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Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high *MTP1* transcript levels

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Summary

The zinc hyperaccumulator plant *Arabidopsis halleri* is able to naturally accumulate 100-fold higher leaf zinc concentrations when compared with non-accumulator species such as the closely related *A. lyrata* and *A. thaliana*, without showing toxicity symptoms. A novel member of the cation diffusion facilitator (CDF) protein family, an *A. halleri* metal tolerance protein 1 (MTP1), and the homologous *A. thaliana* Zn transporter (ZAT)/AtMTP1 metal-specifically complement the zinc hypersensitivity of a *Saccharomyces cerevisiae zrc1 cot1* mutant strain. A fusion of the AhMTP1 protein to green fluorescent protein (GFP) localizes to the vacuolar membrane of *A. thaliana* protoplasts. When compared with *A. lyrata* and *A. thaliana*, the total *MTP1* transcript levels are substantially higher in the leaves and upregulated upon exposure to high zinc concentrations in the roots of *A. halleri*. The high *MTP1* transcript levels in *A. halleri* can be primarily attributed to two genetically unlinked genomic *AhMTP1* gene copies. The two corresponding loci co-segregate with zinc tolerance in the back-cross 1 generation of a cross between the zinc-tolerant species *A. halleri* and the zinc-sensitive species *A. lyrata*. In contrast, a third *MTP1* gene in the genome of *A. halleri* generates only minor amounts of *MTP1* transcripts and does not co-segregate with zinc tolerance. Our data suggests that zinc tolerance in *A. halleri* involves an expanded copy number of an ancestral *MTP1* gene, encoding functional proteins that mediate the detoxification of zinc in the cell vacuole. At the transcript level, *MTP1* gene copies of *A. halleri* are regulated differentially and in response to changes in zinc supply.

Keywords: ZAT, cation diffusion facilitator, Zn, hyperaccumulator, tonoplast.

Introduction

Transition metal ions, like those of iron (Fe), zinc (Zn), manganese (Mn) and copper (Cu) are essential for all organisms. However, excess accumulation or inaccurate localization of metal ions can cause severe damage to cells. Transition metal ions can be harmful because of their high binding affinities for organic molecules, their ability to displace cationic cofactors of proteins and the ability of some transition metals to catalyse redox reactions. Plants possess a tightly knit and intricately regulated metal homeostasis network to control acquisition, uptake, distribution and storage of metals both at the organism and cellular levels (Clemens *et al*., 2002b). *Arabidopsis halleri* ssp. *halleri* is a Zn and cadmium (Cd) hyperaccumulator plant, which exhibits naturally selected tolerance to both metals (Ernst, 1974). *Arabidopsis halleri* belongs to about 440 known taxa of hyperaccumulator plants, which have been recognized based on the accumulation in the above-ground tissues of more than 1% Mn or Zn, or more than 0.1% arsenic, cobalt (Co), Cu, nickel (Ni), lead, antimony, selenium or thallium, or more than 0.01% Cd in the dry biomass of plants grown in their natural environment (Baker and Brooks, 1989). For comparison, critical Zn toxicity levels in crop plants are

generally between 0.01 and 0.03% Zn in dry leaf biomass (Marschner, 1995). Metal hyperaccumulator plants are considered models for the development of plant-based technologies for the clean-up of metal-contaminated soils, termed phytoremediation (Chaney *et al.*, 1997; Clemens *et al.*, 2002b; Salt *et al.*, 1998).

The species most closely related to the metal hyperaccumulator *A. halleri* is *A. lyrata*, which is generally known as a non-hyperaccumulator that does not exhibit metal tolerance. The *A. halleri*/*A. lyrata* lineage diverged from the last common ancestor with *A. thaliana* approximately 5 Ma ago (Koch *et al.*, 2000). Both *A. halleri* and *A. lyrata* are herbaceous perennials with self-incompatible breeding systems and $2n = 16$ chromosomes. Laboratory experiments have confirmed a pronounced Zn and a moderate Cd tolerance in *A. halleri*, as well as the accumulation of high Zn concentrations in the leaves, when compared with *A. lyrata* ssp. *petraea* or *A. thaliana* (Becher *et al.*, 2004; Bert *et al.*, 2000, 2003; Cho *et al.*, 2003; Küpper *et al.*, 2000; Macnair *et al.*, 1999; Weber *et al.*, 2004; Zhao *et al.*, 2000). In addition, some *A. halleri* accessions have been found to hyperaccumulate Cd (Bert *et al.*, 2002, 2003; Dahmani-Muller *et al.*, 2000). In *A. halleri*, Zn concentrations are higher in the leaves than in the roots (Dahmani-Muller *et al.*, 2000), which is characteristic of hyperaccumulator plants of at least Zn and Ni (Baker *et al.*, 1994; Dahmani-Muller *et al.*, 2000; Krämer *et al.*, 1996). The hyperaccumulation of metals, specifically in the leaves of *A. halleri*, necessitates metal detoxification, for instance by metal sequestration or metal chelation (Clemens *et al.*, 2002b). In *A. halleri*, the highest Zn concentrations were reported in the vacuoles of leaf mesophyll cells and in a narrow ring-shaped region at the base of trichomes (Küpper *et al.*, 2000). In leaf mesophyll vacuoles, Zn was proposed to occur predominantly in the form of a malate complex (Sarret *et al.*, 2002; Zhao *et al.*, 2000).

Recently, cross-species microarray expression profiling led to the identification of a number of candidate genes for metal hyperaccumulation and tolerance, which are constitutively more highly expressed in *A. halleri* when compared with *A. thaliana* (Becher *et al.*, 2004; Weber *et al.*, 2004). In the shoots of *A. halleri*, the identified genes support the predominance of metal sequestration, implicating, among others, a protein of the cation diffusion facilitator (CDF) family (Becher *et al.*, 2004; Paulsen and Saier, 1997).

