Metal binding to the N-terminal cytoplasmic domain of the \( P_{1B} \) ATPase HMA4 is required for metal transport in *Arabidopsis*.

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Abstract

P_{IB} ATPases are metal cation pumps that transport metals across membranes. These proteins possess N- and C-terminal cytoplasmic extensions that contain Cys- and His-rich high affinity metal binding domains, which may be involved in metal sensing, metal ion selectivity and/or in regulation of the pump activity. The P_{IB} ATPase HMA4 (Heavy Metal ATPase 4) plays a central role in metal homeostasis in *Arabidopsis thaliana* and has a key function in zinc and cadmium hypertolerance and hyperaccumulation in the extremophile plant species *Arabidopsis halleri*.

Here, we examined the function and structure of the N-terminal cytoplasmic metal-binding domain of HMA4. We mutagenized a conserved CCTSE metal-binding motif in the domain and assessed the impact of the mutations on protein function and localization in planta, on metal-binding properties in vitro and on protein structure by Nuclear Magnetic Resonance spectroscopy.

The two Cys residues of the motif are essential for the function, but not for localization, of HMA4 in planta, whereas the Glu residue is important but not essential. These residues also determine zinc coordination and affinity. Zinc binding to the N-terminal domain is thus crucial for HMA4 protein function, whereas it is not required to maintain the protein structure.

Altogether, combining in vivo and in vitro approaches in our study provides insights towards the molecular understanding of metal transport and specificity of metal P-type ATPases.

**Keywords:** metal P-type ATPase, metal binding domain, zinc transport, structure-function analysis, Arabidopsis.
Introduction

Zinc is an essential transition metal for development and growth of photosynthetic organisms. It plays important roles as enzyme or structural cofactor in many biochemical processes (Broadley et al. 2007; Palmer and Guerinot 2009; Nouet et al. 2011). However, zinc becomes toxic when present in excess in tissues, through unspecific binding or competition with other metals for the active sites in proteins (Goyer 1997; Gaither and Eide 2001; Hall and Williams 2003; Tuerk and Fazel 2009). To maintain zinc concentration in tissues within an optimal range, plants have developed a complex and tightly controlled zinc homeostasis network. This network relies in part on zinc membrane transporters that ensure zinc uptake, distribution and storage (Krämer et al. 2007; Palmer and Guerinot 2009; Nouet et al. 2011).

In Arabidopsis thaliana, HMA4 (Heavy Metal ATPase 4) encodes a zinc and cadmium efflux pump of the IB subfamily of P-type ATPases (or CPx-ATPases) (Williams and Mills 2005; Palmgren and Nissen 2011; Pedersen et al. 2012; Hanikenne and Baurain 2014) and is an essential node of the metal homeostasis network (Mills et al. 2003; Hussain et al. 2004; Verret et al. 2004). Together with its paralog AtHMA2, the HMA4 transporter is localized at the plasma membrane and is expressed in vascular tissues in roots and shoots (Hussain et al. 2004; Verret et al. 2004; Siemianowski et al. 2013). AtHMA2 and AtHMA4 are responsible for the translocation of zinc from roots to shoots. A hma2hma4 double A. thaliana mutant displays stunted growth resulting from severe zinc deficiency in shoots (Hussain et al. 2004). AtHMA2 and AtHMA4 are also responsible for cadmium translocation to shoots (Wong and Cobbett 2009; Cun et al. 2014). In addition, the HMA4 protein plays a key role in the zinc and cadmium hyperaccumulation and hypertolerance syndrome in the Brassicaceae Arabidopsis halleri (Talke et al. 2006; Courbot et al. 2007; Hanikenne et al. 2008; Hanikenne et al. 2013) and Noccaea caerulescens (O’ Lochlainn et al. 2011; Craciun et al. 2012), an extreme trait enabling these species to colonize metal-polluted soils (Krämer 2010; Hanikenne and Nieuw 2011). In A. halleri, high expression of HMA4 supports high rates of root-to-shoot translocation of zinc mediated by xylem loading (Hanikenne et al. 2008). Increased expression of HMA4 in A. halleri results from tandem triplication and cis-activation of expression of all three gene copies that were selected for during the evolutionary history of A. halleri (Hanikenne et al. 2008; Hanikenne et al. 2013).

P-type ATPases constitute a superfamily of pumps using the energy of ATP to transport cations, and possibly phospholipids (Kühlbrandt 2004; Palmgren and Nissen 2011). P-type
ATPases can be divided into five major classes, I–V, based on ion transport specificities and clustering in phylogenetic trees (Axelsen and Palmgren 1998; Palmgren and Nissen 2011). Despite a low sequence conservation, all P-type ATPases share a set of structural and mechanistic features (Toyoshima and Nomura 2002; Toyoshima and Inesi 2004; Toyoshima 2008; Toyoshima 2009; Palmgren and Nissen 2011). These proteins are characterized by the phosphorylation of an invariant Asp residue by ATP during the ion transport cycle. During this so-called Post-Albers cycle, the pumps undergo a series of conformational changes upon ion binding/release and phosphorylation/dephosphorylation. These conformational changes allow transport of the ion across the membrane. P-type ATPases possess specific cytoplasmic catalytic domains, the Actuator, Nucleotide and Phosphorylation domains, which are essential for the transport cycle. The transmembrane segments (TM) of P-type ATPases constitute the transport domain, which, determines ion selectivity and thanks to a high flexibility, allows binding and release of the ion (Kühlbrandt 2004; Williams and Mills 2005; Palmgren and Nissen 2011).

Proteins of the IB subfamily of P-type ATPases are involved in metal cation transport across membranes (Williams and Mills 2005; Palmgren and Nissen 2011; Pedersen et al. 2012; Hanikenne and Baurain 2014). These proteins possess 8 TMs responsible for metal coordination during transport, notably including a specific metal binding site located in TM6 with the conserved Cys-Pro-(Cys/His/Ser) motif and several other conserved residues (Argüello 2003; Pedersen et al. 2012; Hanikenne and Baurain 2014; Wang et al. 2014). Out of 45 P-type ATPases in A. thaliana, eight are IB metal ATPases (AtHMA1-8), which can be further divided in subgroups based on metal transport specificity (Axelsen and Palmgren 1998; Argüello 2003; Hanikenne et al. 2005; Chan et al. 2010; Pedersen et al. 2012; Hanikenne and Baurain 2014). (i) AtHMA5-AtHMA8 transport monovalent metal cations (e.g. Cu\(^{+}\)) and belong to the ubiquitous IB-1 subclass of metal ATPases found in all domains of life. This subclass includes the two human IB P-type ATPases, ATP7A and ATP7B, which both transport monovalent copper and whose mutations determine Menkes and Wilson diseases, respectively (Lutsenko and Petris 2003). In plants, the PAA1 (AtHMA6) and PAA2 (AtHMA8) proteins are responsible for copper transport across the inner envelope and thylakoid membranes, respectively, which is required for copper delivery to plastocyanin (Shikanai et al. 2003; Abdel-Ghany et al. 2005; Bernal et al. 2007). (ii) AtHMA2-4 transport divalent metal cations (e.g. Zn\(^{2+}\) and Cd\(^{2+}\)) and belong to subclass IB-2 of metal ATPases found in plants and in prokaryotes. (iii) AtHMA1 belongs to subclass IB-4 of metal ATPases...
and has a broad ion specificity (Ca\(^{2+}\), Cd\(^{2+}\), Zn\(^{2+}\), Cu\(^{2+}\)) (Seigneurin-Berny et al. 2006; Moreno et al. 2008; Kim et al. 2009; Boutigny et al. 2014). HMA1 orthologs in plants originate from a horizontal gene transfer from Chlamydiae into the common ancestor of Plantae and all share a non-canonical Ser-Pro-Cys in TM6 (Baum 2013; Hanikenne and Baurain 2014).

