Heavy metal transport by AtHMA4 involves the N-terminal degenerated metal binding domain and the C-terminal His_{11} stretch

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Abstract **The *Arabidopsis thaliana* AtHMA4 is a P_{1B}-type ATPase that clusters with the Zn/Cd/Pb/Co subgroup. It has been previously shown, by heterologous expression and the study of *AtHMA4* knockout or overexpressing lines in Arabidopsis [1–3], that AtHMA4 is implicated in zinc homeostasis and cadmium tolerance. Here, we report the study of the heterologous expression of AtHMA4 in the yeast *Saccharomyces cerevisiae*. AtHMA4 expression resulted in an increased tolerance to Zn, Cd and Pb and to a phenotypic complementation of hypersensitive mutants. In contrast, an increased sensitivity towards Co was observed. An AtHMA4::GFP fusion protein was observed in endocytic vesicles and at the yeast plasma membrane. Mutagenesis of the cysteine and glutamate residues from the N-ter degenerated heavy metal binding domain impaired the function of AtHMA4. It was also the case when the C-ter His_{11} stretch was deleted, giving evidence that these amino acids are essential for the AtHMA4 binding/translocation of metals.**

Keywords: P_{1B}-type ATPase; AtHMA4; Metal binding domain; *Arabidopsis*

1. Introduction

It has been recently shown that AtHMA4, a P_{1B}-type ATPase, is involved in the long distance transport of Zn and Cd in *Arabidopsis thaliana* [2,3]. Among the eight P_{1B}-type ATPases present in the *A. thaliana* genome [4], four are predicted to be Cu/Ag transporters. Such function has been demonstrated for AtHMA7 (RAN1) and AtHMA6 (PAA1) [5–7]. The four others, AtHMA1–4, are putative Zn/Cd/Pb/Co ATPases. Recent studies using heterologous expression and knockout or overexpressing mutant lines of *A. thaliana* have confirmed such a role in Zn and Cd transport for AtHMA2–4 [2,3,8].

AtHMA2, 3 and 4 proteins are highly similar over the first 750 amino acids (aa). On this part, they exhibit all the characteristics of P_{1B}-ATPases, i.e., eight transmembranous segments and at the N-ter hydrophilic extension a putative 70 aa HMA domain (referred as PS01047 by PROSITE) including a metal binding domain (MBD). Several studies have demonstrated the role of HMA domains in the allosteric control of cation transport rate [9,10]. A peculiar characteristic of AtHMA2–4 is the substitution of the highly conserved GMxCxxC motif – usually found in MBDs – by a degenerated sequence GICC(T/S)SE. This has led some authors to suspect this domain as non-functional [1]. Moreover, AtHMA2 and AtHMA4 exhibit long C-ter hydrophilic extensions which are very unusual among P-ATPases and seem specific of plant P_{1B}-ATPases. Among the P_{1B}-ATPases identified so far, AtHMA4 possesses the longest sequence resulting from a 450-aa hydrophilic extension including as much as 44 cysteines and a terminal stretch of 11 consecutive histidines (Fig. 1).

A preliminary study has shown that AtHMA4 induces an increased tolerance to Cd when expressed in yeast (underlining heterologous expression as a valuable tool to characterize the role of the specific domains shared by AtHMA2–4 proteins [1]). This tool was used in the present study to determine the AtHMA4 substrate specificity. An AtHMA4D401A altered protein, substituted in the ATPase strictly conserved sequence DKTGT, allowed us to demonstrate that metal tolerance enhancement in yeast was linked to the ATPase activity and not to a simple metal chelation. Finally, mutagenesis studies were performed to investigate the involvement of the N-ter degenerated MBD, the CPC domain and the C-ter histidine stretch in the metal transport function.

2. Materials and methods

2.1. Cloning of the AtHMA4 cDNA

Cloning of the *AtHMA4* cDNA was performed as previously described in [11] except the primer pair used: 1HMA4 as 5′-end primer, and Rev14HMA4 as 3′-end reverse primer, both designed on the BAC sequence At2g19110. The full-length *AtHMA4* cDNA was obtained in a unique fragment by a two steps RT-PCR, cloned in the pCR®–XL-TOPO vector (Invitrogen™), and sequenced (GenBank Accession No. AF412407). Then, *AtHMA4* cDNA was excised from the pCR®–XL-TOPO vector and cloned into the yeast expression vector pYES2 under a galactose-inducible promoter. To obtain the *AtHMA4ΔHis* altered version (deleted of the last 16 aa at the C-ter end), a Stop codon was introduced by PCR in the *AtHMA4* cDNA using the primer pair: 22HMA4 as forward and HMA4Stop as reverse oligonucleotides, and the fragment was subcloned into the plasmid pYES2-*AtHMA4*. To create the *AtHMA4::EFGP* fusion, the *AtHMA4* cDNA was excised from the pCR®–XL-TOPO vector and cloned in frame into the pYES2-GFP vector [11].

