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A novel CPx-ATPase from the cadmium hyperaccumulator *Thlaspi caerulescens* ☆

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Abstract ***Thlaspi caerulescens*** **exhibits a unique capacity for cadmium tolerance and accumulation. We investigated the molecular basis of this exceptional Cd^{2+} tolerance by screening for *T. caerulescens* genes, which alleviate Cd^{2+} toxicity upon expression in *Saccharomyces cerevisiae*. This allowed for the isolation of a cDNA encoding a peptide with homology to the C-terminal part of a heavy metal ATPase. The corresponding *TcHMA4* full-length sequence was isolated from *T. caerulescens* and compared to its homolog from *Arabidopsis thaliana* (*AtHMA4*). Expression of *TcHMA4* and *AtHMA4* cDNAs conferred Cd sensitivity in yeast, while expression of *TcHMA4-C* and *AtHMA4-C* cDNAs encoding the C-termini of, respectively, *TcHMA4* and *AtHMA4* conferred Cd tolerance. Moreover, heterologous expression in yeast suggested a higher Cd binding capacity of TcHMA4-C compared to AtHMA4-C. In planta, both *HMA4* genes were expressed at a higher level in roots than in shoots. However, *TcHMA4* shows a much higher constitutive expression than *AtHMA4*. Our data indicate that HMA4 could be involved in Cd^{2+} transport and possibly in the Cd hyperaccumulation character.**

Keywords: HMA; Heavy metal; Zinc

1. Introduction

P-type ATPases form a large family of transporter proteins, which hydrolyze ATP for transporting substances across cell membranes. These membrane proteins, which have been identified in almost all organisms (except some Bacteria and Archea), have been classified into five groups according to their substrate specificity [1]. One group, P_{1B} ATPases [1], also known as HMAs [1], CPx-ATPases [2] or soft metal P-type ATPases [3] transports heavy metals. Their physiological functions include metal ions homeostasis, resistance to toxic metal concentrations and the delivery of essential metals to target enzymes. This group of pumps can be further divided into two subgroups exhibiting Cu^{+}/Ag^{+} or $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ substrate specificity. Pumps that belong to the first group are found in a variety of organisms, from *Escherichia coli* to humans [4–7], while those in the second group are widespread only in prokaryotes. Cd^{2+} CPx-ATPases are for instance common in bacteria, (e.g., CadA from *Staphylococcus aureus* [8] or ZntA from *E. coli* [9]) where they play an essential role in resistance by extruding Cd^{2+} from the cell.

Analysis of the *Arabidopsis* complete genome sequence allowed for the first identification of putative eukaryotic CPx-ATPases proposed to bear a $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ specificity, which so far were not detected in any other sequenced eukaryotic genome [1]. Recently, the characterization of the first eukaryotic HMA gene, *AtHMA4* from *Arabidopsis thaliana*, has been published and supports a role in Zn and Cd transport [10].

Thlaspi caerulescens is a plant well known for its capacity to hyper-accumulate zinc and cadmium in its shoot (hyper-accumulation being defined as 100 mg Cd kg^{-1} in the shoot dry weight [11]). Some *T. caerulescens* populations from Ganges (southern France) present the remarkable property to accumulate up to 10 000 mg Cd kg^{-1} in shoots in hydroponic culture without toxicity symptoms [12,13]. The mechanisms involved in hyper-accumulation are poorly understood. In higher plants, non-essential heavy metals such as cadmium are likely transported across membranes via nutrient transporters or channels that are not completely selective [14]. Whether the same transporters are involved in cadmium uptake and translocation in the hyper-accumulator *T. caerulescens* remains an open question. Depending on the population studied, cadmium was proposed to get inside the cell via either a high affinity uptake system for iron or a low affinity system for calcium or zinc uptake [13,15–18].

Thlaspi caerulescens provides a unique source of genes involved in cadmium transport and detoxification. Using a functional strategy based on the transformation of *Saccharomyces cerevisiae*, we attempted to identify *T. caerulescens* cDNAs that confer cadmium tolerance. In the present study, we describe the isolation and characterization of one cDNA (*TcHMA4*), which encodes a protein highly similar to *Arabidopsis* HMA4.

2. Materials and methods

2.1. Yeast cultures, transformation and growth assays

The *S. cerevisiae* wild-type strains, *BY4741* (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and *CM100* (*MATa can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52*), and the *zrc1* mutant (parental wild-type strain

☆ The *TcHMA4* nucleotide sequence reported is available in the EMBL database under the accession No. AJ567384.

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BY4741) were used in this study. They were grown at 30 °C in SD medium (0.17% yeast nitrogen base (Sigma) without amino acids and ammonium sulfate, containing 2% glucose, 0.5% ammonium sulfate) supplemented with the appropriate amino acids. For the growth tests, cells were incubated, at a density adjusted to OD_{600} of 0.2 in the presence of 20 and 50 μM of $CdSO_4$ for 24 h. Additional tests were performed (in *BY4741*) in the presence of 11 mM $ZnSO_4$ (7 mM in *zrc1* mutant), 700 μM $CoSO_4$, 250 μM $PbCO_3$ and 500 μM $NiSO_4$.

2.2. Library screening

To identify plant cDNAs able to increase cadmium tolerance, the *BY4741* yeast strain was transformed with a leaf *T. caerulescens* (Ganges population) cDNA library constructed in the pYX212 (Ingenius, Madison, WI) yeast expression vector under the control of the strong constitutive TPI promoter [19]. Transformations were performed by the standard lithium acetate method [20]. Cd^{2+} tolerant Ura^+ transformants were selected after plating the transformants on SD medium, supplemented with the appropriate amino acids, containing 15 μM $CdSO_4$. Recombinant pYX212 plasmids that conferred cadmium tolerance were isolated and retransformed in *BY4741* to confirm the functional tolerance. On 430 000 transformants, 139 recombinant pYX212 plasmids were confirmed. The most frequently identified insert was encoding PCS1 homologous sequence. HMA4-C homologous sequences were identified four times.