IB metal ATPases possess N- and C-terminal extensions that contain high affinity metal binding domains (MBDs) rich in Cys and sometimes His residues. For instance, the C-terminal domain of AtHMA4 contains multiple di-Cys motifs and an extended His stretch. This domain may act as a zinc and cadmium sensor regulating the export capacity of the pump and is required for the full function of the protein in planta (Baekgaard et al. 2010; Mills et al. 2010).

The N-terminal domains of IB-1 copper ATPases are characterized by Cys-x-x-Cys motifs, which are required for copper delivery by metallochaperones, protein activation and/or protein intracellular trafficking. These processes have been described in detail for ATP7A and ATP7B (Barry et al. 2010). In contrast, the N-terminal domain of plant IB-2 zinc ATPases possess a Cys-Cys-x-x-Glu conserved metal-binding motif (CCxxE) within a \(\beta\alpha\beta\beta\alpha\beta\) ferredoxin fold. This non-canonical site binds one zinc atom (Eren et al. 2007; Zimmermann et al. 2009). Deletion of the AtHMA2 N-terminal domain results in decreased ATPase activity (Eren et al. 2007) and in failure to complement the phenotype of the hma2hma4 A. thaliana mutant (Wong et al. 2009). Mutation of the two Cys residues of the motif into Ala equally impairs the function of the protein in vivo (Wong et al. 2009). The N-terminal domain of AtHMA2 is thus essential for function in planta. Finally, the N-terminal domain of AtHMA4 was characterized using Nuclear Magnetic Resonance (NMR) and metal probes, which revealed zinc binding by the Cys and Glu residues of the C\(^{27}\)CTSE\(^{31}\) motif and showed that its affinity for zinc was in the subnanomolar range (Zimmermann et al. 2009). The two Cys residues of the motif were required for function of AtHMA4 in yeast (Verret et al. 2005).

Conserved MBD motifs in the N-terminal domain of copper IB P-type ATPases have distinct functions in different proteins or even different functions when present in tandem in the same protein ((Tsivkovskii et al. 2001; Mana-Capelli et al. 2003; Mandal et al. 2003; Argüello et al. 2007; Veldhuis et al. 2009; Palmgren and Nissen 2011; Drees et al. 2015). It is thus important to examine differences and commonalities in the role of the N-terminal domains.
and their conserved MBDs for several IB P-type ATPases. Moreover, many examples exist in the literature illustrating the fact that the functional analysis of IB P-type ATPase MBDs may result in differing observations between *in vivo* and *in vitro* experiments, and also depending on the experimental setup (see for instance, Eren et al. 2007; Wong et al. 2009; Bäckgaard et al. 2010; Mills et al. 2010; Drees et al. 2015). Here, to further advance our understanding of structure/function relationship in the HMA4 N-terminal domain, a range of mutants of the C27CTSE31 conserved motif were characterized by complementary *in vivo* and *in vitro* approaches. Our data highlight the key function of the domain *in planta* and reveal predominance of the two Cys residues for zinc coordination and affinity.

Materials and methods

**Plant material, transformation and growth conditions**

*A. thaliana* L. Heynhold (accession Columbia, Col-0) and the *A. thaliana hma2hma4* double mutant (Hussain et al. 2004) were used in all experiments. For genetic transformation of the *hma2hma4* mutant, plants were cultivated on soil watered with a 1mM ZnSO₄ solution in a controlled climate room at 22°C and a 8h day⁻¹ photoperiod (short days) during 8 weeks. Plants were then transferred in a 16h day⁻¹ photoperiod (long days) growth chamber to induce flowering and were watered with 3mM ZnSO₄ for 5 weeks. The *hma2hma4* plants were then transformed using *Agrobacterium tumefasciens* by floral dipping (Clough and Bent 1998). GFP fusions were transformed into Col-0 wild-type plants.

For experiments, homozygous transgenic seeds (T3 generation) were germinated on 1/2 MS agar medium containing 1% sucrose in short days, after a 5 day incubation at 4°C. After 18 days, seedlings were transferred on soil for phenotyping or into hydroponic trays (Araponics, Tocquin et al. 2003) containing modified Hoagland solution as described (Talke et al. 2006; Charlier et al. 2015; Nouet et al. 2015). For soil experiments, plants were watered with distilled water and grown for 2 weeks in short days followed by 5 weeks in long days. For hydroponic experiments, plants were grown for 2 weeks in control conditions (1 µM ZnSO₄) in short days. Plants were then transferred in long days to initiate the treatments: 0.05µM CdSO₄ or 0.2µM ZnSO₄ (Nouet et al. 2015). Nutrient solutions were changed weekly during
4 weeks. Root and shoot samples were then harvested separately before processing for ICP-AES analyses.

**Cloning**

To construct the pAtHMA4-AhHMA4 cassette and the C\textsuperscript{27}CTSE\textsuperscript{31} variants, the AtHMA4 promoter (pAtHMA4, 2595 bp, Hanikenne et al. 2008) was amplified from Col-0 genomic DNA by PCR using primers harbouring 5’-AscI and 3’-AcyI restriction sites (Table S1A), respectively. The promoter fragment was cloned into the AscI and AcyI sites of a pAhHMA4-1-AhHMA4 pBluescript II KS+ vector (Hanikenne et al. 2008) in replacement of pAhHMA4-1. This vector served as a template for the site-directed mutagenesis of the conserved N-terminal C\textsuperscript{27}CTSE\textsuperscript{31} motif as described (Talke et al. 2006) using mutagenic primers (Table S1B). The wild-type and variant versions of pAtHMA4-AhHMA4 were then excised by digestion with AscI and PacI and cloned at the corresponding sites of a promoter-less variant of the pMDC32 vector (Curtis and Grossniklaus 2003; Hanikenne et al. 2008). For localization experiments, the wild-type and variant versions of AhHMA4 were cloned at the PacI and AscI sites (Table S1A) in fusion with GFP into the pMDC83 vector allowing expression under the control of a double 35S promoter (Curtis and Grossniklaus 2003).

