The plant P_{1B}-type ATPase AtHMA4 transports Zn and Cd and plays a role in detoxification of transition metals supplied at elevated levels

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Abstract **The transition metal Zn is essential for many physiological processes in plants, yet at elevated concentrations this, and the related non-essential metal Cd, can be toxic. *Arabidopsis thaliana* HMA4, belonging to the Type P_{1B} subfamily of P-type ATPases, has recently been implicated in Zn nutrition, having a role in root to shoot Zn translocation. Using *Arabidopsis* insertional mutants, it is shown here that disruption of AtHMA4 function also results in increased sensitivity to elevated levels of Cd and Zn, suggesting that AtHMA4 serves an important role in metal detoxification at higher metal concentrations. AtHMA4 and a truncated form lacking the last 457 amino acids both confer Cd and Zn resistance to yeast but a mutant version of the full-length AtHMA4 (AtHMA4-C357G) does not; this demonstrates that the C-terminal region is not essential for this function. Evidence is presented that AtHMA4 functions as an efflux pump.**

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1. Introduction

Mechanisms are required by all organisms to ensure that they have an adequate supply of essential nutrients and also to control accumulation of toxic solutes. Transition metals can pose a serious problem for plant growth; whereas some such as Cu, Zn, and Mn are essential micronutrients, others such as Cd and Pb can be toxic to most plants [1]. Indeed, even the essential micronutrients can have deleterious effects at elevated concentrations, due to their reactive nature [2]. Therefore, tight control of metal concentrations within narrow physiological limits is required and various mechanisms have been implicated depending on the metal and plant species in question. These include the control of metal uptake by the roots, the distribution or partitioning to different tissues, and cellular processes controlling the trafficking, sequestration and compartmentalization of metal ions within cells [3]. Membrane transport proteins play important roles in these processes and a number of gene families have been identified with members functioning in transition metal transport [4,5].

The P_{1B}-type ATPases, also known as the heavy metal ATPases (HMAs), are thought to play an important role in transition metal transport in plants [4–11]. They are sometimes referred to as CPx-type ATPases due to the presence of a distinctive CPx motif in the sixth transmembrane domain; this is conserved in all members of this sub-family apart from a few examples where there is an SPC motif (5). HMAs cluster into two main sub-classes in phylogenetic analysis, referred to as the Cu/Ag group and the Zn/Co/Cd/Pb group [7,9]. AtHMA4 from *Arabidopsis* was the first plant P_{1B}-type ATPase of the Zn/Co/Cd/Pb group to be cloned and characterized [7]. *AtHMA4* confers Cd resistance when heterologously expressed in a wild-type (wt) strain of *S. cerevisiae* [7] and it also rescues the Zn sensitivity of the *E. coli zntA* mutant [7], suggesting a role in Cd and Zn transport. These metals also regulate *AtHMA4* expression in *Arabidopsis* [7]. Evidence for a role in Zn nutrition was provided recently when it was shown that an *Arabidopsis hma2 hma4* double mutant showed a phenotype consistent with Zn deficiency and that this phenotype could be suppressed by adding Zn to the growth medium [11]. This phenotype was not observed in the single mutants, suggesting partially redundant functions of AtHMA4 and AtHMA2 [11]. Expression studies using HMA-promoter-GUS constructs indicated that *AtHMA2* and *AtHMA4* were predominantly expressed in the vascular tissues of roots, stems and leaves, and that AtHMA2 was localized to the plasma membrane [11]. It therefore appears that AtHMA2 and AtHMA4 may play a role in the translocation of Zn and may function more specifically in the loading or unloading of Zn in the xylem and in remobilization of Zn from shoot to root in the phloem [11]. In [11], no altered sensitivity to Zn and Cd was observed for the single *hma4* mutant, although the concentration range over which these metals were tested was not stated. However, in a

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Abbreviations: ANOVA, analysis of variance; EDTA, ethylenediaminetetraacetic acid; FW, fresh weight; HMA, heavy metal P-type ATPase; LB, left border; OD, optical density; ORF, open reading frame; SC, synthetic complete; S.E., standard error; TM, transmembrane; wt, wild type

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phytochelatin-deficient mutant background, *hma2* and *hma4* mutations increased the sensitivity to low levels of Cd [11]. A further mutant allele for *AtHMA4* (*athma4-3*) has been isolated here and additional phenotypes in the single *hma4* mutants are reported that are consistent with possible detoxification roles for AtHMA4 when metals are supplied at high concentration. Evidence is presented using heterologous expression in yeast to show that AtHMA4 functions as an efflux pump conferring both Cd and Zn resistance.