2.3. Cloning of cDNAs

The full-length *TcHMA4* cDNA was amplified by RT-PCR. Total RNA was extracted from *T. caerulescens* shoots as described in [21]. The first strand cDNA was synthesized by random hexamer primer from 2 μg of total RNA with the Superscript II reverse transcriptase (Invitrogen Life Technologies). The full-length coding sequence was isolated in two stages. Degenerate oligonucleotide primer designed from 5′ conserved regions of *HMA* cDNAs from *A. thaliana* (primer 1 = 5′-GTISTIGGAATITGYTGTWCATCGGA-3′) and *TcHMA4* specific primers designed from partial cDNA isolated from screening (primer 2: 5′-GCTCAGTGAAGCCATATCTCCCTCTGG-3′ and primer 3: 5′-CTTTTCACCACAACAACCAGAGTGAAG-3′) were used for nested PCR amplification from first strand cDNA. Products from RT-PCRs were cloned and the longest recovered cDNA was sequenced and used to design primers for 5′RACE amplification (Invitrogen Life Technologies). Based on the latter results, a full-length ORF was amplified using primers in the 5′ and 3′ ends of *TcHMA4*, which included the *Mfe*I, *Eco*RI compatible, and *Xho*I sites. (forward primer: CCGCAATTGCGTCTTTTCTCCGAAATGGCG and reverse primer: CCGCTCGAGTCAAGCAGCCCCAACATGGTG). The resulting 3.5-kb PCR fragment was cloned in pUC18 for sequencing and further cloned in pYX212 (Ingenius) in the *Eco*RI–*Xho*I restriction sites for expression in yeast. The homologous *AtHMA4* and *AtHMA4-C* cDNAs were amplified by RT-PCR. The truncated and full-length cDNAs were amplified using *AtHMA4* specific primers (*AtHMA4* forward: GGAATTCATGGCGTTACAAAAC, *AtHMA4-C* forward: GGAATTCCAAGAGAATGTTGTGATGATG and reverse: CCGCTCGAGTCAAGCAGCCCCAACATGGTG). The two resulting PCR products (full-length *AtHMA4* and fragment *AtHMA4-C*) were cloned in pUC18 for sequencing and further cloned in the *Eco*RI–*Xho*I restriction sites of pYX212 for expression in yeast. All PCRs were performed with the Pfx polymerase (Invitrogen) at 94 °C for 2 min, followed by 30 cycles at 94 °C denaturation for 1 min, at 58 °C annealing for 1 min, at 68 °C extension for 3 min or 1 min 30 s, and finished by an extension at 68 °C for 7 min.

2.4. Sequence analysis

Alignment of HMA4 sequences was performed using ClustalW [22], which was manually adjusted using the PSBLAST pairwise alignments. Putative transmembrane domains were identified by using TMHMM [23] and hydropathy analysis [24].

2.5. Plant material, growth conditions and treatments

Thlaspi caerulescens populations, described in [13], Prayon (Belgium), St Félix-de-Pallières (Ganges region, Southern France) and Puente Basadre (Spain) and *A. thaliana* (ecotype Columbia) were grown in hydroponic conditions in 1/10 Hoagland medium with a temperature cycle of 20 °C/17 °C and a light (100 μE) cycle of 16 h light/8 h dark. The hydroponic solutions used were continuously aerated and changed every week. After 6 weeks in nutrient solution, 10 and 100 μM $CdSO_4$ were added. Roots and shoots were collected after 24 h of treatment.

2.6. TcHMA4 and AtHMA4 expression

For Northern blot analysis, total RNA was isolated from shoots and roots of *T. caerulescens* and *A. thaliana* treated with cadmium as described in [21]. RNA samples (15 μg) were separated by electrophoresis on a 1% formaldehyde–agarose gel, transferred to a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, Little Chaflont, UK) and hybridized with ^{32}P-labeled probes made from C-terminal TcHMA4 encoding cDNA (residues 758–1186 in TcHMA4) and AtHMA4 encoding cDNA (residues 766–1172 in AtHMA4) using the Rediprime II Random Prime labelling system (Amersham Pharmacia Biotech). Equal loading of RNA was verified by probing the RNA blots with a constitutively expressed 18S rRNA fragment from *Arabidopsis*. To reveal any difference in the intensity of 18S rRNA hybridization between the two species, *Thlaspi* and *Arabidopsis* RNA blots were together hybridized and quantified with a phosphoimager system (Storm 860, Molecular Dynamics).

2.7. Determination of intracellular Cd^{2+} content

Yeast strains were grown overnight at 30 °C in liquid SD medium. Cells were incubated, at a density adjusted to OD_{600} 0.2 in the presence of 20 μM of $CdSO_4$ for 24 h. After incubation, cells collected by centrifugation were washed twice with distilled water, once with EDTA 50 mM and were dried at 95 °C for 48 h to determine the dry weight. Cells resuspended in distilled water were mixed with an equal volume of concentrated HNO_3, incubated at 95 °C for 2 h and diluted with 2 volumes of distilled water. The Cd content in the cells was determined with an atomic absorption spectrophotometer (Perkin–Elmer AAS 3110). All the above centrifugations were carried out at $5500 \times g$ for 5 min at 4 °C.

2.8. Statistical analysis

Statistical analysis was performed by one-way ANOVA using SYSTAT version 5.0 (Systat Software Inc., Richmond, CA, USA).

3. Results

3.1. Isolation of T. caerulescens HMA4 cDNA

A *T. caerulescens* cDNA library was screened in *S. cerevisiae BY4741* for higher Cd^{2+} tolerance, on agar medium containing Cd^{2+} at a concentration that is toxic for the wild-type strain. This allowed for the recovery of cDNAs encoding proteins known to play a role in heavy metal chelation as metallothioneins and phytochelatin synthase (*TcPCS*). cDNAs, without clear relationship to cadmium ion stress, as well as abiotic stress responsive cDNAs were also identified. Our attention was particularly focused on one cDNA for which in silico analysis of nucleotide sequence and conceptual translation product were similar to the C-terminus of AtHMA4. The corresponding full-length cDNA was isolated from *T. caerulescens* by RT-PCR, which was subsequently sequenced. The deduced 1186 amino-acid sequence aligned well with several *A. thaliana* HMAs polypeptides (Fig. 1). Since the best alignment was found with AtHMA4, the *T. caerulescens* cDNA was named *TcHMA4*. The truncated cDNA encoding the C-terminal fragment, which was identified during the screening, corresponded to TcHMA4 aa 752–1186, with a first methionine at residue 758, and was named *TcHMA4-C*. The TcHMA4 deduced amino acid sequence displayed 69% identity and 76% similarity with the AtHMA4 sequence, while TcHMA4-C was more divergent and shared only 43% identity to the corresponding AtHMA4-C sequence.