For production in *E. coli*, a synthetic gene encoding the N-terminal part of the *A. halleri* HMA4 protein (AhHMA4n, residues 1-95) with optimized codon usage was obtained from GeneArt. The fragment was subsequently cloned into the pET9a expression vector (Novagen) using NdeI and BamHI restriction sites. Variants with mutations in the C\textsuperscript{27}CTSE\textsuperscript{31} motif were obtained as above using the pET9a-AhHMA4n vector as template (see mutagenic primers in Table S1B).

All final constructions were verified by sequencing.

**RNA Extraction, cDNA Synthesis, and Quantitative RT-PCR**

Total RNAs were prepared using the RNeasy Plant Mini kit with on column DNAse treatment (Qiagen), and cDNAs were synthesized using the RevertAid H Minus First Strand cDNA Synthesis kit with Oligo dT (Thermo Scientific). Transcript levels were determined by real-time RT-PCR in 384-well plates with an ABI Prism 7900HT system (Applied Biosystems) using MESA GREEN qPCR MasterMix (Eurogentec) as described (Talke et al., 2006; Nouet et al. 2015) including 4 technical replicates for each sample/primer pair (Online
The quality of the PCRs was checked visually through analysis of dissociation and amplification curves. Relative gene expression levels were determined by normalization using multiple reference genes with the qBase software (Biogazelle, Hellemans et al. 2007). Three reference genes (At1g18050, UBQ10, EF1a) were selected from the literature (Czechowski et al. 2005). Their adequacy to normalize gene expression in our experimental conditions was verified using the geNorm software in qBase (gene stability measure M=0.404, pairwise variation CV=0.155) (Vandesompele et al. 2002).

ICP-AES analyses

For plant samples, shoot tissues were rinsed in milliQ water, whereas root tissues were desorbed and washed as described (Talke et al. 2006). Tissues were then dried at 60°C for 2 days. For protein samples, proteins were dialyzed against the purification buffer A without zinc (see below). Samples (10-50 mg of tissues, 5-10 µM purified proteins) were then acid-digested in DigiPrep tubes with 3 ml ≥65% (w/w) HNO₃ (Sigma-Aldrich) on a DigiPrep Graphite Block Digestion System (SCP Science) as follows: 15 min at 45°C, 15 min at 65°C and 90 min at 105°C. After cooling, sample volumes were adjusted to 10 ml with milliQ water and 200 µl ≥65% HNO₃ (Sigma-Aldrich). Metal concentrations were determined by ICP-AES (Inductively Coupled Plasma-Atomic Emission Spectroscopy, Vista AX, Varian).

GFP imaging

Leaves of eighteen day-old T1 seedlings expressing the GFP fusions described above were analyzed (3 independent lines per construct). Images were collected using a SP2 inverted confocal microscope (Leica). An Argon/Ion laser (488nm) was used for excitation of the GFP protein and the emission light was dispersed and recorded at 500 to 540 nm, as described (Rausin et al. 2010). To induce plasmolysis, seedlings were incubated in a 6% (w/v) NaCl solution for 5 minutes prior observation.

Production and purification of non-labelled and isotope-labelled N-terminal domains

E. coli cells (strain BL21 (DE3)) transformed with the pET9a/AhHMA4n expression vector were grown at 37°C in 2 L of LB (Luria-Bertani) medium containing 50µM ZnCl₂ and 50µg/mL kanamycin. At an OD₆₀₀ of ~ 0.8, the production was directly induced with 1mM IPTG (isopropyl β-D-thiogalactopyranoside). The culture was then incubated for 18h at 18°C.
The cells, collected by centrifugation, were resuspended in 50 mL of 10 mM Tris/HCl pH 8 supplemented by 1 mM TCEP and 50 µM ZnCl₂ (buffer A). A protease inhibitor cocktail (mini complete EDTA-free, Roche) was added to avoid the degradation of the protein.

For isotope labelling, prior induction of expression, cells were harvested an OD₆₀₀ of ~0.8 by centrifugation at 11000g for 20 min and resuspended in 500 ml of M9 medium containing [¹⁵N]NH₄Cl and/or [¹³C]Glucose (Cambridge Isotope Laboratories, Inc.), 50µM ZnCl₂ and 50µg/mL kanamycin. After 1h incubation at 37°C, the expression of the protein was induced by 1mM IPTG. Labelled proteins were then collected as described above.

In all cases, cells were lysed after harvest using an EmulsiFlex-C3 cell disrupter (Avestin). The cellular extracts were clarified by centrifugation at 48000g for 40 min at 4°C. The soluble fraction was then loaded onto a cation exchange sepharose column (24 mL, GE Healthcare) equilibrated in 10mM Tris/HCl pH 8 supplemented with 1mM TCEP and 50 µM ZnCl₂. The bound proteins were eluted over a 250 mL linear NaCl gradient (0-300mM). The fractions containing AhHMA4n were pooled, dialyzed overnight against buffer A (see above) and then further purified on a 5 mL Poros H column (Applied Biosystems). The proteins were eluted thanks to a linear NaCl gradient as above.

The N-terminal domain variants were purified as described for the native AhHMA4 N-terminal domain. However, for variants C²⁷A, C²⁸A and C²⁷A/C²⁸A/E³¹A, purification conditions were slightly different: (i) 10mM Tris/HCl pH 8, 1mM TCEP, 50 µM ZnCl₂ and 0.02% n-Dodecyl β-D-Maltopyranoside was used for equilibration and (ii) the elution gradient was increased to 1 M NaCl.

The AhHMA4n fractions were concentrated by ultrafiltration on a 3 kDa molecular-mass cut-off column (Vivaspin). The protein purity was assessed by SDS/PAGE (18% gels), and the final protein concentration was determined by using the molar absorption coefficient at 280 nm (ε=8480 M⁻¹·cm⁻¹), which was calculated with the help of ProtParam (ExPASy Proteomics Server, http://expasy.org/).

**Determination of the affinity of HMA4n for zinc**

The AtHMA4n control was produced as reported previously (Zimmermann et al. 2009). The oxidised AhHMA4n protein with an internal disulphide bond (i.e. AhHMA4n-SS) was produced by quantitative oxidation of AhHMA4n with stoichiometric amount of
[Fe$^{III}$]$_6$(CN)$_3$$^{3+}$ and purified by an ion-exchange column. The AhHMA4n native and variant proteins were produced as described above. To ensure a complete removal of all metal ions from the samples, they were incubated with excess EDTA, followed by a gel-filtration separation.