2. Materials and methods

2.1. Plant growth

Seeds of wt and transgenic *Arabidopsis thaliana*, ecotype Columbia (Col 0) were sterilized in 10% (v/v) bleach for 20 min, rinsed five times with sterile water and inoculated onto plates containing half-strength Murashige and Skoog medium [12] (Sigma, UK), 1.5% w/v Difco technical agar, 1% w/v sucrose and various metal salts. Seeds were stratified at 4 °C for 48 h prior to transfer to a controlled environment cabinet (23 °C 16 h light, 18 °C 8 h dark) and incubated vertically.

2.2. Isolation of insertional mutants for AtHMA4

Seeds for a T-DNA insertion line for *AtHMA4* (168_C10) were obtained from GABI-kat (http://www.mpiz-koeln.mpg.de/GABI-Kat/db/). Seeds were sown in soil (1 part Levingtons F5 No. 2: 1 part John Innes No. 2: 1 part Vermiperl® graded horticultural vermiculite, medium grade) sterilized by autoclaving at 121 °C for 15 min at 1 bar pressure and plants were grown in a controlled-environment cabinet (22 °C 16 h light, 20 °C 8 h dark cycle). Genomic DNA was isolated from the plants using the Dnamite Plant kit for genomic DNA extraction (Microzone Ltd. Haywards Heath, UK). To genotype the plants with respect to the T-DNA insert, PCR was carried out on genomic DNA using a primer for the T-DNA (GK-LB-PCR$_1$ 5′CCCATTTGGACGTGAATGTAGACA) and a gene-specific primer (HMA4-F$_1$ 5′AAACGAAGCTAGGTTAGAAGCAAACG). The PCR conditions were 94 °C for 4 min and 36 cycles of: 94 °C for 45 s, 60 °C for 45 s, 72 °C for 1 min 20 s followed by 72 °C for 7 min. This detected a band of 0.6 kb (the expected size for an intact left border (LB) junction being 612 bp). In addition, gene-specific primers were used to detect plants that also contained a wt copy of the gene: HMA4-F$_1$ and HMA4R nested 5′GAGATTTGGTTTTACTGCTCTGAGC. The PCR conditions were 94 °C for 4 min and 38 cycles of: 94 °C for 45 s, 57 °C for 45 s, 72 °C for 3 min 10 s followed by 72 °C for 7 min. This detected a band of 2391 bp. Using this method and genotyping by segregation patterns (see below), mutants homozygous for the insert were obtained. The mutant allele was designated *hma4-3*. A similar procedure was carried out to isolate a mutant (N550924) from the SALK collection (http://signal.salk.edu/) homozygous for the insert using a primer for the T-DNA (Salk-LBa1 5′TGGTTCACGTAGTGGGCCATCG) and the gene-specific primer HMA4R nested. The PCR conditions were 95 °C for 4 min and 38 cycles of: 95 °C for 50 s, 57 °C for 50 s, 72 °C for 1 min 10 s followed by 72 °C for 7 min. This produced a band of ≈0.75 kb after agarose gel electrophoresis and subsequent sequencing showed the product to be 745 bp (the expected size for an intact LB junction being 839 bp). In addition, the gene-specific primers HMA4Int2F 5′GCAGCAGTTGTGTTCCTATTCACC and HMA4Seq1R 5′TGAGAGTGTGTCAAGATAATCAGC were used to detect plants that also contained a wt copy of the gene. The PCR conditions were 95 °C for 4 min and 37 cycles of: 95 °C for 50 s, 60 °C for 48 s, 72 °C for 1 min 20 s followed by 72 °C for 7 min. This amplified a band of 985 bp. Sequencing confirmed both the original SALK and GABI-Kat predicted T-DNA locations. The mutant allele was designated *hma4-2* and has been described previously [11].

Segregation patterns were analyzed by germinating progeny seed on plates containing half-strength Murashige and Skoog salts (Sigma, UK), 0.8% agar, 2% w/v sucrose plus and minus the antibiotic (11 μg ml^{-1} sulfadiazine for the GABI-KAT line and 50 μg ml^{-1} kanamycin for the SALK line). For both lines, selfed heterozygotes segregated according to a 3:1 ratio, indicative of a single insert.