Besides features typical of CPx-ATPases (Fig. 1), the conceptual TcHMA4 amino acid sequence also displayed significant differences from those, which it shared with AtHMA4.

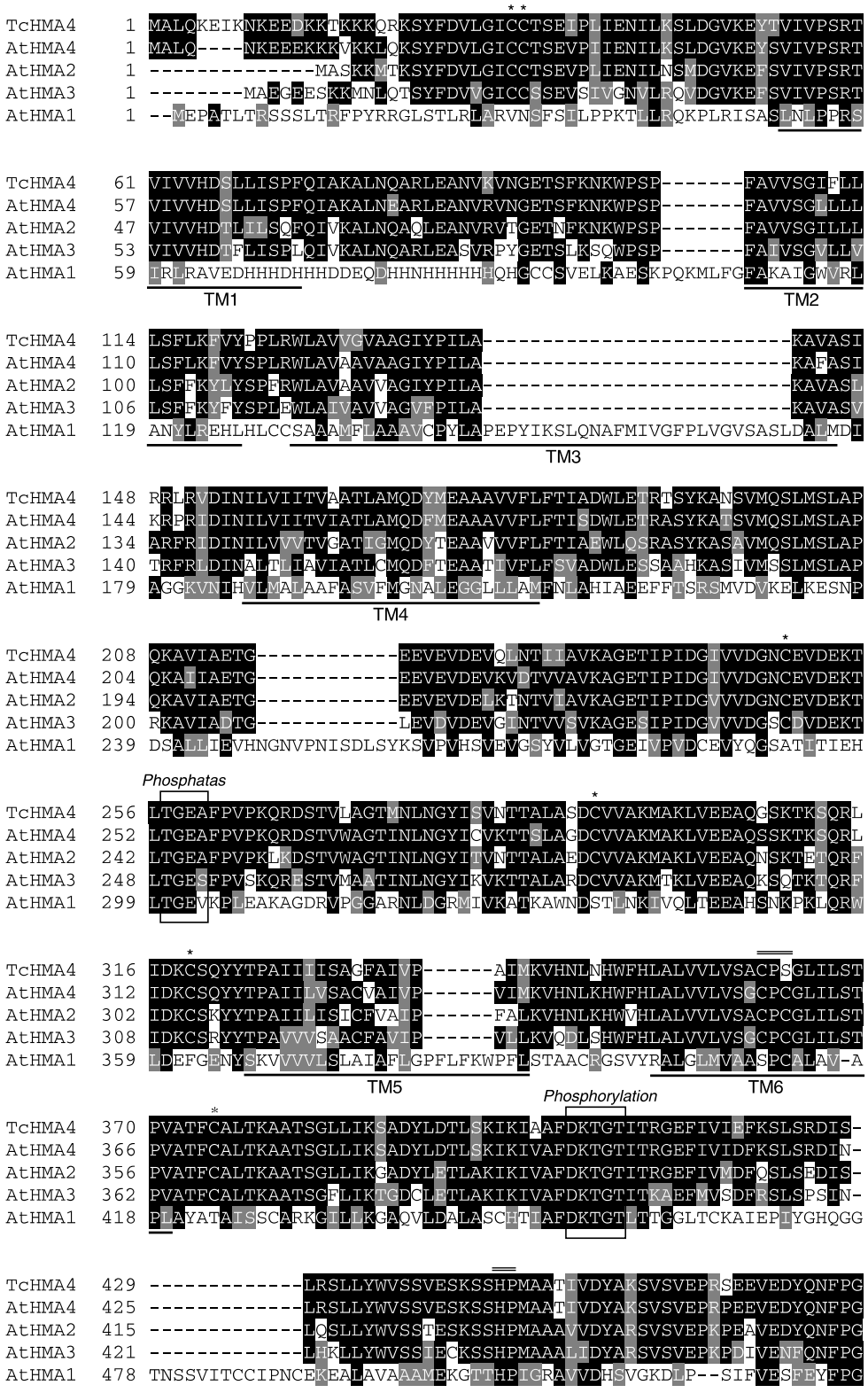

Fig. 1. Alignment of TcHMA4 with AtHMA1-4. ClustalW (1.8) alignment of TcHMA4 deduced sequence with HMA from *A. thaliana*. EMBL accession No. TcHMA4, AJ567384 and Swiss-Prot accession Nos. AtHMA1, Q9M3H5; AtHMA2, Q9SZW4; AtHMA3, Q9SZW5; AtHMA4, O64474. Identical residues are in black and similar residues are shaded. The eight putative transmembrane domains (TM1–TM8) for TcHMA4 are underlined. Conserved sequences found in all P-type ATPases are boxed: TGES, part of the phosphatase domain; DKTGT, site of aspartyl phosphate formation; GDGxNDx, ATP-binding motif. Motifs unique to P_{1B}-ATPases are indicated by double lines above the alignment: CPx, ion translocation; HP, unknown function. Possible metal binding motifs and cysteine residues are indicated by *asterisks* above the alignment.