The determination of zinc (Zn$^{II}$) affinity was conducted via the competition reaction 1 and the data analysed via equation 2 (Xiao et al. 2013):

$$[\text{Zn}^{II}(\text{Par})_2] + P \rightleftharpoons \text{Zn}^{II}$$.P + 2 Par \quad K_{ex} \quad (P = \text{protein or EGTA}) \quad \text{(eq 1)}$$

$$\frac{[P]_{tot}}{[\text{Zn}]_{tot}} = K_D \beta_2 \left( \frac{[\text{Par}]_{tot}}{[\text{Zn}^{II}(\text{Par})_2]} - 2 \right)^2 \left( \frac{1 - [\text{Zn}^{II}(\text{Par})_2]}{[\text{Zn}]_{tot}} \right) + \left( \frac{1 - [\text{Zn}^{II}(\text{Par})_2]}{[\text{Zn}]_{tot}} \right) \quad \text{(eq 2)}$$

The term [Zn$^{II}$(Par)$_2$] is the equilibrium concentration of probe complex [Zn$^{II}$(Par)$_2$] in eq 1 and may be determined directly from the solution absorbance at 500 nm after subtracting the minor contribution from the Par ligand. The other terms in eq 2 are the known total concentrations of the relevant species. The term $K_D \beta_2 = (K_{ex})^{-1}$ is a constant under fixed conditions and may be derived by curve-fitting of the experimental data to eq 2. However, the accumulated formation constant $\beta_2$ for Zn$^{II}$(Par)$_2$ varies considerably with experimental conditions and this will affect the reliability of $K_D$ for Zn$^{II}$-P (Zimmermann et al. 2009). To control such variation, the EGTA ligand, whose affinities for Zn$^{II}$ at various pH values are known, was used as a control affinity calibrator under each experimental condition. Consequently, the $K_D$ for Zn$^{II}$-P may be obtained reliably via eq 3 relative to a control experiment in the same reaction medium with P = EGTA in eq 1:

$$K_D(\text{Zn}^{II}$$.P) = [K_{ex}(\text{EGTA}) / K_{ex}(P)] x K_D(\text{Zn}^{II}$$.EGTA) \quad \text{(eq 3)}$$

The experiments were conducted in MOPS buffer (50 mM, pH 7.3, 100 mM NaCl) with the detailed procedure following that reported previously (Zimmermann et al. 2009). To ensure a complete reduction of all protein thiols, reductant TCEP (100 µM) was included in all reaction media (except for the experiments with the oxidised HMA4n sample). Control experiments showed that excess TCEP had no discernible impact on the estimated Zn(II) affinity.

Nuclear Magnetic Resonance analysis
The spectral assignment experiment of the native and C27A/C28A/E31A triple mutant HMA4n domains were performed on 0.5 mM 13C- and 15N-labelled samples while the HSQC experiments on the single mutants (C27A, C28A and E31A) were conducted on 0.07-0.14 mM 15N-labelled samples. The apo form of AhHMA4n was obtained by treating 100µM of protein with 400µM EDTA for 10 minutes. All samples were prepared in a buffer containing 10mM NH4Ac, 1mM TCEP, 100mM NaCl at pH 6.6 with 5% D2O and DSS 10 µM. The different spectra were acquired at 293 K on a Bruker AVI 500MHz spectrometer equipped with a TCI cryogenically cooled probe. The spectra of the 2D (1H–15N HSQC) and 3D [HNCA, HNCO, HNCACB and CBCA(CO)NH] (Cavanagh 1996) experiments were processed using TOPSPIN (Bruker) and analyzed with CCPNmr.

NMR Minimum Chemical Shift Perturbation

The effects of N-terminal domain mutations on the amide NH resonances were analysed as follows. For each cross-peak in the 1H-15N HSQC spectrum of the wild-type protein, the nearest cross-peak (in terms of 1H and 15N chemical shifts) in the spectrum of each mutant was identified. The 1H and 15N chemical shift differences, ΔH and ΔN, between each such pair of cross-peaks were measured and used to calculate a “minimum Chemical Shift Perturbation” (mCSP) (Lian et al. 2000).

\[ mCSP = \sqrt{(6.7\Delta H)^2 + \Delta N^2} \]

While the individual chemical shifts may be underestimated, the perturbed amide NH can be reliably identified (Williamson et al. 1997). The minimum chemical shift is plotted as a function of residue number. The calculations were performed using TOPSPIN (*xpk) peak lists and a custom made tcl script.

A threshold value was estimated in order to determine significant CSP. In a first step, all CSP are considered and the average (<CSP>) plus three times the standard deviation (σ) is calculated. Then, the highest CSP (CSP≥<CSP>+3σ) are removed from the data and new average and new standard deviation are calculated. The operation is repeated until convergence is reached. The final value <CSP>+3σ for the residues not significantly perturbed corresponds to the threshold (Tavel et al. 2012).

Statistical analysis

All statistical analyses of the data were carried out using STATISTICA (Statsoft).
Results

In this study, we selected the *A. halleri* HMA4 protein as an experimental system to examine *in vivo* and *in vitro* the function of the conserved N-terminal C\textsuperscript{27}CTSE\textsuperscript{31} metal binding motif. The AtHMA4 and AhHMA4 protein share 96.8% identity over the 95 residue N-terminal domain (Online Resource 1).

The C\textsuperscript{27}CTSE\textsuperscript{31} motif of the N-terminal domain is essential for the function of the HMA4 protein *in vivo*

To examine *in vivo* the function of the C\textsuperscript{27}CTSE\textsuperscript{31} N-terminal motif of HMA4 (Zimmermann et al. 2009), we separately mutated the two Cys and the Glu residues into Ala. A triple mutant, where the two Cys and the Glu were mutated into Ala, was also generated (Fig. 1a). The native (AhHMA4) and mutated genes were expressed under the control of the endogenous *A. thaliana* HMA4 promoter (pAtHMA4) in the loss-of-function *hma2hma4* *A. thaliana* mutant (Hussain et al. 2004). Several independent homozygous transgenic lines (T3 generation) were obtained for each construct. The HMA4 gene variants were expressed at similar levels in plant tissues (Online Resource 2).