Members of the ubiquitous CDF protein family catalyse the efflux of transition metal ions, like Zn^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+} or Ni^{2+}, from the cytoplasm to the outside of the cell or into subcellular compartments (Gaither and Eide, 2001; Palmiter and Huang, 2004). CDF proteins or domains exhibit six predicted membrane-spanning segments, a histidine-rich cytoplasmic loop between transmembrane domains IV and V, a signature sequence between transmembrane domains I and II, and a large cation efflux domain with scattered sequence similarities encompassing transmembrane domains I–VI and most of the cytoplasmic C-terminus (Anton *et al.*, 1999; Gaither and Eide, 2001; Paulsen and Saier, 1997). For example, the *Schizosaccharomyces pombe* CDF protein Zhf1p can transport Zn and most probably other transition metal ions into the endoplasmatic reticulum (Borrelly *et al.*, 2002; Clemens *et al.*, 2002a). Mutant cells devoid of Zhf1p are hypersensitive to Zn and Co, but exhibit enhanced tolerance to Cd and Ni, a phenotype that stresses the metal-specific role of metal compartmentation in metal homeostasis (Clemens *et al.*, 2002a). The *Bacillus subtilis* CDF protein CzcD has been shown to operate alternatively as a proton or potassium-coupled metal exporter (Guffanti *et al.*, 2002). The Zrc1p-dependent transport of Zn^{2+} into vacuole-enriched membrane vesicles of *Saccharomyces cerevisiae* is ATP-dependent, requires a proton gradient across the membrane and exhibits a K_M of 160 nM (MacDiarmid *et al.*, 2002). The available data thus suggest that at least some CDF proteins function as metal-proton antiporters.

The first plant CDF protein, which was identified according to a cloned cDNA, was the Zn transporter (ZAT) of *A. thaliana* (Van der Zaal *et al.*, 1999), which was later renamed metal tolerance protein 1 (AtMTP1; Delhaize *et al.*, 2003). *Arabidopsis thaliana*, ectopically overexpressing *AtMTP1* under the control of a *CaMV* 35S promoter, displayed enhanced Zn tolerance and root Zn accumulation. The *A. thaliana* genome is predicted to encode 12 highly divergent CDF proteins (Delhaize *et al.*, 2003).

Here, we describe cloning of the *MTP1-3* cDNA from the Zn-tolerant Zn hyperaccumulator species *A. halleri*. We show evidence that AhMTP1-3 is localized in the vacuolar membrane and is capable of cellular Zn detoxification. Whereas constitutively low *MTP1* transcript levels are found in the Zn sensitive species *A. thaliana* and *A. lyrata*, total *MTP1* transcript levels are constitutively high and metal-regulated in *A. halleri*. High *MTP1* expression levels result from both the amplification of *MTP1* gene copy number and an enhanced expression primarily of two *MTP1* loci in the *A. halleri* genome, both of which co-segregate with Zn tolerance in the back-cross 1 (BC1) generation of a cross between *A. halleri* and *A. lyrata*.

Results

Expression of the A. thaliana *or* A. halleri *MTP1 protein increases Zn tolerance of the* S. cerevisiae zrc1 cot1 *double mutant*

To identify genes involved in naturally selected Zn tolerance in *A. halleri*, we screened an *A. halleri* cDNA library for cDNAs that allow growth of the Zn hypersensitive *zrc1 cot1* mutant of *S. cerevisiae* at 3 mM Zn within 6 days

(MacDiarmid *et al.*, 2000). The screen identified an *AhMTP1* cDNA. As additional *AhMTP1* cDNAs were later cloned from *A. halleri* (see below), the cDNA isolated in the screen was originally termed *AhCDF1-3* (Becher *et al.*, 2004) and later renamed *AhMTP1-3*. Within the coding sequence, *AhMTP1-3* exhibited 92% identity with the homologous *A. thaliana ZAT/AtMTP1*. Both cDNAs encode members of group III (Blaudez *et al.*, 2003) of the so-called CDF family of metal transport proteins. Within 48 h, *zrc1 cot1* cells expressing the cDNAs of either *AhMTP1-3*, *AtMTP1*, or wild-type *ScZRC1* under the control of a *GAL1* promoter were able to grow in a liquid minimal medium containing 0.5 mM $ZnSO_4$, whereas empty vector transformants were unable to grow (Figure 1a). These results were replicated at least 10 times with two independently cloned *AtMTP1* cDNAs and *AhMTP1-3*, using the vectors pYES2, pFL61 and pDR196 (data not shown). This suggests that both AtMTP1 and AhMTP1-3 were functionally expressed in yeast and have a role in cellular Zn detoxification. Earlier, AtMTP1 was reported to functionally complement a Zn hypersensitive *Schizosaccharomyces pombe zhf1* mutant, but not a *S. cerevisiae zrc1 cot1* mutant (Bloss *et al.*, 2002; Clemens *et al.*, 2002a). A green fluorescent protein (GFP) fusion of AhMTP1-3, expressed under the control of a methionine-repressible *MET25* promoter, was localized exclusively in the vacuolar membrane of *S. cerevisiae* cells (data not shown). This suggests that in yeast cells AhMTP1-3 functions primarily in vacuolar Zn sequestration.