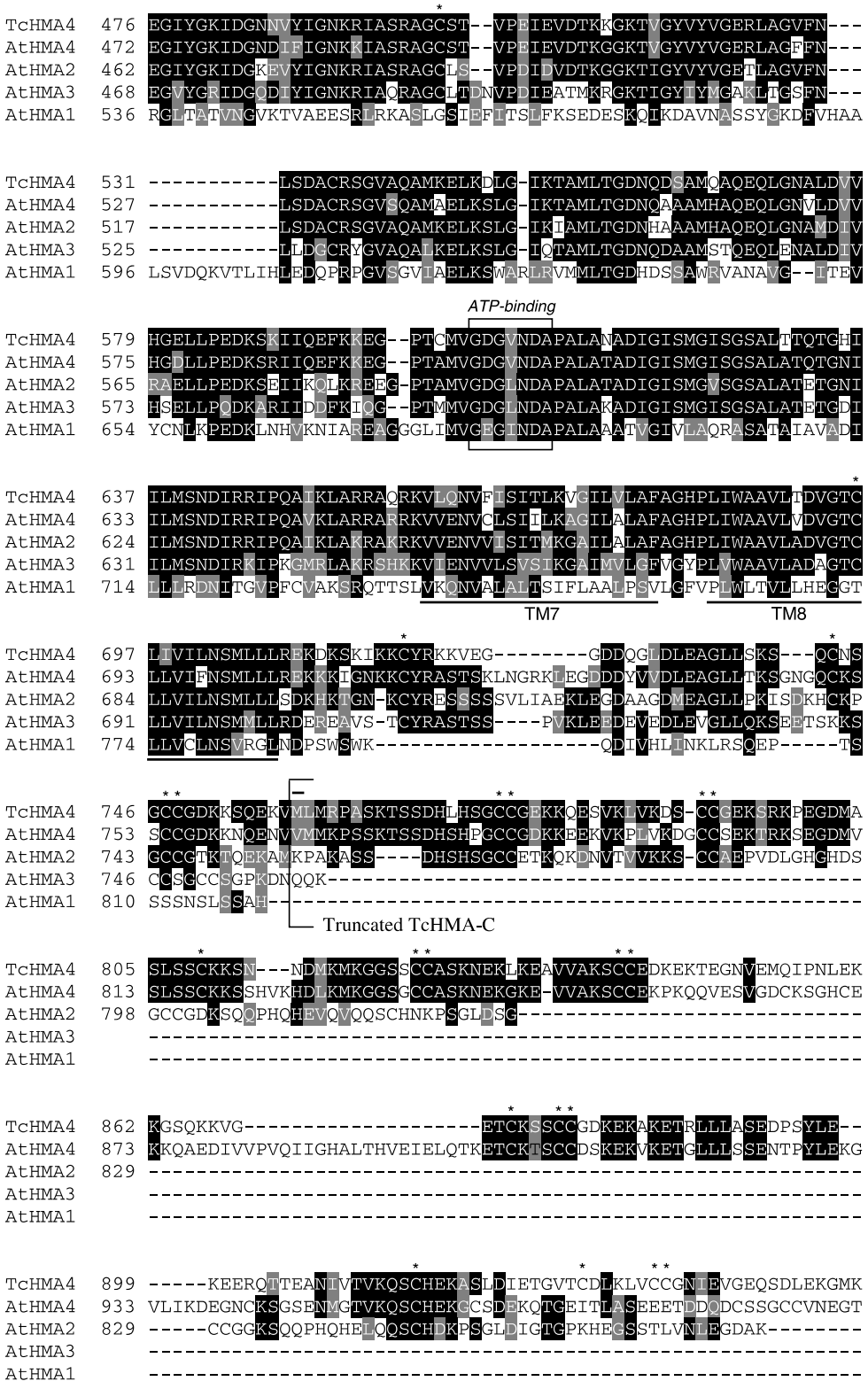

Fig. 1 (*continued*)

The N-terminal sequence of TcHMA4 and AtHMA4 lacked the metal associated domain (GMTCxxC) but contained a single CC dipeptide ($C_{31}C_{32}$ in TcHMA4 and $C_{27}C_{28}$ in AtHMA4). The presence of a long C-terminal extension after the eighth transmembrane domain was another particular feature that TcHMA4 shared with AtHMA4 (478 amino acids for TcHMA4 and 470 for AtHMA4) and to a lesser extent with AtHMA2 (267 amino acids). All these three peptides also contained three additional cysteine motifs – $C(x)_4C$, $C(x)_{3-5}CC$, CC – and a His rich domain within their extended

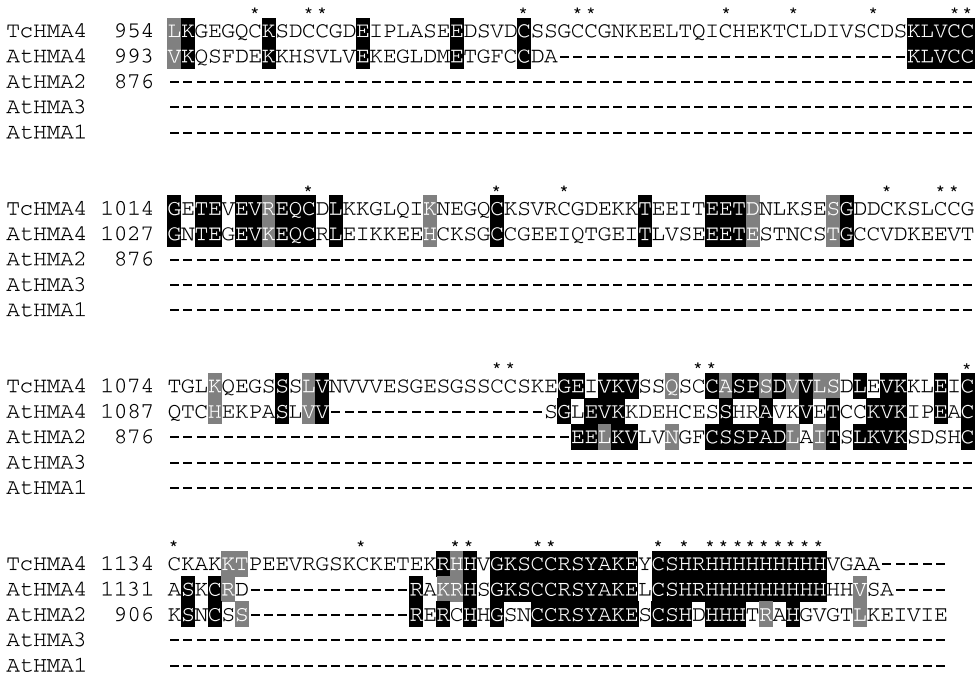

Fig. 1 (*continued*)

C-terminus which could be involved in heavy metal binding. Further examination of the two putative HMA4 COOH tails revealed that the $C(x)_3CC$ motifs were in a larger cysteine motif, $C(x)_{14-28}C(x)_3CC$ for *Thlaspi* and $C(x)_{20-35}C(x)_3CC$ for *Arabidopsis*. This sequence is similar to the recently described TRASH domain, $C(x)_{19-22}C(x)_3C$, predicted to be involved in heavy metal sensing, trafficking and resistance [25]. A His rich domain is also present in AtHMA1, where it is associated with a single CC dipeptide, but in this case in the N-terminal domain. The TcHMA4-C fragment lacked the putative catalytic domains while keeping the putative heavy metal binding domains (see Fig. 1).

3.2. Metal tolerance and Cd content in yeast expressing truncated and full-length HMA4 cDNAs

BY4741 and *CM100* yeast strains were constructed, which overexpressed *TcHMA4-C* and *TcHMA4* cDNAs under the control of the *TPI* (triose phosphate isomerase) promoter. Control cells contained the pYX212 cloning vector.