In our growth conditions on standard soil watered with tap water, the expression of AhHMA4 rescued the phenotype of the *hma2hma4* mutant. The plants developed normally and were able to flower and set seeds without additional zinc supply in the soil, as Col-0 wild-type plants (Fig. 1b-e). In contrast, expression of the C\textsuperscript{27}CTSE\textsuperscript{31} motif variants resulted in the absence of (C\textsuperscript{27}A, C\textsuperscript{28}A, triple mutant) or in partial (E\textsuperscript{31}A) complementation, respectively (Fig. 1f-j). Indeed, plant expressing the C\textsuperscript{27}A, C\textsuperscript{28}A and triple mutants displayed the stunted growth and chlorotic phenotype typical of the *hma2hma4* mutant (Hussain et al. 2004; Wong and Cobbett 2009; Mills et al. 2010). These plants could complete their life cycle and set seeds only upon massive external zinc supply. The plants expressing the E\textsuperscript{31}A mutant presented a phenotype intermediate between Col-0 and *hma2hma4* plants, with a larger rosette and the development of flowers (Fig. 1f). The plants however required additional zinc supply to complete their life cycle. Note that similar results were obtained upon expression of the variants under the control of the *A. halleri* HMA4-1 promoter (data not shown, Hanikenne et al. 2008).

Mutations in the C\textsuperscript{27}CTSE\textsuperscript{31} motif alter zinc and cadmium distribution in plant tissues
We next determined if the mutation in the C^{27}CTSE^{31} motif altered metal accumulation in plant tissues. Zinc and cadmium concentrations were measured by ICP-AES in roots and rosette leaves of 8 week old wild-type (Col-0) and hma2hma4 plants as well as transgenic plants expressing the C^{27}CTSE^{31} variants after cultivation for four weeks in Hoagland hydroponic medium containing either 0.2 µM Zn or 0.05 µM Cd (Nouet et al. 2015). The hma2hma4 mutant accumulated about 6-fold higher zinc and 4-fold higher cadmium in roots and 2.2-fold lower zinc and 6-fold lower cadmium in shoots than the wild-type, respectively (Fig. 2). This reflected the inability of the hma2hma4 mutant to translocate zinc and cadmium from root to shoot. Expression of the native AhHMA4 gene in the hma2hma4 genetic background almost completely restored wild-type levels of zinc accumulation in root and shoot tissues. The plants expressing the C^{27}A, C^{28}A and triple mutants accumulated zinc at levels identical to the hma2hma4 mutant. In contrast, expression of the E^{31}A mutant partially restored shoot zinc accumulation to levels intermediate between Col-0 and the hma2hma4 mutant, but only marginally reduced root accumulation (Fig. 2a-b). Identical observations were made upon 0.05 µM Cd exposure (in the presence of 1 µM Zn): only the expression of the E^{31}A mutant resulted in moderate increase of cadmium accumulation in shoots compared to the hma2hma4 mutant (Fig. 2c-d).

The C^{27}CTSE^{31} motif is not required for plasma membrane localization

Mutations in the N-terminal domain of AhHMA4 might impact its intracellular localization. The inability of the C^{27}CTSE^{31} motif variants to complement the phenotype of the hma2hma4 mutant may therefore results from a mis-localization of the protein in cells rather than a loss of function. To exclude this hypothesis and to ascertain that the variants are expressed at the protein level, we expressed GFP fusions of the C^{27}CTSE^{31} AhHMA4 variants under the control of a double 35S promoter in the Col-0 genetic background.

Leaves of 18-day-old seedlings expressing the GFP fusions were imaged by confocal microscopy. All three simple mutants (C^{27}A, C^{28}A and E^{31}A) and the triple mutant of the AhHMA4 protein were expressed and localized in the plasma membrane of leaf epidermal cells (Fig. 3). The induction of a plasmolysis of leaf cells confirmed the plasma membrane localization of the protein: a characteristic detachment of the membrane from the cell-wall, with the exception of plasmodesmata, was observed (Fig. 3f). No fluorescence was detected in leaves of Col-0 seedlings using identical settings (Fig. 3a). GFP imaging experiments also
suggests that the C\textsuperscript{27}CTSE\textsuperscript{31} motif variant proteins are stable in planta, as we did not detect any GFP aggregation in cells (Fig. 3).

**Mutations in the C\textsuperscript{27}CTSE\textsuperscript{31} motif alter zinc binding properties in vitro**

The native N-terminal domain of AhHMA4 (residues 1-95 residues, AhHMA4n) and the C\textsuperscript{27}CTSE\textsuperscript{31} variants were expressed in *E. coli* and purified. After dialysis, we assessed the stoichiometry of zinc binding to the proteins by ICP-AES measurements (Table 1). The native AhHMA4n bound \( \approx 1 \) zinc ion per protein. Zinc binding was strongly reduced for the C\textsuperscript{27}A and C\textsuperscript{28}A mutant proteins as well as for the triple mutant. The E\textsuperscript{31}A mutant retained an intermediate zinc binding capacity (Table 1).

Using the Par zinc probe (Zimmermann et al. 2009), we next quantitatively estimated the binding affinity of the AhHMA4n variants for zinc (Table 2 and Online Resource 3). The native AhHMA4n protein had a \( K_D \) for zinc in the nanomolar range identical to that for the AtHMA4n protein (previously determined by Zimmermann et al. 2009), despite 3 polymorphic positions in the N-terminal domain (see Online Resource 1). Oxidation of the two Cys thiols to an internal disulfide bond in the binding motif completely abolished zinc binding. The C\textsuperscript{27}A mutation, taken as a representative of the most affected mutant variants, decreased affinity for zinc by 1.8 orders of magnitude, whereas the E\textsuperscript{31}A mutation only reduced the affinity for zinc by 1.4 orders of magnitude.

**Structural impact of the C\textsuperscript{27}CTSE\textsuperscript{31} motif mutations by NMR spectroscopy**

To examine the structural consequences of mutations in the C\textsuperscript{27}CTSE\textsuperscript{31} motif of AhHMA4, 2D 15N-1H HSQC NMR experiments were recorded for the native and C\textsuperscript{27}CTSE\textsuperscript{31} variant AhHMA4n proteins. Backbone NH NMR signal chemical shifts and intensities are very sensitive probes to study protein structural and dynamic modifications. The HSQC spectrum of the native AhHMA4n protein was similar to previously published data for AtHMA4n (Zimmermann et al. 2009). The HSQC spectra of the C\textsuperscript{27}CTSE\textsuperscript{31} motif variants revealed that the AhHMA4n domains were structured, despite (strongly) reduced zinc binding capability (Table 1), and displayed limited variations compared to the spectrum of the native AhHMA4n domain (Fig. 4 and Online Resource 4). These observations suggested that mutations in the C\textsuperscript{27}CTSE\textsuperscript{31} motif had limited impact on the domain structure. To statistically support these conclusions, an analysis of the minimal chemical shift perturbations (mCSP) was used to compare the HSQC spectra of the native and C\textsuperscript{27}CTSE\textsuperscript{31} variant AhHMA4n
proteins (Fig. 5a and Online Resource 5). The observed mCSPs corresponded to the mutated amino acid residues and to amino acid residues interacting with the mutated residues (Fig. 5). A very similar mCSP profile was also obtained for the apo form of the protein obtained by treatment with EDTA prior to NMR analysis (Online Resource 6).