The predicted amino acid sequence of AhMTP1-3 shares 91% overall identity with *A. thaliana* MTP1 and 97% identity with *A. lyrata* MTP1-1 (Figure 2). AhMTP1-3 differs from AtMTP1 by a short deletion of four amino acids close to the N-terminus of the protein. In addition, the three MTP1 sequences diverge in the histidine-rich cytoplasmic loop between the predicted transmembrane domains IV and V. In the N-terminal part of this loop, AhMTP1-3 exhibits a short deletion of 11 amino acids. Eleven amino acids downstream, there is an $(HD)_5$ motif, which includes a short insertion of two residues in comparison with the two non-accumulator MTP1 proteins. Both histidine and aspartate residues are found in Zn binding sites of proteins and the histidine-rich region of AtMTP1 has been proposed to act in metal binding (Bloss *et al.*, 2002; Paulsen and Saier, 1997). In order to exclude that the mere binding of Zn^{2+} to the histidine and aspartate-rich region is responsible for complementation of the yeast double mutant, we exchanged an aspartate residue in the second transmembrane domain of AhMTP1-3 (Paulsen and Saier, 1997) for an alanine residue. The mutated aspartate residue is conserved in all CDF proteins and is essential for protein function (Blaudez *et al.*, 2003). Although the AhMTP1-3 D90A protein was made (Figure 1c), it did not complement the *zrc1 cot1* double mutant (Figure 1b). Neither AtMTP1 nor AhMTP1-3 increased Ni tolerance in wild-type yeast,

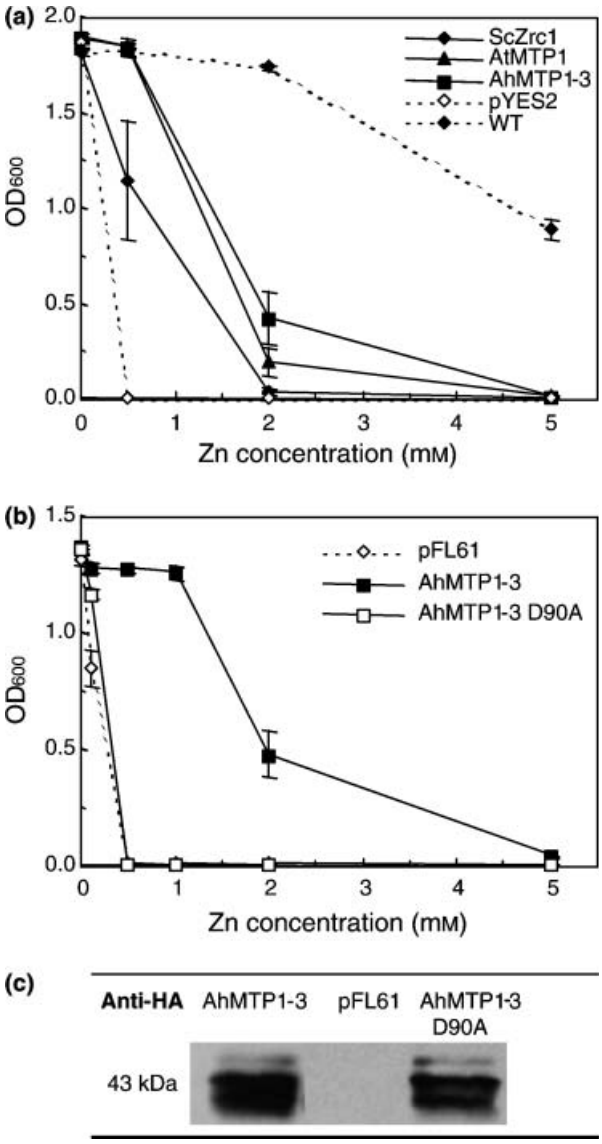

Figure 1. Functional analysis of the *MTP1* cDNAs from *A. halleri* and *A. thaliana* by expression in the Zn-hypersensitive *zrc1 cot1* double mutant of *S. cerevisiae*.

(a) Complementation of the *zrc1 cot1* double mutant. Yeast cell densities are given after 48 h of growth in a minimal medium (LSP-URA) with galactose as carbon source, supplemented with different concentrations of $ZnSO_4$. Values are mean ± SD ($n = 3$). Transformants were the wild-type yeast strain transformed with an empty pYES2, the *zrc1 cot1* double mutant transformed with pYES2, the *AhMTP1-3* cDNA in pYES2 as obtained in the library screen, the open-reading frame of *AtMTP1* or the open-reading frame of *ScZRC1* cloned into pYES2, respectively.

(b) The AhMTP1-3 D90A mutant protein is unable to complement the *zrc1 cot1* double mutant. Given are yeast cell densities after growth in minimal medium, with glucose as carbon source. Values are mean ± SD ($n = 3$). Transformants were the *zrc1 cot1* double mutant transformed with the empty pFL61, the open-reading frame of *AhMTP1-3* in pFL613 and *AhMTP1-3*(D90A) in pFL613. The plasmid pFL613 encodes a 3xHA tag that is translationally fused to the N-terminus of the protein encoded by the cloned cDNA.

(c) Signals on a Western blot of total protein extracts from the transformants described in (b), after incubation with an anti-HA antibody.