Growth was monitored in liquid medium containing various Cd^{2+} concentrations (Fig 2). Similar results were observed in both strains. Expression of *TcHMA4-C* allowed *S. cerevisiae* cells to grow better than controls in the presence of Cd^{2+} (Fig. 2(a) and (b)). Differences were visible at lower Cd concentrations in *CM100*, which is more sensitive than the *BY4741* strain. In contrast, cells expressing *TcHMA4* were far more sensitive to $CdSO_4$ than the control cells (Fig. 2(a) and (b)).

To compare TcHMA4 with its *Arabidopsis* homolog, the full-length *AtHMA4* cDNA and its truncated version, *AtHMA4-C*, coding from the 766–1172 C-terminal residues, were cloned in pYX212 and expressed in BY4741 and CM100. In both yeast strains, the expression of *TcHMA4-C* and *AtHMA4-C* conferred cadmium tolerance, yet the tolerance conferred by *AtHMA4-C* was much lower, Fig. 2(c) and (d). On the contrary, there was no significant difference in the Cd sensitivity conferred by the entire HMA4 protein from *Thlaspi* or *Arabidopsis*. These results were confirmed on solid medium (Fig. 3).

Cadmium accumulation in *S. cerevisiae* cells that expressed *TcHMA4-C* and *TcHMA4* was examined after exposition to a mild cadmium stress. To avoid massive passive entry arising from damaged or dead cells, treatment was with 20 μM $CdSO_4$. After 24 h in the presence of 20 μM Cd^{2+}, *BY4741* control cells accumulated cadmium to a higher extent than the *CM100* ones, with a difference of 27-fold. *BY4741* cells expressing *TcHMA4*, which were more Cd sensitive than control ones, accumulated, over 2-fold ($P < 0.01$) less cadmium than the control cells (see Fig. 4(a)). In contrast, *CM100* cells expressing *TcHMA4*, which were also more sensitive to cadmium than control ones, accumulated significantly ($P < 0.01$), over 3-fold more cadmium than the control cells (see Fig. 4(b)). Both *TcHM4-C*-overexpressing yeast strains, which were more resistant to cadmium, did accumulate similar cadmium amounts than control yeast cells (Fig. 4(a) and (b)).

Yeast strains expressing *AtHMA4* or *TcHMA4* were also assayed for zinc tolerance and accumulation. Expression of partial or full-length *TcHMA4* cDNAs in wild-type yeast did not result in a cross-tolerance or a cross-sensitivity to Zn^{2+} (data not shown). Because growth tests in wild-type strain require high Zn^{2+} concentrations, Zn^{2+}-related phenotypes were also tested in the zinc sensitive, *zrc1*, yeast mutant. This strain lacks a vacuolar Cd^{2+}/Zn^{2+} transporter and was indeed more sensitive to Zn^{2+} than the corresponding wild-type strain [26]. Neither *TcHMA4* nor *AtHMA4* did complement *zrc1*. However, *zrc1* mutants that expressed *TcHMA4* or *AtHMA4* were slightly more sensitive to zinc than the control strain (Fig. 5). Growth of *zrc1* cells expressing *HMA4-C* of either *Thlaspi* or *Arabidopsis* did not differ from that of the controls. Zinc accumulation was monitored upon 1 mM $ZnSO_4$ treatment. No significant difference was observed whether cells