Backbone assignment (N, H, CO, Ca, Cb) was performed for the native AhHMA4n protein and the triple C27A/C28A/E31A mutant, which represented the most extreme modification of the C27CTSE31 motif. This confirmed that perturbed amino acid residues are located at the vicinity of the zinc binding site or correspond to more distant residues in the primary sequence that are spatially close in the 3D structure. Structure predictions using the backbone chemical shift and the Talos+ software (Shen et al. 2009) confirmed that the mutations in the C27CTSE31 motif did not have a major impact on the AhHMA4n domain secondary structure (data not shown).

Discussion

IB P-type ATPase proteins play essential roles in metal homeostasis in Arabidopsis species (Williams and Mills 2005; Nouet et al. 2011). Hence, HMA4 is a major actor in Zn hyperaccumulation as well as Zn and Cd tolerance in Arabidopsis halleri (Talke et al. 2006; Courbot et al. 2007; Willems et al. 2007; Hanikenne et al. 2008; Hanikenne et al. 2013). If the catalytic mechanism of transport by P-type ATPases is well described, establishing the roles and functions of N- and C-terminal extremities and their MBDs still require further investigations. Depending on the organisms and proteins, N-terminal MBDs have been involved in multiple functions, including regulatory roles, controlling catalytic activities, dephosphorylation and metal ion release possibly via interactions with the cytoplasmic ATP binding domain, or the intracellular targeting of the protein (Tsivkovskii et al. 2001; Mana-Capelli et al. 2003; Mandal et al. 2003; Argüello et al. 2007; Veldhuis et al. 2009).

Zimmermann et al. (2009) determined the 3D solution structure of the N-terminal domain of AtHMA4, which binds one zinc atom at the C27CTSE31 motif. Here, we examined the function of this motif in planta, assessed the contribution of the conserved Cys and Glu residues to zinc binding and evaluated the impact of mutations in the motif on protein structure and function. Combining both in vivo and in vitro analyses allowed an integrated analysis of the structure/function relationship for the N-terminal MBD of HMA4.
The HMA4 protein localizes to the plasma membrane in plant tissues (Verret et al. 2004; Courbot et al. 2007; Siemianowski et al. 2013; Nouet et al. 2015). The N-terminal domain of HMA4 was not involved in protein intracellular localization or in protein stability (Fig. 3). Indeed, all mutant variants in fusion with GFP localized in the plasma membrane of stable A. thaliana transformants (Fig. 3), in agreement with previous results for AtHMA2 (Wong et al. 2009). In contrast, the C-terminal domain of AtHMA2 possibly contains a signal important for the subcellular localization of the protein in planta (Wong et al. 2009), whereas the C-terminal domain of AtHMA4 is not required for correct localization in yeast cells (Baekgaard et al. 2010).

Expression of the AhHMA4 gene under the control of the pAtHMA4 promoter only partially complemented the defect in root to shoot zinc translocation of the hma2hma4 mutant: zinc shoot accumulation level was lower than in the wild-type, but was however sufficient to sustain normal development (Figs. 1 and 2). When expressed under the control in the HMA4 endogenous promoter, HMA4 is thus not sufficient alone to fully compensate for the loss of both HMA2 and HMA4. In contrast, expressing the same gene under the control of promoters of the A. halleri HMA4, which are stronger than the pAtHMA4 promoter (Hanikenne et al. 2008) fully complemented the mutant (Nouet et al. 2015).

In complementation experiments, the C$^{27}$A, C$^{28}$A and C$^{27}$A/C$^{28}$A/E$^{31}$A mutations abolished the ability of the AhHMA4 protein to complement the strong zinc deficiency phenotype and to restore zinc and cadmium root-to-shoot translocation in the hma2hma4 A. thaliana mutant (Fig. 1 and 2). Reduced zinc binding and affinity (Tables 1 and 2, Online Resource 3) are thus accompanied by a loss of function in planta. Previous studies analysing the N-terminal MDB of AtHMA2 (~ 82% sequence identity with the AtHMA4 N-terminal domain, see Online Resource 1) suggested that the CCxxE motif is required for zinc and cadmium binding in vitro and for maximum enzyme turnover but were not essential for activity or metal binding to transmembrane metal binding sites in yeast cells (Eren et al. 2007). However, the two Cys residues of the motif were required for function of AtHMA2 in planta (Wong et al. 2009) and AtHMA4 in yeast (Verret et al. 2005).

In contrast, the E$^{31}$A mutation sustained partial complementation of the hma2hma4 mutant phenotype (Fig. 1 and 2) and retained higher zinc binding and affinity than the Cys-->Ala mutants (Tables 1 and 2, Online Resource 3). Note that this mutant retained a higher capacity for zinc translocation to the shoot than for cadmium (Fig. 2).
The NMR HSQC spectrum of the amide NHs of the purified AhHMA4n protein was nearly identical to the data obtained by Zimmermann et al. for AtHMA4n (Zimmermann et al. 2009). Since NMR chemical shifts are very sensitive to the structural environment, the very similar NMR spectrum was a strong evidence for a very similar 3D structure. This confirmed the global ferredoxin βαβαβ fold of the domain, in which the thiols of Cys residues and carboxy group of the Glu residue contributed to the coordination of zinc. The three polymorphic residues between AtHMA4n and AhHMA4n had thus no major impact on protein structure. Furthermore, we showed here that the mutations in the C27CTSE31 motif of AhHMA4n, which drastically reduced zinc binding, had no significant effect either on the secondary or on the tertiary structure of the protein. This observation was also confirmed by the structural analysis of the apo form of AhHMA4n: the structure of the apo form is not altered.

Altogether, our data indicated that the two Cys residues of the C27CTSE31 motif of the N-terminal MBD are essential for the function of HMA4, whereas the Glu residue is important but not essential. Moreover, zinc binding to the N-terminal MBD is crucial for HMA4 protein function, whereas it is not required to maintain the N-terminal domain structure. Based on these observations arises the following question: what is the requirement of zinc binding for the protein function? It may be required for intramolecular interactions controlling the pump’s activity and/or conformational changes during metal transport (Tsivkovskii et al. 2001).