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          1                                                  50
AhMTP1-3 MESSS----HIVEVNVGKSEEERIIVASKVCGEAPCDFSDSKNASGDAKE
AlMTP1-1 MESSSPHHSHIVEVNVGKSEEERIIVASKVCGEAPCGFSDSKNASGDAQE
  AtMTP1 MESSSPHHSHIVEVNVGKSDEERIIVASKVCGEAPCGFSDSKNASGDAHE

                MMMMMMMMMMMMMMMMMM                MMM▼MMMMMM
AhMTP1-3 RSASMRKLSIAVVMCLVFMTVEVVGGIKANSLAILTDAAHLLSDVAAFAI
AlMTP1-1 RSAFMRKLCIAVVLCLVFMSVEVVGGIKANSLAILTDAAHLLSDVAAFAI
  AtMTP1 RSASMRKLCIAVVLCLVFMSVEVVGGIKANSLAILTDAAHLLSDVAAFAI

           MMMMMMMMMM                  MMMMMMMMMMMMMMMMMMM
AhMTP1-3 SLFSLWAASWEATPTQTYGFFRIEILGALVSIQLIWLLTGILVYEAIIRI
AlMTP1-1 SLFSLWAAGWEATPRQTYGFFRIEILGALVSIQLIWLLTGILVYEAIIRI
  AtMTP1 SLFSLWAAGWEATPRQTYGFFRIEILGALVSIQLIWLLTGILVYEAIIRI

                    MMMMMMMMMMMMMMMMMMMMM
AhMTP1-3 VTETSEVNGFLMFLVAVFGLVVNIIMAVLLGHDHGHSHGHGHS-------
AlMTP1-1 VTETSEVNGFLMFLVAAFGLVVNIIMAVLLGHDHGHSHGHGHSHGHDHHN
  AtMTP1 VTETSEVNGFLMFLVAAFGLVVNIIMAVLLGHDHGHSHGHGHGHDHHN

AhMTP1-3 ----HSHGVTDTTHPHDHDHDHDHGHS--HGEDQHHAHGDVTEQLLDK
AlMTP1-1 HGGNHSHGVTVTTHHHHHDHEHD----HSHGHGEDKHHAHGDVTEQLLDK
  AtMTP1 ----HSHGVTVTTHHHHHDHE----HGHSHGHGEDKHHAHGDVTEQLLDK

                                MMMMMMMMMMMMMMMMMMM      MM
AhMTP1-3 SKTQVAAKEKRKRNINVQGAYLHVLGDSIQSVGVMIGGAIIWYNPEWKIV
AlMTP1-1 SKTQVAAKEKRKRNINVQGAYLHVLGDSIQSVGVMIGGAIIWYNPEWKIV
  AtMTP1 SKTQVAAKEKRKRNINLQGAYLHVLGDSIQSVGVMIGGAIIWYNPEWKIV

          MMMMMLMMMMMMLMMMM  L       L
AhMTP1-3 DLICTLVFSVIVMGTTINMSRNILEVLMESTPREIDATKLEKGLLEMEEV
AlMTP1-1 DLICTLVFSVIVLGTTINMIRNILEVLMESTPREIDATKLEKGLLEMEEV
  AtMTP1 DLICTLAFSVIVLGTTINMIRNILEVLMESTPREIDATKLEKGLLEMEEV

             HExHxWxITxxxxxxxxH
AhMTP1-3 VAVHELHIWAITVGKVLLACHVNIRPEADADMVLNKVMDYIRREYNISHV
AlMTP1-1 VAVHELHIWAITVGKVLLACHVNIRPEADADMVLNKVIDYIRREYNISHV
  AtMTP1 VAVHELHIWAITVGKVLLACHVNIRPEADADMVLNKVIDYIRREYNISHV

AhMTP1-3 TIQIER
AlMTP1-1 TIQIER
  AtMTP1 TIQIER
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Figure 2. Alignment of amino acid sequences of *A. halleri*, *A. lyrata* and *A. thaliana* MTP1 proteins. Sequence differences are marked in bold letters, and fully conserved regions are blocked in grey. Predicted transmembrane domains (M) are marked in the line above the sequences, as well as the conserved aspartate (▼) at position 90 of AhMTP1-3, and the leucine residues (L) of the proposed leucine zipper motif (Blaudez *et al.*, 2003). This is not conserved in AhMTP1-3 as the third apolar leucine residue is replaced by a polar serine residue, as in the corresponding position of ScZrc1p. The putative Zn binding site at the C-terminus HD(E)XHXWXL(I)TX_8H is conserved in AhMTP1-3 (Gaither and Eide, 2001), and indicated. The *A. halleri* sequence was obtained by screening a cDNA library, and the sequences from *A. thaliana* and *A. lyrata* were obtained by RT-PCR with primers designed according to the *A. thaliana* genomic sequence. Note that the primers correspond to the seven N-terminal and the seven C-terminal amino acids of the *A. lyrata* sequence, respectively.

zrc1 mutants or *zrc1 cot1* mutants, nor did they complement Cd hypersensitivity of the YYA4 double mutant (data not shown; Gaedeke *et al.*, 2001).

A GFP–AhMTP1-3 fusion protein localizes to the vacuolar membrane in A. thaliana *protoplasts*

Arabidopsis MTP1 proteins are homologous to the Zn transporter ScZrc1p, which is the principal protein that transports Zn from the cytoplasm into the vacuole of *S. cerevisiae* (MacDiarmid *et al.*, 2002). The vacuoles of mesophyll cells have been proposed to constitute the major site for Zn storage in *A. halleri* (Küpper *et al.*, 2000). We hypothesized that AhMTP1-3 participates in vacuolar sequestration of Zn in *A. halleri*. Transient expression of a *GFP:AhMTP1-3* reporter construct in *A. thaliana* protoplasts indicated a tonoplast localization of the GFP signal (Figure 3). This is consistent with a function of AhMTP1-3 in vacuolar Zn sequestration.