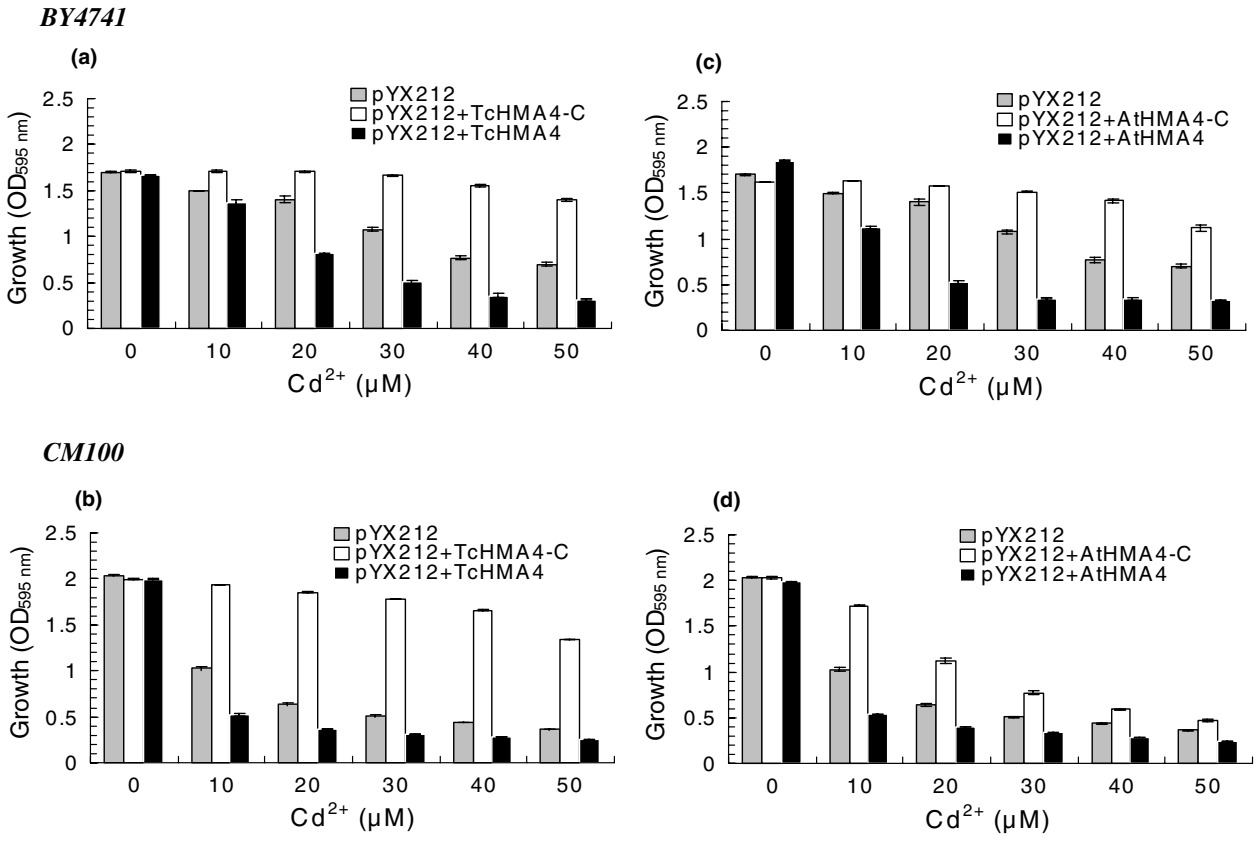

Fig. 2. Effect of *HMA4-C* and *HMA4* expression on the cadmium tolerance of two yeast strains. Yeast *BY4741* and *CM100* cells transformed with the pYX212 plasmid (gray columns) and with pYX212 containing the *T. caerulescens* (a, b) and *A. thaliana* (c, d) 5′ truncated cDNA, *HMA4-C* (white columns), and full-length cDNA, *HMA4* (black columns), were grown in liquid SD medium without or with 20 and 50 μM $CdSO_4$. Cells were incubated at 30 °C for 24 h. Results are averages (±S.E.) from three replicates. This experiment was repeated three times independently.

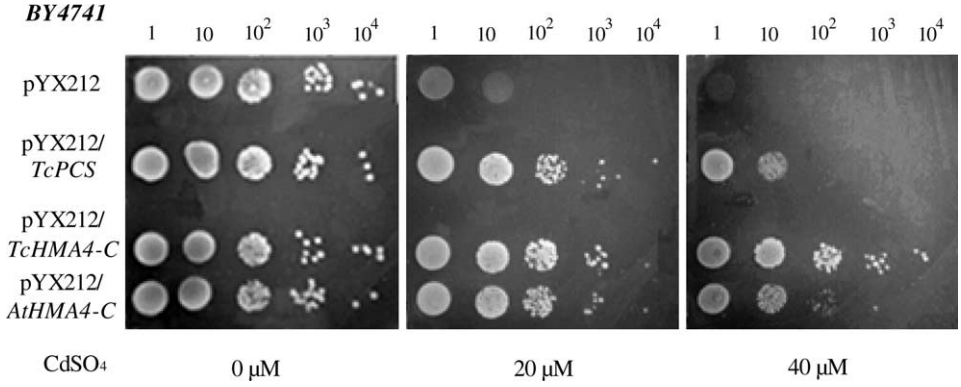

Fig. 3. Growth of *BY4741* expressing *Thlaspi* or *Arabidopsis HMA4-C*. *BY4741* transformants containing pYX212 (negative control), pYX212/*TcPCS* (expressing *Thlaspi caerulescens* phytochelatin synthase cDNA, accession No. AY540104, positive control), pYX212/TcHMA4-C or pYX212/*AtHMA4* expressing, respectively, *Thlapsi* or *Arabidopsis* 5′ truncated *HMA4* cDNA were grown in liquid SD medium overnight. Cultures were adjusted to A_{600} of 1 and serially 10-fold diluted in water. 5 μl aliquots of each dilution were spotted either on non-selective cadmium plates or on plates with 40 μM $CdSO_4$. After 3 days of incubation at 30 °C, plates were photographed. Dilutions are indicated above the figures. Two individual clones of each yeast transformants were analyzed (data not shown).

did or did not express either *HMA4-C* or *HMA4* (data not shown).

Furthermore, under our experimental conditions, partial or full-length *TcHMA4* expression in yeast had no effect on the toxicity caused by other metals such as Co^{2+}, Pb^{2+} and Ni^{2+} (data not shown).

3.3. Expression of HMA4 in plants

The expression of *TcHMA4* was studied in planta by Northern blot analysis under stringent conditions (Fig. 6(a)). The probe was designed to hybridize in the 3′ part of the RNA, predicted to be the most divergent among the different *HMAs*. The steady-state level of *TcHMA4* transcripts was about three

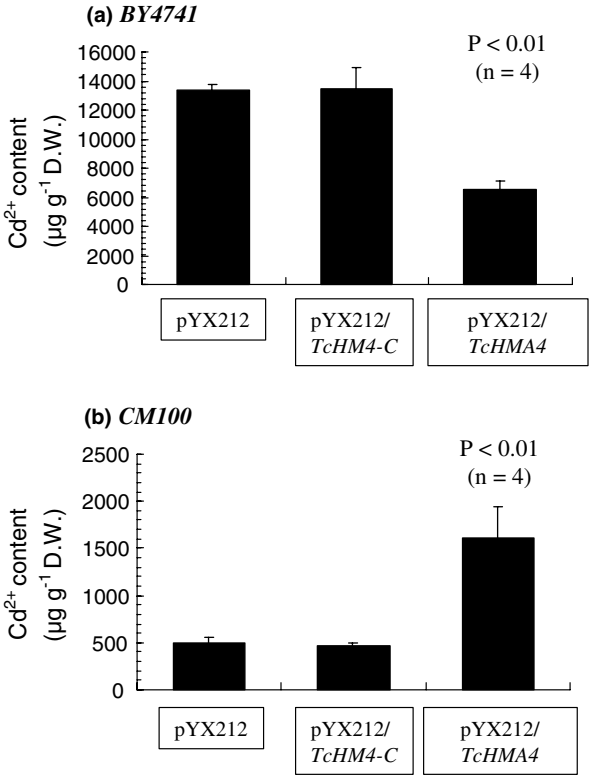

Fig. 4. Cadmium contents of *BY4741* and *CM100* expressing *TcHMA4-C* or *TcHMA4*. Yeast *BY4741* (a) and *CM100* (b) cells transformed with the pYX212 plasmid and with pYX212 containing the *T. caerulescens* cDNAs were grown in liquid SD medium (without uracyl) with 20 µM $CdSO_4$. Cells were incubated at 30 °C for 24 h. Metal contents of samples were analyzed with an AAS. Results are averages (±S.E.) from four independent experiments done with four different colonies.

