enzymes (ATP7B, ATP7A, Ccc2p, AthMA6/PAA1, AtHMA7/RAN1) or to the vacuole system and to extracellular copper release to the environment (56). Our study provides the first evidence for the involvement of P$_{1B}$-ATPases in the copper detoxification of yeast. The subcellular localization of CsHMA5.2 and increased tolerance of expressing cucumber pumps to copper clearly indicates the function of both proteins in detoxification of copper through the vacuolar sequestration of copper excess. The expression of homologous proteins in A. thaliana and rice was up-regulated by excess copper, confirming the putative function of AthMA5 and OsHMA5 in copper detoxification (17, 18). Interestingly, CsHMA5.1 and CsHMA5.2 expression and protein levels were differentially affected by copper stress. However, CsHMA5.1 was not affected by different copper availability, whereas CsHMA5.2 was significantly up-regulated or down-regulated upon copper excess or deficiency, respectively. Similarly, the ATP-dependent copper transport into tonoplast membranes isolated from cucumber roots was also strongly increased under copper excess and markedly reduced upon copper deficiency. These findings indicate that CsHMA5.2 might be responsible for the increased vacuolar copper sequestration in cucumber root cells under copper toxicity and reveal different regulation of the two CsHMA5 isoforms by copper. Hence, we propose that the functional diversity of CsHMA5.1 and CsHMA5.2 comes from variation in their regulatory mechanisms (different expression patterns) and possibly from some structural differences between both proteins (different affinities for copper and different ATPase activities).

Acknowledgments—We greatly appreciate Beata Kuligowska and Anna Szawlowska-Kubik (Wrocław University, Institute of Experimental Biology, Department of Molecular Plant Physiology) for technical assistance.
Characterization of Cu-ATPases CsHMA5.1 and CsHMA5.2