Three AhMTP1 *loci segregate independently in the back-cross 1 population of a cross between* A. halleri *and* A. lyrata

To determine whether *AhMTP1-3* is present in the genome of *A. halleri* as a single copy or as multiple copies, we performed genomic DNA blots (Figure 4). The single band observed in *A. thaliana* after digestion with *Eco*RI, which does not cut within the coding sequence of *AtMTP1* or *AhMTP1-3*, corresponds to a single *MTP1* gene, located at the end of the long arm of chromosome 2 of the *A. thaliana* genome (At2g46800, BAC clone AC005310). Upon digestion with *Eco*RI, four fragments were detected in *A. halleri* and two in *A. lyrata* (Figure 4a). As both these species are outcrossing, groups of two fragments may correspond to two alleles of a single locus. All *Arabidopsis MTP1* sequences exhibit an *Nco*I cleavage site located centrally in the open-reading frame. DNA gel blot analysis of *Nco*I-digested genomic DNA yielded a total number of nine discernible

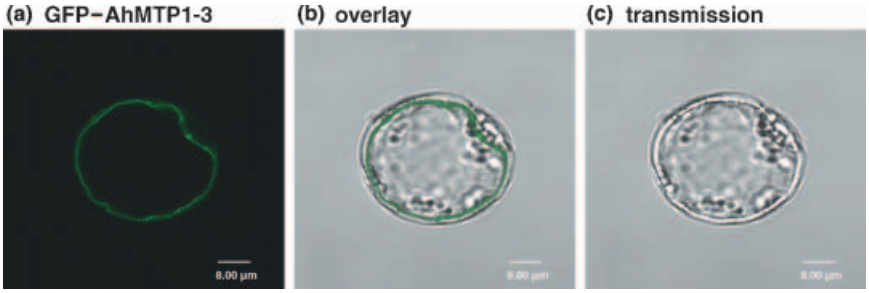


Figure 3. Subcellular localization of the AhMTP1-3 protein.
(a) GFP fluorescence image of GFP–AhMTP1-3
(b) overlay of GFP fluorescence and transmission image and
(c) transmission image of an *A. thaliana* protoplast 24 h after transfection.

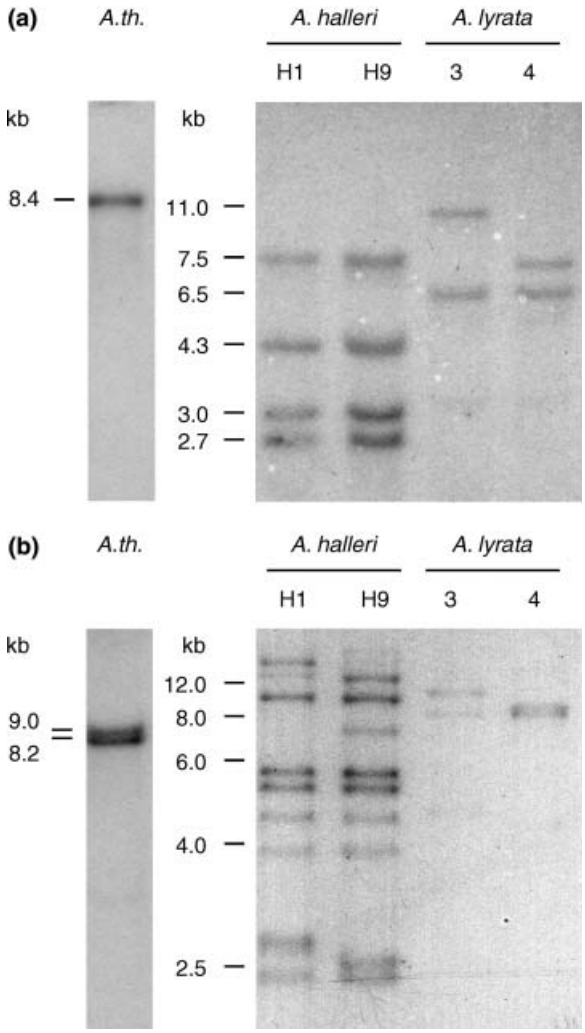


Figure 4. Southern analysis of single individuals of *A. thaliana*, *A. halleri* and *A. lyrata*.
(a) Genomic DNA was digested with *Eco*RI or
(b) *Nco*I, and blots were hybridized with a probe encompassing the full length coding sequence of the *AtMTP1* cDNA. *Eco*RI restriction sites are outside the coding region of the *MTP1* gene in all three *Arabidopsis* species. *Nco*I is predicted to cut the coding region of all *MTP1* sequences into two fragments of approximately equal length. Characters above the lanes designate the individuals of *A. halleri* and *A. lyrata*, from which genomic DNA was extracted for the respective lane.

bands in each of two *A. halleri* individuals and two bands in *A. lyrata* (Figure 4b). In additional experiments, a minimum of seven and a maximum of 10 signals were detected in *A. halleri* individuals (*Nco*I digest, 12 individuals analysed; data not shown). In *A. thaliana*, the closest homologues of *AtMTP1* are *AtMTP2* and *AtMTP3*, with 66 and 65% nucleotide identity within the coding sequence, respectively. In a gel blot of *A. halleri* genomic DNA hybridized with an *AhMTP2* probe, the observed signals were distinct from those detected in Figure 4 (data not shown). Conversely, in *A. thaliana* genomic DNA blots hybridized with an *AtMTP1* probe, no fragments derived from *AtMTP2* (At3g61940, 9.5 kb for *Eco*RI and 20.5 kb for *Nco*I) or *AtMTP3* (At3g58810, 9 kb for *Eco*RI, and 3.3 and 16.5 kb for *Nco*I) were detected. Instead, only the expected fragments generated *AtMTP1*-specific signals of 8.4 kb for *Eco*RI, and of 9 and 8.2 kb for *Nco*I (Figure 4a,b). This confirms the absence of any cross-hybridization with other CDF family genes. DNA gel blot analysis thus suggested that the *MTP1* gene has undergone copy number expansion in the genome of *A. halleri*, when compared with the genomes of *A. lyrata* and *A. thaliana*.