Copper IB pType ATPases and many bacterial zinc IB pType ATPases possess a highly conserved CxxC motif in their N-terminal MBDs. This motif can bind both monovalent (Cu+) or divalent (Cu2+, Zn2+, Cd2+) metal ions in vitro and, for instance, metal selectivity for Cu+ is determined in vivo by electrostatic and hydrophobic interactions with specific copper chaperones (Argüello et al. 2007). However, in all plant zinc IB pType ATPases, this conserved motif is replaced by a CCxxE motif. It was initially suggested that the CCxxE motif could confer selectivity for Zn(II) but not for Cu(I) due to its higher binding affinity for Zn(II) than for Cu(I) (Eren et al. 2007). However, this conclusion has been questioned when both CCxxE and CxxC motifs were showed to bind Cu(I) with affinities at least 6 orders of magnitude higher than Zn(II). Both motifs can also bind Zn(II) with moderate affinities in the nanomolar range (Zimmermann et al. 2009). Quantitative evaluations under identical conditions revealed that the MBDs containing the CCxxE motif bind Zn(II) with affinities 20-30 times stronger than do those MBDs containing CxxC while relative affinities for Cu(I)
are inverted by a factor of 30-50 (Zimmermann et al. 2009). Consequently, it was proposed that, under metal-limiting conditions, zinc selectivity is conferred by relative affinities and not absolute affinities. Under these conditions, Cu(I) ions are confined to their native high affinity sites and are not available to compete for native Zn(II) sites (Zimmermann et al. 2009). Interestingly, the affinity for zinc of the E31A domain of HMA4 featuring a CCxxA metal binding motif is essentially identical to that of the N-terminal domain of the copper ATPase HMA7 containing a CxxC metal binding motif (Zimmermann et al. 2009). Yet, in the hma2hma4 A. thaliana mutant, the endogenous copper ATPases, such as HMA5 (Andrés-Colás et al. 2006; Kobayashi et al. 2008) or HMA7, do not seem to substitute HMA2 and HMA4 for Zn/Cd transport while the AhHMA4 E31A variant did partially sustain the Zn/Cd transport function. It appears that the functional specificity of different metal transporters must also depend on factors other than the metal-binding affinity and that these may include specific intra-molecular interactions (Tsivkovskii et al. 2001) or expression patterns. Interestingly, bacterial zinc (ZntA) and cadmium (CadA) IB pType ATPases also use a carboxylate ligand for zinc and cadmium, respectively, with the presence of a DCxxC motif found in the E. coli ZntA and of a Glu residue in a loop more distant of the CxxC motif in the CadA N-terminal domain (Banci et al. 2002; Banci et al. 2006).

In conclusion, our analyses highlight the importance of zinc binding to the N-terminal MBD of HMA4 for the protein function in planta. This work further establishes the value of combining in planta and in vitro studies to reveal the structure/function relationships for transmembrane metal transporters. Future developments may possibly include analysing the interaction of the N-terminal domain and its MBD variants with other cytoplasmic domains of HMA4.
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transporters: selectivities match the relative, but not the absolute, affinities of their
Table 1. Stoechiometry of zinc binding to AhHMA4 C<sup>27</sup>CTSE<sup>31</sup> N-terminal variants.

<table>
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<tr>
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<th>Ratio protein/Zn</th>
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<tbody>
<tr>
<td>AhHMA4n</td>
<td>0.86</td>
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<tr>
<td>AhHMA4n C&lt;sup&gt;27&lt;/sup&gt;A</td>
<td>0.06</td>
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<tr>
<td>AhHMA4n C&lt;sup&gt;28&lt;/sup&gt;A</td>
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<tr>
<td>AhHMA4n E&lt;sup&gt;31&lt;/sup&gt;A</td>
<td>0.37</td>
</tr>
<tr>
<td>AhHMA4n C&lt;sup&gt;27&lt;/sup&gt;A/C&lt;sup&gt;28&lt;/sup&gt;A/E&lt;sup&gt;31&lt;/sup&gt;A</td>
<td>&lt;0.01</td>
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Table 2. Affinity of zinc binding to AhHMA4 C27CTSE31 N-terminal variants.

<table>
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<tr>
<th>Protein</th>
<th>$\log K_D^{a}$ ([Par]$^{\text{tot}}$, 50 $\mu$M)</th>
<th>$\log K_D^{a}$ ([Par]$^{\text{tot}}$, 100 $\mu$M)</th>
<th>average $\log K_D$</th>
<th>$\log (K_D/K_D^{(\text{wt})})$</th>
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<tr>
<td>AtHMA4n$^{b}$</td>
<td>$&lt;- 9.40^a$</td>
<td>$- 9.63$</td>
<td>$- 9.63$</td>
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<td>AhHMA4n</td>
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<td>AhHMA4n C27A</td>
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<td>$- 7.83$</td>
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<tr>
<td>AhHMA4n E31A</td>
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<td>$- 8.19$</td>
<td>$- 8.20$</td>
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<td>AhHMA4n SS</td>
<td>N.D.$^c$</td>
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$^a$The affinity for zinc was determined at two Par concentrations (50 and 100 $\mu$M) (see Zimmermann et al. 2009).

$^b$Used as control (described in Zimmermann et al. 2009).

$^c$Not Detectable.
Figure legends

Fig. 1. Complementation of the hma2hma4 A. thaliana mutant by the A. halleri HMA4 protein and C\textsuperscript{27}CTSE\textsuperscript{31} variants expressed under the control of the pAtHMA4 promoter. a Partial sequences of the AhHMA4 N-terminal (AhHMA4n) domain. Mutated residues in the C\textsuperscript{27}CTSE\textsuperscript{31} motif are shown in bold font. b-j Phenotype of the plants after 8 weeks of growth on standard soil. Plants were grown without Zn supplementation for phenotyping. Wild-type A. thaliana plants (Col-0 accession) (b) and the hma2hma4 mutant (c-d) are shown as controls. Mutant plants expressing a native AhHMA4 protein (e) or C\textsuperscript{27}A (g-h), C\textsuperscript{28}A (i), E\textsuperscript{31}A (f) and triple C\textsuperscript{27}A/C\textsuperscript{28}A/E\textsuperscript{31}A (j) variants display wild-type (e) or mutant phenotypes (g-j). Scalebars: 1 cm.

Fig. 2. Zinc and cadmium accumulation in plants expressing AhHMA4n variants. Wild-type A. thaliana plants (Col-0 accession), hma2hma4 mutant and mutant plants expressing a native AhHMA4 protein or C\textsuperscript{27}A, C\textsuperscript{28}A, E\textsuperscript{31}A and triple C\textsuperscript{27}A/C\textsuperscript{28}A/E\textsuperscript{31}A variants were grown for 4 weeks in Hoagland hydroponic medium containing 0.2 µM ZnSO\textsubscript{4} (a-b) or 0.05 µM CdSO\textsubscript{4} (c-d). Metal contents (mg kg\textsuperscript{-1} DW) were measured by ICP-AES from root and shoot tissues. Values relative to the wild-type (Col-0) are means ± SEM of 2-3 independent lines from two independent experiments, each including two replicates of 3 plants per line. The data were analyzed with a Kruskal-Wallis non-parametric ANOVA followed by multiple comparison tests. Statistically significant differences (p<0.01) between means are indicated by different superscripted letters.