2.3. Preparation of AtHMA4 constructs and heterologous expression in yeast

The *S. cerevisiae* reference strain BY4741 (*MATa;his3Δ1;leu2Δ0;met15Δ0;ura3Δ0*) and the Cd-sensitive *ycf1* mutant (*MATα;his3Δ1;leu2Δ0;lys2Δ0;ura3Δ0;YDR135c::kanMX4*) lacking a vacuolar ABC transporter of glutathione-S conjugates [13] were obtained from Euroscarf (Frankfurt, Germany). The *zrc1cot1* mutant which is hypersensitive to Zn (*MATa;his3Δ1;leu2Δ0;met15Δ0;ura3Δ0;zrc1::natMX cot1::kanMX4)* was obtained from Dr. U. Kramer (Golm, Germany). Synthetic complete (SC) medium lacking specific nutrients was used for the selection and maintenance of yeast transformed with plasmids [14]. Cloning of the full-length cDNA for *AtHMA4* (referred to as *AtHMA4*$_{(\text{Full Length})}$) into the yeast shuttle vector p426, yeast transformation and isolation of transformants was described previously [7]. The truncated version of *AtHMA4* lacking the C-terminal region (referred to as *AtHMA4*$_{(\text{truncated})}$) was prepared by amplifying the N-terminal region of the cDNA using the forward primer HMA4-F-core (5′CTCTTCTCCGAAAATGGCGTTAC) and the reverse primer HMA4-R-core (5′AGCCCTGTATCACTTTTTGTTCC). The reverse primer introduces a stop codon. This sequence was initially cloned into the pGEM-T easy vector and then excised using *Eco*RI sites and inserted into the *Eco*RI site of p426. The correct orientation of the insert was confirmed. This gives a 2142 bp truncated *AtHMA4* sequence (from the start ATG codon to the end of the introduced stop codon) coding for a 713 amino acid protein, which terminates just after the final predicted transmembrane domain, effectively removing the C-terminal 459 amino acids. Cloning of *AtHMA4*$_{(\text{truncated})}$ in p426 and transformation of yeast was carried out as described previously for the full-length construct [7]. A mutated version of the full-length *AtHMA4* sequence (AtHMA4-C357G), in which the initial cysteine of the CPx motif (C357), was changed to glycine was also transformed into yeast.

To determine the effect of a range of metal concentrations on the growth of transformed yeast, the cells were first grown in liquid culture overnight at 30 °C in SC without uracil (pH 5.0) and containing 20 g l^{-1} raffinose as the carbon source. The cultures were diluted to an OD_{600} of 0.8 with the same medium but with galactose (20 g l^{-1}) as the carbon source and grown for a further 4 h. The cultures were adjusted to the same OD_{600} and aliquots were inoculated onto 2% (w/v) agar plates containing the SC-uracil medium (pH 4.9) with galactose and various concentrations of metal supplied as the sulfate salt. Plates were incubated at 30 °C for 3–5 days.

2.4. Uptake of labeled Cd and Zn in yeast

Yeast were grown as described above except that they were grown in SC-uracil, 20 g l^{-1} galactose medium (pH 5.5) for 12 h before harvesting. Cultures were centrifuged at 2000 × *g* for 5 min to pellet the cells, washed three times in ice-cold glucose/galactose (18/2 g l^{-1}) solution and resuspended to 0.1 g ml^{-1} in the same solution. To measure uptake, cells were incubated in a glucose/galactose buffer [104 mM glucose, 11.5 mM galactose, 10 mM Mes-NaOH (pH 5.5)] with 10 μM ^{109}Cd-labeled Cd (10 MBq μmol^{-1}) or 20 μM ^{65}Zn-labeled Zn (2.275MBq μmol^{-1}) at 10 mg ml^{-1}. Assays were conducted at 30 °C. Aliquots were removed at appropriate times and transferred to a glass fiber filter and washed with 3 × 5 ml of ice-cold wash buffer (1 mM EDTA, 1 mM NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM KH_2PO_4, and 20 mM sodium citrate, pH 4.2). Filters were placed in scintillation vials with 5 ml OptiPhase Hi-Safe scintillation fluid and counted in a scintillation counter (Beckman LS 6500). Assays were conducted in duplicate and the whole experiment was replicated at least three times.

2.5. Sequencing

The '*BigDye*' method (PE Biosystems, Cheshire, UK), based on the chain termination method of [15], was used for sequencing using the Perkin Elmer ABI 337 automated sequencer.

2.6. Sequence analysis

The first 800 amino acids of AtHMA4 (064474) from *Arabidopsis thaliana* were aligned to the CadA sequences from *S. aureus* (P20021) and *Helicobacter pylori* (Q59465) [16–18] using Clustal W 1.82 [19].

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