The segregation of *AhMTP1* genes was examined by RFLP analysis in a segregating BC1 population of a cross between *A. halleri* and *A. lyrata*, which was established previously to examine the inheritance of Zn and Cd tolerance and accumulation in *A. halleri* (Bert *et al.*, 2003). To generate the BC1 population, one *A. halleri* individual (P1, male) was first crossed with an *A. lyrata* individual (*A. lyrata 1*, P2, female). One F1 individual (P3, male) was then back-crossed with a second *A. lyrata* individual (*A. lyrata 2*, P4, female) to obtain the BC1 seed. In individuals of the BC1 population, three *AhMTP1* loci, termed *A*, *B* and *C*, were found to segregate as single bands or as pairs of two bands after digestion of genomic DNA with *Eco*RI (Figure S1) or *Nco*I (data not shown), respectively. This suggests that each of these three loci harbours one single copy of *AhMTP1*. Chi-square goodness-of-fit tests confirmed that *AhMTP1* loci *A* and *C* segregate in a Mendelian fashion (Table 1). However, only approximately one third of BC1 individuals harboured locus *B*, which was confirmed to exhibit a significant segregation distortion (χ^2 of 23.4; $P < 0.001$, Table 1). Pair-wise recombination frequencies of *AhMTP1* loci *A*, *B* and *C* were between 0.48 and 0.57, suggesting that the *A. halleri MTP1* loci are not genetically linked (Table 2).

The RFLPs of the BC1 population indicated that in the *A. lyrata* genome there is a single *MTP1* locus, termed *a* (Figure S1). Of the two alleles a_1 and a_2 present in the *A. lyrata* 1 grandmother of the BC1 (P2), the F1 individual P3, which was the father of the BC1, inherited a_2. Alleles of the

Table 1 Segregation of *A. halleri MTP1* genes in the back-cross 1 (BC1) population

	Observed					
Locus	*n*A	*n*P	Expected ratio	χ^2	*P*	*n*
A	156	163	1:1	0.1536	0.7	319
B	197	112	1:1	23.3819	<0.001	309
C	159	149	1:1	0.3247	0.6	308

For the three *AhMTP1* loci chi-square values (χ^2) are given for goodness-of-fit to Mendelian inheritance ratios, followed by associated *P*-values. The observed numbers of BC1 individuals carrying (*n*P-present) or not carrying (*n*A-absent) the respective A*hMTP1* locus are also given (*n* – number of BC1 individuals analysed).

Table 2 Recombination frequencies of *A. halleri MTP1* loci *A*, *B*, and *C* in the BC1 generation

	*Nco*I (210)	*Eco*RI (91)	Total (301)
A/*B*	0.57	0.58	0.57
A/*C*	0.55	0.60	0.56
B/*C*	0.49	0.46	0.48

Recombination frequencies were calculated from RFLP analyses performed with *Eco*RI or *Nco*I as restriction enzymes, and in total. Values ≥0.5 indicate independent segregation. Values <0.5 indicate linkage. The number of individuals analysed are shown in parentheses.

MTP1 loci *A, B and C* of the *A. halleri* grandfather of the BC1 (P1) were also present in the F1 individual P3. After crossing P3 with *A. lyrata* 2 (P4, alleles a_1 and a_3), the *AhMTP1* locus *A* and the *A. lyrata* allele a_2 from P3 never occurred together in any BC1 individual. This strongly suggests that the *A. halleri* locus *A* and *A. lyrata* locus *a* are at equivalent positions in the *A. lyrata* and the *A. halleri* genomes (Figure S1). Alternatively, the two loci may occupy positions on corresponding chromosomes that are too close together to exhibit significant recombination in the analysed BC1 population.

Thus, our RFLP analysis showed that three *A. halleri MTP1* genes segregate independently in the analysed BC1 population. In contrast, the genome of *A. lyrata* hosts only a single *MTP1* gene.

Two A. halleri MTP1 *loci co-segregate with Zn tolerance in the BC1*

In the parents of the cross and in the BC1 individuals, Zn tolerance was determined as the EC_{100} Zn concentration which is the lowest Zn concentration that fully inhibits root elongation in a sequential exposure test, as previously described and analysed (Bert *et al.*, 2003; Macnair *et al.*, 1999). Compared with the *A. halleri* parent individual (P1, EC_{100} of 2500 μM Zn), metal tolerance was significantly reduced in the F1 individual (P3, EC_{100} of 1500 μM Zn, $P < 0.05$, *G*-test) used to generate the BC1, whereas the *A. lyrata* parent individuals P2 and P4 (EC_{100} of 25 and 75 μM Zn, respectively) were dramatically less Zn tolerant (Bert *et al.*, 2003). We concluded that it is possible to analyse segregation of metal tolerance and metal tolerance genes in the BC1 population. The heterozygous status of all *A. halleri* genes in the F1 may explain the difference in Zn tolerance between P1 and P3.

To investigate whether any of the *AhMTP1* loci *A*, *B*, or *C* co-segregate with Zn tolerance in the BC1 generation, one-way analysis of variance (ANOVA) was performed. This revealed a significant association of the *A. halleri MTP1* locus *A* with Zn tolerance ($F_{1,183} = 18.09$; $P < 0.001$). Moreover, *AhMTP1* locus *B* was also found to be associated with Zn tolerance ($F_{1,182} = 15.24$; $P < 0.001$). In contrast, there was no difference in the Zn tolerance between BC1 individuals harbouring locus *C* and those that do not harbour locus *C* ($F_{1,183} = 0.07$; $P = 0.8$).