Fig. 3. Cellular localization of C\textsuperscript{27}CTSE\textsuperscript{31} AhHMA4 variants. GFP fusions of C\textsuperscript{27}A (b), C\textsuperscript{28}A (c), E\textsuperscript{31}A (d) and triple C\textsuperscript{27}A/C\textsuperscript{28}A/E\textsuperscript{31}A (e) variants were imaged by confocal microscopy in leaves of 18 day-old seedlings. The fusions were expressed in the Col-0 background under the control of a double 35S promoter. Non transformed Col-0 seedlings served as controls (a). (f) Plasmolysis on leaf of AhHMA4 C\textsuperscript{28}A expressing plants confirmed plasma membrane localization. The arrow indicates a plasmodesmata. Scalebars: 20 µM.
**Fig. 4.** Superposition of the 2D HSQC $^1$H-$^{15}$N NMR spectra of the native (in black) and triple C$^{27}$A/C$^{28}$A/E$^{31}$A mutant (in red) AhHMA4$n$ proteins at pH 6.6. The marked peaks (arrows) represent the most affected residues.

**Fig. 5.** Chemical shift perturbation resulting from the C$^{27}$A/C$^{28}$A/E$^{31}$A mutation in the AhHMA4$n$ protein. a A threshold of 3 standard deviations (green line) was selected to identify significant shift perturbations when comparing the native and triple C$^{27}$A/C$^{28}$A/E$^{31}$A mutant AhHMA4$n$ proteins. The green and orange boxes represent the alpha helices and beta sheets in the sequence, respectively. b The shift perturbations were localized on the structure of AtHMA4$n$ (2KKH (Zimmermann et al. 2009)) using Pymol. A color code for residues was used to represent the level of perturbation: red $\geq$ 2ppm; 2ppm $\leq$ purple $\leq$ 1ppm; blue $\leq$ 1ppm.
Figure 1

(A)

WT  KKKVKKLQKSYFDVLGICTSEVPIIENILKSL
C7A  KKKVKKLQKSYFDVLGI
C28A  KKKVKKLQKSYFDVLGI
E31A  KKKVKKLQKSYFDVLGICTSEVPIIENILKSL
C10A/C10A  KKKVKKLQKSYFDVLGI

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|                               |
|                               |
|                               |
Figure 2

(A) Relative root Zn concentration

(B) Relative shoot Cd concentration

(C) Relative root Cd concentration

(D) Relative shoot Zn concentration
Figure 3
Figure 4
Figure 5

(A)

(B)

(20 30 40 50 60)

70 80

KKVRLQDYYTVPTTIESQFQKVTPSVVHSLLLPFPQIAKANVNLKTVHEET

KKVRLQDYYTVPTTIESQFQKVTPSVVHSLLLPFPQIAKANVNLKTVHEET

Cys28  Cys27

Glu31  α1

β2  β3

β4  β1  α2
Metal binding to the N-terminal cytoplasmic domain of the P_{1B} ATPase HMA4 is required for metal transport in *Arabidopsis*.

Plant Molecular Biology

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Tel: +32-4-3663844
Fax: +32-4-3662960
**Table S1.** Primers used in the study.

**(A) Cloning primers.**

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<td>pAtHMA4 AscI</td>
<td>TATAGAATTCGGCGCCACTTATCCCGATGTTGATGCCATG</td>
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<tr>
<td>AhHMA4 AscI</td>
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<tr>
<td>AhHMA4 PacI</td>
<td>CTTAATTAAGCGATGCGTACAAAAACAAAGGAAGAAG</td>
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Restriction enzyme sites are indicated in bold font.

**(B) Mutagenic primers.**

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<th>Name</th>
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<td><strong>Full length protein (in vivo experiments)</strong></td>
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<tr>
<td>AhHMA4 Nter C27A Fwd</td>
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<td><strong>N-terminal domain (in vitro experiments)</strong></td>
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<td>GGGTATTTGCTGTAACGAGTCGGATGTTGCCGATTCGAAAAACATTCTG</td>
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Mutated bases are indicated in bold font.
(C) Real-time RT-PCR primers.

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Online Resource 2. Expression of *AhHMA4* in seedlings expressing *AhHMA4* variants. *hma2hma4* mutant plants expressing a native AhHMA4 protein or C27A, C28A, E31A and triple C27A/C28A/E31A variants were grown for 2 weeks on 1/2 MS medium. Expression levels of *AhHMA4* relative to three reference genes (see Methods) and to *AhHMA4* lines are means ± SEM of 2-5 independent lines (>10 seedlings/line). The expression levels are not statistically different (t-test, *p*<0.05). RTL: Relative Transcript Level.
Online Resource 3. Determination of Zn\textsuperscript{II} dissociation constants ($K_D$) of AhHMA4n native and C\textsuperscript{27}CTSE\textsuperscript{31} variant proteins in MOPS buffer (50 mM, pH 7.3) with the Par ligand as a detection probe and the EGTA ligand as an affinity standard. Variation of [Zn\textsuperscript{II}Par\textsubscript{2}] with the HMA4n : Zn ratio in the presence of [Par]\textsubscript{total} = 50 \mu M (a) or 100 \mu M (b): (i) native AhHMA4n (in red) and control AhHMA4n (in black, Zimmermann et al. 2009); (ii) control EGTA affinity standard; (iii) E\textsuperscript{31}A variant; (iv) C\textsuperscript{27}A variant; (v) oxidized AhHMA4n with an internal disulfide bond. Curve-fittings of the experimental data to eq 2 (see Methods) allowed derivation of $K_D$ ($\beta_1 = (K_D)^\frac{1}{2}$) and the calculation of $K_D$ for Zn\textsuperscript{II}-P via eq 3 (see Methods) with a control reaction involving EGTA and with known $K_D$ for Zn\textsuperscript{II}-EGTA.
Online Resource 4. Superposition of the 2D HSQC $^1$H-$^{15}$N NMR spectra of the native (in black) and C27A (a), C28A (b) and E31A (c) mutant (in red) AhHMA4n proteins at pH 6.6.
Online Resource 5. Chemical shift perturbation at pH 6.6 resulting from the C27A (a), C28A (b) and E31A (c) mutations in the AhHMA4n protein. A threshold of 3 standard deviations (green line) was selected to identify significant shift perturbations when comparing the native and mutant AhHMA4n proteins. The mutated residues are in red.
Online Resource 6. Chemical shift perturbation at pH 7.3 resulting from the treatment of the native AhHMA4n protein with EDTA. A threshold of 3 standard deviations (green line) was selected to identify significant shift perturbations when comparing the holo- and apo-forms of the AhHMA4n protein.