References

1. Koch, K. A., Peña M. M., and Thiele, D. J. (1997) Copper-binding motifs in catalysis, transport, detoxification and signalling. Chem. Biol. 4, 549–560
2. Yruela, I. (2005) Copper in plants. Braz. J. Plant Physiol. 17, 145–156
3. Axelsen, K. B., and Palmgren M. G. (2001) Inventory of the superfamily of P-type ion pumps in Arabidopsis. Plant Physiol. 126, 696–706
4. Cobbett, C. S., Hussain, D., and Haydon, M. J. (2003) Structural and functional relationships between type 1B heavy metal-transporting P-type ATPases in Arabidopsis. New Phytol. 159, 315–321
5. Argüello, J. M. (2003) Identification of ion-selectivity determinants in heavy-metal transport P-type ATPases. J. Membr. Biol. 195, 93–108
6. Hall, J. L., and Williams, L. E. (2003) Transition metal transporters in plants. J. Exp. Bot. 54, 2601–2613
7. Williams, L. E., and Mills, R. F. (2005) P(1B)-ATPases—an ancient family of transition metal pumps with diverse functions in plants. Trends Plant Sci. 10, 491–502
8. Seigneurin-Berny, D., Gravot, A., Auryp, A., Pizarro, C., Kraut, A., Finazzi, G., Grunwald, D., Rappart, F., Vavasseur, A., Joyard, J., Richaud, P., and Rolland, N. (2006) HMA1, a new Cu-ATPase of the chloroplast envelope, is essential for growth under adverse light conditions. J. Biol. Chem. 281, 2882–2892
9. Moreno, I., Norambuena, L., Maturana, D., Chali, D., Fiala, J., and Ordenes, V. R. (2008) AtHMA1 is a thapsigargin-sensitive Ca^{2+}/heavy metal pump. J. Biol. Chem. 283, 9633–9641
10. Mikkelsen, M. D., Pedas, P., Schiller, M., Vincze, E., Mills, R. F., Borg, S., Moller, A., Schoering, J. K., Williams, L. E., Backgaard, L., Holm, P. B., and Palmgren, M. G. (2012) Barley HvHMA1 is a heavy metal pump involved in mobilizing organellar Zn and Cu and plays a role in metal loading into grains. PLoS One 7, e49027
11. Voskoboinik, I., Mar, J., Strausak, D., and Camakaris, J. (2001) The regulation of catalytic activity of the Menkes copper-transporting P-type ATPase. Role of high affinity copper-binding sites. J. Biol. Chem. 286, 28620–28627
12. Catty, P., Boutigny, S., Miras, R., Joas, H., and Berny, D. (2011) Biochemical characterization of a chloroplast envelope Cu(I)-ATPase. Plant Cell 23, 435–445
13. Hirayama, T., Kieber, J. J., and Staskawizh, S., Alonso, J. M., and Vantao, P. (2011) RESPONSE-TO-ANTAGONIST1, a Menkes/Wilson disease–related copper transporter, is required for normal growth under different nitro-
physiological role for *Saccharomyces cerevisiae* copper/zinc superoxide dismutase in copper buffering. *J. Biol. Chem.* **270**, 29991–29997
43. Thiele, D. J. (1988) ACE1 regulates expression of the *Saccharomyces cerevisiae* metallothionein gene. *Mol. Cell. Biol.* **8**, 2745–2752
44. Portnoy, M. E., Schmidt, P. J., Rogers, R. S., and Culotta, V. C. (2001) Metal transporters that contribute copper to metallochaperones in *Saccharomyces cerevisiae*. *Mol. Genet. Genomics* **265**, 873–882
45. Kobayashi, Y., Kuroda, K., Kimura, K., Southron-Francis, J. L., Furuzawa, A., Kimura, K., Iuchi, S., Kobayashi, M., Taylor, G. J., and Koyama, H. (2008) Amino acid polymorphisms in strictly conserved domains of a P-type ATPase HMA5 are involved in the mechanism of copper tolerance variation in *Arabidopsis*. *Plant Physiol.* **148**, 969–980
46. Mitra, B., and Sharma, R. (2001) The cysteine-rich amino-terminal domain of ZntA, a Pb(II)/Zn(II)/Cd(II)-translocating ATPase from *Escherichia coli*, is not essential for its function. *Biochemistry* **40**, 7694–7699
47. Voskoboinik, I., Brooks, H., Smith, S., Shen, P., and Camakaris, J. (1998) ATP-dependent copper transport by the Menkes protein in membrane vesicles isolated from cultured Chinese hamster ovary cells. *FEBS Lett.* **435**, 178–182
48. Rensing, C., Fan, B., Sharma, R., Mitra, B., and Rosen, B. P. (2000) CopA, an *Escherichia coli* Cu(II)-translocating P-type ATPase. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 652–656
49. Tsvikovskii, R., MacArthur, B. C., and Lutsenko, S. (2001) The Lys1010, Lys132 fragment of the Wilson's disease protein binds nucleotides and interacts with the N-terminal domain of this protein in a copper-dependent manner. *J. Biol. Chem.* **276**, 2234–2242
50. Barry, A. N., Shinde, U., and Lutsenko, S. (2010) Structural organization of human Cu-transporting ATPases: learning from building blocks. *J. Biol. Inorg. Chem.* **15**, 47–59
51. Bani, L., Bertini, I., Ciofi-Baffoni, S., Gonnelli, L., and Su, X. C. (2003) Structural basis for the function of the N-terminal domain of the ATPase CopA from *Bacillus subtilis*. *J. Biol. Chem.* **278**, 50506–50513
52. Bissig, K.-D., Voegelin, T. C., and Solioz, M. (2001) Tetrathiomolybdate inhibition of the *E. hirae* CopB copper ATPase. *FEBS Lett.* **507**, 367–370
53. Mana-Capelli, S., Mandal, A. K., and Argüello, J. M. (2003) *Archeoglobus fulgidus* CopB is a thermophilic Cu2+/H11001-ATPase: functional role of its histidine-rich N-terminal metal binding domain. *J. Biol. Chem.* **278**, 40534–40541
54. Su, Y., Hu, F., Hong, M. (2012) Paramagnetic Cu(II) for probing membrane protein structure and function: inhibition mechanism of the influenza M2 proton channel. *J. Am. Chem. Soc.* **134**, 8693–8702
55. Lee, A. M., Singleton, S. F. (2004) Inhibition of the *Escherichia coli* RecA protein: zinc(II), copper(II) and mercury(II) trap RecA as inactive aggregates. *J. Inorg. Biochem.* **98**, 1981–1986
56. Phung, L. T., Aijani, G., and Haselkorn, R. (1994) P-type ATPase from the cyanobacterium *Synechococcus 7942* related to the human Menkes and Wilson disease genes. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9651–9654
57. Tottey, S., Rich, P. R., Rondet, S. A., and Robinson, N. I. (2001) Two Menkes-type ATPases supply copper for photosynthesis in *Synechocystis PCC 6803*. *J. Biol. Chem.* **276**, 16941–16944