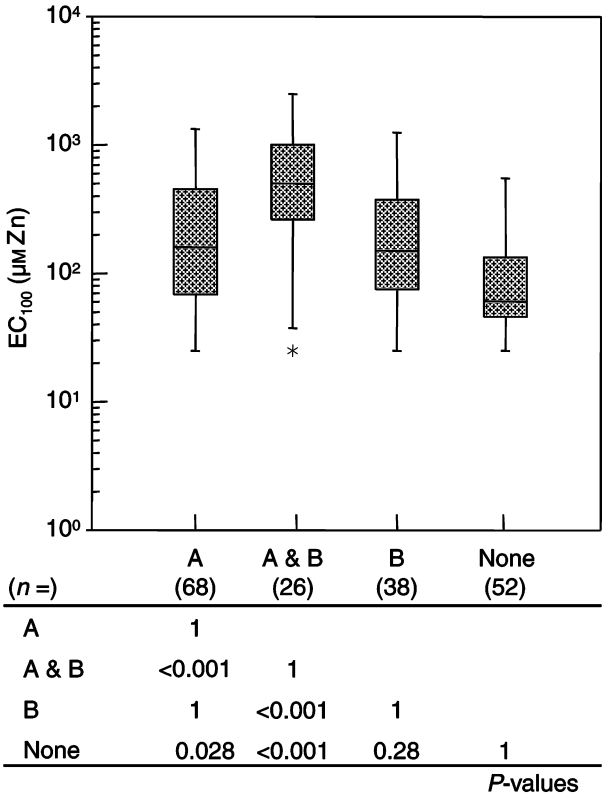
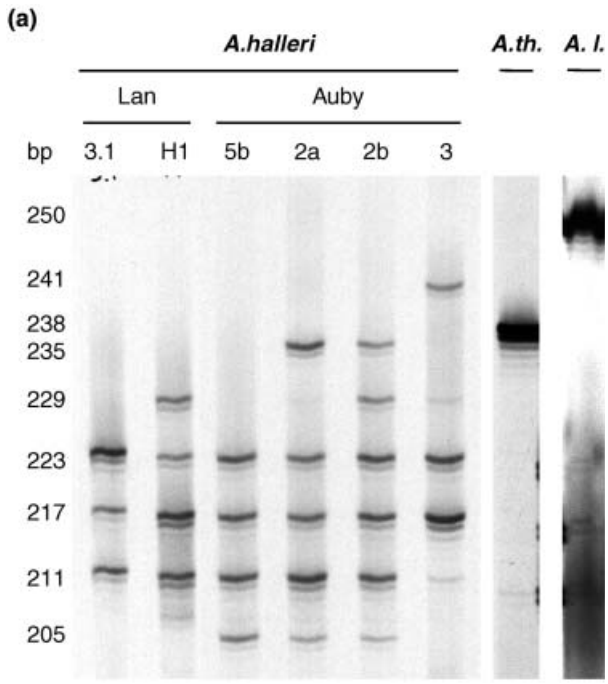
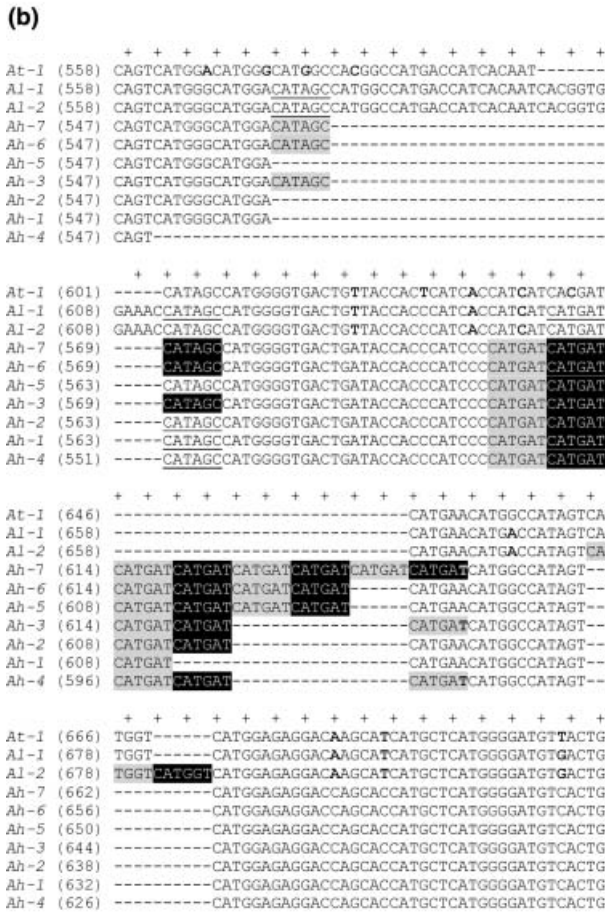


Figure 5. Box plot of Zn tolerance by genotypes of the BC1 population. The boxes represent the inter-quartile range, with geometric means indicated by horizontal lines. Vertical lines (whiskers) connect the nearest observations within 1.5 times the inter-quartile ranges of the lower and upper quartiles. One outlier in group '*A* & *B*' is represented by an asterisk. Zn tolerance is shown on a logarithmic scale. *P*-values of pair-wise comparisons of mean tolerance between groups are given below the diagram.