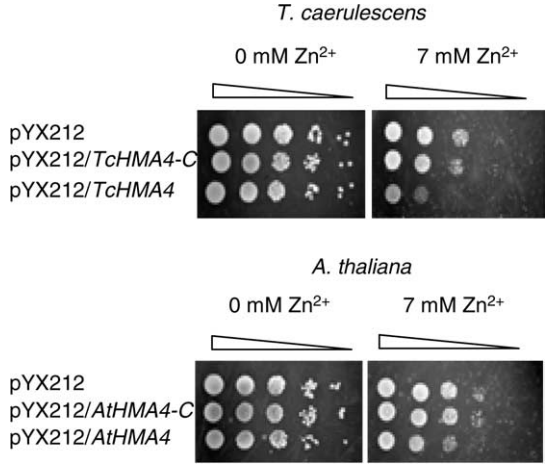

Fig. 5. Growth of the *zrc1* mutant expressing *HMA4-C* and *HMA4* cDNAs. *S. cerevisiae zrc1* mutant transformed with pYX212 or pYX212/*TcHMA4-C*, pYX212/*TcHMA4*, pYX212/*AtHMA4*, and pYX212/*AtHMA4-C* recombinant vectors was grown on minimal medium overnight, adjusted to A_{600} of 1 and serially 10-fold diluted in water. 5 µl aliquots of each dilution were spotted onto SD medium containing 0 and 7 mM $ZnSO_4$, and incubated for 3 days at 30 °C. Triangles indicate gradation from higher to lower cell densities.

times more abundant ($P < 0.05$) in the roots than in the shoots. It was weakly induced (less than 2-fold, NS $P > 0.05$) by treating the plants with 10 or 100 µM $CdSO_4$ for 24 h. The expression of *AtHMA4* was studied in parallel, using a probe complementary to the 3′end sequence part of *AtHMA4* (Fig. 6(a)). Here, no expression could be detected in shoots and low expression was observed in roots. Using 18S rRNA to standardize the signals, *TcHMA4* mRNA was about 20 times more abundant in the roots of *T. caerulescens* than *AtHMA4* mRNA was in the roots of *A. thaliana*. No significant change in *AtHMA4* transcript level was observed after a 24-h treatment with cadmium.

Because the capacity for cadmium accumulation and tolerance was shown to differ between *T. caerulescens* populations [13], we studied the expression of *TcHMA4* in the roots of the

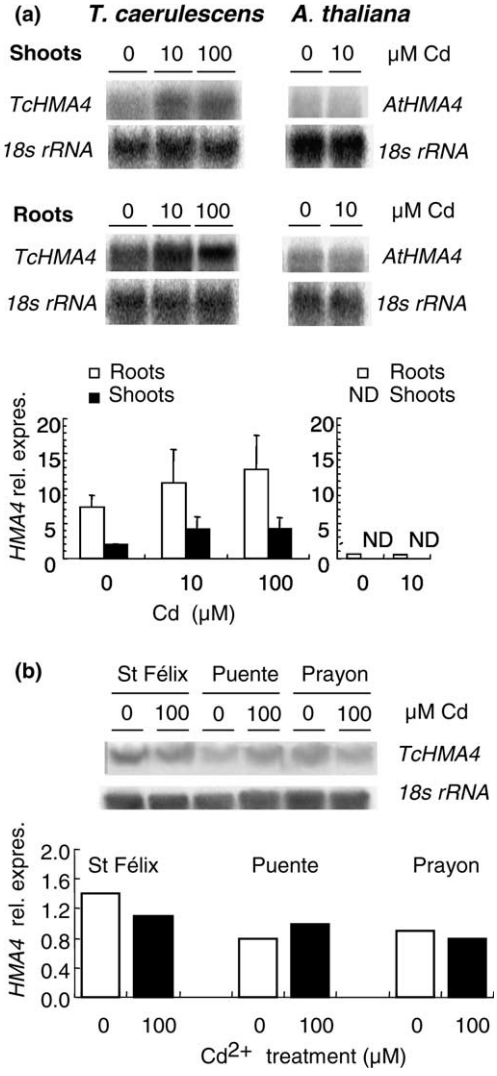

Fig. 6. Northern blot analysis of *HMA4* in *T. caerulescens* and *A. thaliana*. (a) Total RNA was isolated from shoots and roots of the hyperaccumulator *T. caerulescens* and the non-accumulator *A. thaliana*. Plants were exposed to 10 and 100 µM $CdSO_4$ for 24 h. Northern blots equally loaded with 15 µg of total RNA were probed with, respectively, 3′ terminal part of *TcHMA4* and *AtHMA4* (±1.2 kb) and after stripping with 18S rRNA as a loading control. Expression levels were normalized to 18S rRNA. Results are averages (±S.E.) from three independent experiments. (b) Total RNA was isolated from roots of three contrasting populations of *T. caerulescens* different in their cadmium tolerance and accumulation: Prayon (Belgium), St Félix-de-Pallières (France) and Puente Basadre (Spain). Plants were exposed to 100 µM $CdSO_4$ for 24 h. Method is described above.

following three contrasting populations: Puente Basadre (Spain, Ni-enriched serpentine soil), the most sensitive among the three, but with a high capacity to accumulate Cd^{2+}; St Félix-de-Palliéres (France, Ganges, Zn/Cd enriched soil), the most tolerant population identified so far and with a high level of Cd^{2+} accumulation and Prayon (Belgium, Zn/Cd enriched soil) with an intermediate tolerance and the lowest level of Cd^{2+} accumulation. In the three populations, the constitutively high expression of *TcHMA4* was confirmed. No significant difference in the abundance of *TcHMA4* expression could be detected between these three populations whether by Northern blot (Fig. 6(b)) or by RT-PCR (data not shown).

4. Discussion

Using a functional screening in yeast grown on cadmium, we identified a putative heavy metal transporting P-type ATPase from *T. caerulescens*. Actually, the DNA fragment isolated in the screening encoded a peptide corresponding to the C-terminal domain of the putative ATPase and was devoid of transporter function. Nevertheless, the translated product retained potential cadmium binding motifs and could protect the cell from toxic effects of free cadmium ions. The corresponding full-length cDNA was isolated and examination of the deduced polypeptide revealed high similarity with AtHMA4, therefore the identified *Thlaspi* cDNA was named *TcHMA4*. Such pumps, which display $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$substrate specificity, are common in bacteria but have so far never been reported in fungi and animals [1]. Their presence in plants could be related to the ability of these organisms to adapt to various environmental conditions [10,27].