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Abbreviations: aa, amino acids; EGFP, enhanced green fluorescent protein; ICP-AES, inductively coupled plasma-atomic emission spectrometry; CDF, cation diffusion facilitator; HMA, heavy metal associated

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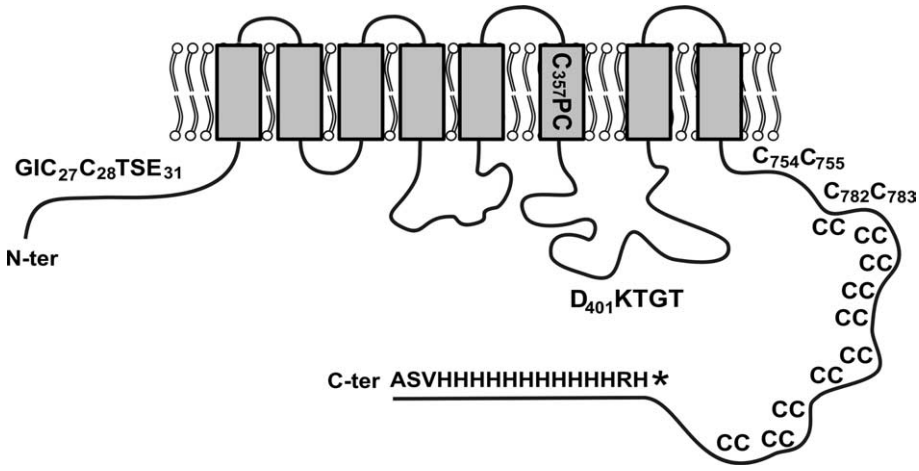

Fig. 1. Schematic representation of the structure of AtHMA4. Site-directed mutagenesis substituted aa are shown with their position numbers in the sequence. Asterisk indicates the insertion of the Stop codon to create the AtHMA4ΔHis altered form.

2.2. Site-directed mutagenesis

The various substituted forms of AtHMA4 were obtained using the Quickchange site-directed mutagenesis kit (Stratagene) using the pYES2-*AtHMA4::EGFP* vector as a template.

2.3. Yeast strain and growth conditions

Saccharomyces cerevisiae reference strain BY4741 (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) and mutant strains *ycf1* (like BY4741 except *YDR135c::kanMX4*), *zcr1* (like BY4741 except *YMR243c::kanMX4*), *cot1* (strain BY4742: *MATα*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*; *YOR316c::kanMX4*) and *end3* (like BY4741 except *YNL084c::kanMX4*) were provided by Euroscarf (Frankfurt, Germany). YCF1::EGFP fusion cloned into the pYES2 vector was used to complement the *ycf1* strain. Yeast transformation and growth conditions were as described in [11].

2.4. Assays for metal tolerance

All metal tolerance assays were performed as previously described in [11]. For the drop-test experiments on solid media, 2 μL of the yeast cultures at $O.D._{600\ nm} = 2.0$ was spread (1.6×10^4 cells) and three 10-fold serial dilutions were made. Metal solutions ($ZnSO_4$; $CdCl_2$; $Pb(CH_3COO)_2$; $CoCl_2$) were added to the liquid or solid media at the concentrations indicated in the figure legends.

2.5. EGFP observations

The yeast strains expressing the AtHMA4::EGFP fusion protein were grown as described in [11]. EGFP excitation was performed at 470–490 nm using a DM505 dichroic mirror. Observations were carried out at a magnification of 100×/1.35 na (UplanApo), under oil immersion. The EGFP fluorescence was monitored with a color camera (Cohu Electronics), and analyzed with the analySIS software.

2.6. Measurement of metal content

Yeast cells were grown as described for metal tolerance assays. Cell mineralization and heavy metal content determination by ICP were as described in [11].

3. Results

3.1. AtHMA4 localizes in the yeast microsomal fraction

Expression of AtHMA4 in yeast was evaluated using an *AtHMA4::EGFP* fusion chimera. Western blots using anti-EGFP horseradish peroxidase antibodies confirmed the presence of the protein in the microsomal fraction (Fig. 2A). Under fluorescence microscopy, AtHMA4::EGFP fluorescence was observed as an intracellular punctuated staining (Fig. 2B). Such localization was unexpected since in planta AtHMA4 is believed to function as an efflux pump and localized at the plasma membrane in transient expression experiments [3]. However, some yeast plasma membrane proteins, such as Ste6p, also exhibit a punctuated distribution due to a rapid turnover resulting from endocytosis [12]. Such process could affect AtHMA4 whose sequence displays numerous dileucine motifs hypothesized to initiate an endocytosis-mediated catabolism of this transporter [13]. Therefore, we monitored AtHMA4::EGFP localization in the *end3* mutant of *S. cerevisiae* which is known to be defective for endocytosis [14]. When expressed in *end3*, AtHMA4::EGFP showed a rim-staining pattern revealing a partial localization at the plasma membrane (Fig. 2C).

3.2. AtHMA4 confers an increased Cd/Zn/Pb tolerance to wild-type and hypersensitive strains of S. cerevisiae

Based on its sequence, AtHMA4 has been predicted as a putative Zn/Cd/Pb/Co transporter. Previous studies have shown that alteration of the level of expression of AtHMA4 in planta or in heterologous systems leads in Zn, Cd and Pb over tolerance/sensitivity [1–3]. When expressed in the wild-type yeast strain, AtHMA4 was able to increase the yeast Cd tolerance up to 150 μM as exemplified by drop-test experiments (Fig. 3). In the *ycf1* context, a yeast mutant extremely sensitive to Cd [15], *AtHMA4* expression was able to restore the tolerance to Cd to the level of the wild-type (Fig. 3). Interestingly, the Cd tolerance enhancement conferred by AtHMA4 was unaffected by the GFP fusion at the C-terminal part of the protein (see Fig. 5e). A series of drop-test experiments were also carried out in presence of a range of Zn concentrations (Fig. 4A). AtHMA4-transformants were slightly more resistant to Zn than the wild-type strain, up to a concentration of 25 mM. AtHMA4 was also expressed in the *zrc1* context. The *zrc1* Zn hypersensitive strain is a deletion mutant of the zinc ZRC1 vacuolar transporter belonging to the CDF family [16]. As shown in Fig. 4A, AtHMA4 expression partially restored the Zn tolerance of the *zrc1* strain. Similar experiments were performed with Pb acetate supplied in solid medium. In agreement with a previous work [17], we observed that the *ycf1* strain was highly sensitive to Pb. When expressed in the *ycf1* context, AtHMA4 was able to partially restore the growth