Similarity of the TcHMA4 deduced amino-acid sequence with AtHMA4 includes the characteristic features of CPx-ATPases and specific features, in the N-terminal domain as well as a very long C-terminal tail downstream of the eighth transmembrane domain, bearing numerous predicted heavy metal binding sites. This domain presents in both plant HMA4 a His-rich track and numerous CC dipeptides, which is quite unusual in CPx-ATPases. Although similar motifs were found in TcHMA4-C and AtHMA4-C, this domain is also the most divergent part between the two sequences. CC dipeptides were only found, together with a His-rich domain, in the N-terminal region of bacterial CPx-ATPases and were associated with Cd^{2+} tolerance [28,29]. The function of these putative heavy metal binding motifs remains to be elucidated. They may be directly involved in ion transport or have a regulatory role. Cysteine-rich polypeptides are known to bind heavy metals, in particular cadmium, and are also found in heavy metal chelators like metallothioneins and phytochelatins [30].

Regulatory domain has not been demonstrated for CPx-ATPases proposed to bear a $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ specificity. However, the identification of N- and C-terminal regulatory domains in non-heavy metal plant P-type ATPases [31,32] is an argument in favor of the existence of a similar regulatory or sensing function. Moreover, *Listeria monocytogenes* CadA truncated from its metal binding domain displayed a lower catalytic rate and a higher cadmium affinity than the corresponding full-length CadA, suggesting an interaction between metal binding domain and membranous transport sites [33]. Last, the presence of a TRASH domain in the C-terminal region of Archeal transcriptional regulators suggests that this motif could function as a metal-sensing regulatory module [25]. Interestingly, the extended C-terminus of plant HMA4 displays a domain similar to the Archeal TRASH one.

The substrate specificity of the two HMA4 was addressed by growing yeast cells that expressed the protein or its truncated version in the presence of Cd^{2+}, Zn^{2+}, Pb^{2+}, Co^{2+} and Ni^{2+}. *Thlaspi* and *Arabidopsis* HMA4 C-terminal fragment conferred tolerance only to Cd^{2+} in wild-type strains and did not protect against the other metals tested. Heterologous expression in yeast also suggests a higher Cd^{2+} binding capacity of TcHMA4-C compared to AtHMA4-C. This difference may be associated with the higher divergence of the C-terminal domain between the *Thlaspi* and *Arabidopsis* sequences. Data support the role of the unusually long C-terminus extension present only in plant HMA4 in heavy metal binding, which is a mark for future analysis of plant CPx-ATPases. This is also consistent with the isolation in our screening of HMA4 sequences only and not of other HMAs. Expression of the full-length cDNA conferred higher sensitivity to Cd^{2+} in wild-type strains as well as to Zn^{2+} in the *zrc1* yeast mutant. HMA4 may be able to transport Zn^{2+} as well. Further experiments are required to investigate the cadmium and zinc transport activity of HMA4.

Most CPx-ATPases described to date confer heavy metal tolerance to a range of organisms by exporting metals from the cytoplasm (as bacterial Cd^{2+}-ATPase, which extrudes Cd^{2+} from the cell) or through intracellular sequestration of the metal ions. Heterologous expression of *TcHMA4* and *AtHMA4* in yeast reported here contrasting data as higher sensitivity to cadmium was observed in two different strains.

Currently, there is only one recent publication reporting a functional study of the *Arabidopsis* HMA4 in *E. coli* and in yeast [10]. In that work, AtHMA4 did confer cadmium tolerance when overexpressed in yeast. It is difficult to explain the differences between their results and ours as the same yeast strain (*BY4741*) and the same *Arabidopsis* cDNA sequence were used. The difference in phenotype may be linked to the localization of the heterologous protein. It is known, e.g., that different expression levels may result in difference in protein localisation. In [10], *AtHMA4* expression was under a galactose-inducible promoter. To investigate whether the different results were due to the expression system, we repeated our heterologous expression with the pYES-DEST52 (Gateway vector, Invitrogen), which expressed the cDNA under the activity of the galactose-inducible promoter and we confirmed the sensitive phenotype in the *BY4741* and *CM100* yeast strains (data not shown). Interestingly, cadmium tolerance was independent of cadmium accumulation, since *TcHMA4* expression resulted in higher cadmium accumulation in the *CM100* strain while it was reduced in *BY4741*. Our data are consistent with a role of TcHMA4 in Cd^{2+} transport. In [10], there is no report of cadmium accumulation. Differences in cadmium accumulation between the strains could be explained by different localizations of the recombinant protein. The observed sensitivity in *BY4741* and in *CM100* and the higher cadmium content in *CM100* is probably associated with a targeting in an endomembrane. In support to this hypothesis, Clemens and collaborators have shown that endoplasmic reticulum is a major site for Cd toxicity in yeast [34]. Lower cadmium amount in *BY4741* is consistent with an extra localization of TcHMA4 in the plasma membrane, which could

function as an efflux pump. Using GFP fusions, we were not able to localize the full-length protein, while TcHMA4-C was clearly localized in the cytosol of yeast cells (data not shown).

In plants, little is known about the molecular basis of cadmium transport across membranes. Several lines of evidence support the view that cadmium enters the cell through either a high-affinity iron transport system or a calcium or zinc lower affinity pathway [13,15–18]. Involvement of HMA4 in cadmium uptake from the soil appears unlikely, as it is a non-essential metal. Recent results about AtHMA4 support a role in Zn translocation [35]. In *Thlaspi*, contrary to most other plants, Cd and Zn are actively translocated from roots to the shoot where it accumulates but no transport system associated with that traffic has yet been identified. High expression of *HMA4* in *Thlaspi* roots, where it is 20-fold higher than in *Arabidopsis*, makes this protein a potential candidate for a role in root-shoot translocation. Reduced accumulation of Cd upon expression in *BY4741* is consistent with a role in Cd export and a localization at the plasma membrane.

The three *Thlaspi* populations analyzed, contrasting in their capacity to tolerate and accumulate cadmium, showed a similar *HMA4* expression level. The higher ability of the St Félix-de-Pallières population (Ganges population) to take up cadmium seems not to be related to *TcHMA4* overexpression. However, post-transcriptional regulation of synthesis or activity of HMA4 cannot be excluded at this stage.

In conclusion, a new metal transporter TcHMA4 has been identified from *T. caerulescens* that belongs to the subgroup of CPx-ATPases proposed to display $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ substrate specificity. Our results are consistent with a role of TcHMA4 in cadmium transport and possibly in zinc in roots. The striking differences observed from heterologous expression in yeast of *HMA4* and *HMA4-C* from *T. caerulescens*, a cadmium hyperaccumulator, and *A. thaliana*, a close non-tolerant non-accumulator relative, and from expression levels in planta, support a role for TcHMA4 in the cadmium hyperaccumulation character.

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