To test for a possible additive effect of the loci found to be in linkage disequilibrium with Zn tolerance in the BC1 generation, four genotypic groups of BC1 individuals were formed, each characterized by the absence or presence of one or both *AhMTP1* loci *A* and *B*. Side-by-side box plots indicated differences in Zn tolerance between the four genotypic classes in the BC1 population (Figure 5). Pair-wise comparisons of arithmetic means of tolerance between the genotypic groups confirmed significant differences between groups '*A* only' and 'none' ($P = 0.028$), '*A* & *B*' and '*A* only' ($P < 0.001$), '*A* & *B*' and '*B* only' ($P < 0.001$), and '*A* & *B*' and 'none' ($P < 0.001$). This confirmed that members of the two genotypic classes described by the presence of either locus *A* or *B* alone exhibited intermediate tolerance, whereas BC1 individuals harbouring both loci *A* and *B* were the most tolerant. In the pair-wise comparisons, the difference between '*B* only' and 'none' was not significant ($P = 0.28$, all *P*-values after Bonferroni corrections), although the ANOVA mentioned above indicated a significant correlation between





locus *B* and tolerance. The number of individuals carrying locus *B* is suppressed by segregation distortion (see above), and the generation and analysis of additional BC1 individuals may reveal a significant effect also in pair-wise comparisons as shown in Figure 5. In agreement with the ANOVA, no significant effect was detected for locus *C*, either alone or in combination with other *AhMTP1* loci, in a second set of pair-wise comparisons (not shown).

A microsatellite within the open-reading frame of A. halleri MTP1 *genes*

To identify potential sequence differences between *AhMTP1* genes, additional partial *AhMTP1* cDNAs were cloned by PCR from the *A. halleri* cDNA library (see above). These partial cDNA sequences were between 98 and 99% identical to *AhMTP1-3* at the nucleotide level (data not shown) and exhibited insertion/deletion polymorphisms within a region that corresponds to the cytoplasmic loop between membrane spanning domains IV and V of the protein. The same region was found to contain insertions/deletions in the comparison of *AhMTP1-3* with *A. lyrata* and *A. thaliana* *MTP1* cDNAs (see also Figure 2). Fragments encompassing this polymorphic region of *MTP1* were amplified by PCR from the genomic DNA of a number of individuals of *A. thaliana*, *A. lyrata* and *A. halleri*. A single PCR fragment of 238 bp was detected in all *A. thaliana* (accession Col-0) individuals analysed (Figure 6a). In individuals of *A. lyrata* one or two fragments of 250 and/or 256 bp were observed (Figure 6a, and data not shown). This is consistent with our earlier observation that the *A. lyrata* genome harbours one *MTP1* locus (see above) with one or two alleles (Figure 6a). In an analysis of 30 additional *A. halleri* plants from the

Figure 6. Length polymorphisms in *MTP1* genes of *A. halleri*, *A. thaliana* and *A. lyrata*.

(a) Fragments including the polymorphic region of *MTP1* genes, which corresponds to the cytoplasmic loop between transmembrane domains IV and V of the MTP1 proteins, were amplified by PCR using *Taq* polymerase. Genomic DNA isolated from one single plant was used as a template for each reaction. The amplified fragments consisted of base pairs 496–718 of the open-reading frame of *AhMTP1-3*, and the corresponding region of other *MTP1* genes or alleles. The species *A. halleri* is represented by individuals from accessions Langelsheim (Lan) and Auby. Amplification products were resolved on a denaturing 6% (v/v) polyacrylamide gel and visualized by autoradiography. Note that double bands occurred because of the non-template-dependent terminal transferase activity of *Taq* polymerase, and were not observed when a proof-reading polymerase was used instead.

(b) Alignment of nucleotide sequences in the polymorphic region of *MTP1* genes of *A. halleri*, *A. thaliana* and *A. lyrata*. Numbers in parentheses correspond to the position in the coding sequence, and the reading frame is marked by '+' over the first position of each codon. Sequence differences are indicated in bold letters. Hexanucleotide repeats are boxed alternating in grey and black, and unrepeated hexanucleotide motifs are underlined. The alignment shows part of the sequenced PCR fragments shown in (a): *At-1* (*AtMTP1* 238 bp), *Al-1* (*AlMTP1-1*: 250 bp), *Al-2* (*AlMTP1-2*: 256 bp), *Ah-1* (*AhMTP1-1*: 211 bp), *Ah-2* (*AhMTP1-2*: 217 bp), *Ah-3* (*AhMTP1-3*: 223 bp), *Ah-4* (*AhMTP1-4*: 205 bp), *Ah-5* (*AhMTP1-5*: 229 bp), *Ah-6* (*AhMTP1-6*: 235 bp), *Ah-7* (*AhMTP1-7*: 241 bp).

populations of Langelsheim, Auby, Wildemann, and Littfeld, a minimum of three and a maximum of six PCR fragments of different sizes were detected in each single individual (Figure 6a, data not shown). Fragment sizes of 211, 217 and 223 bp were present in all individuals. Other fragments of 205, 229 and 235 bp in length occurred less frequently, but were represented in all populations (Figure 6a and data not shown). The fragment of 241 bp was only found in a single individual. Among *A. halleri* individuals from natural populations there is thus a high occurrence of length polymorphisms within the open-reading frame of *AhMTP1* genes.

To investigate the basis of the length polymorphisms, we sequenced the fragments from *A. lyrata* and *A. halleri*. Sequence alignment revealed that differences in fragment length are mainly caused by the different numbers of *CATGAT* hexanucleotide repeats, corresponding to HD repeats in the predicted proteins, of between three and eight units (Figure 6b). Instead, only one single *CATGAT* motif is present in *A. lyrata* and none in *A. thaliana*. To test whether variant AhMTP1 proteins are functional, the fragment of 223 bp, which corresponds to the histidine-rich region of the AhMTP1-3 protein, was replaced by fragments of 205 or 229 bp, respectively, by homologous recombination in yeast. Recombinant constructs were verified by sequencing. All recombinant proteins were equivalent in their ability to complement the Zn hypersensitivity of *S. cerevisiae zrc1 cot1* mutant (data not shown). Moreover, yeast cells transformed with *AhMTP1-3*-pFL61 did not exhi-

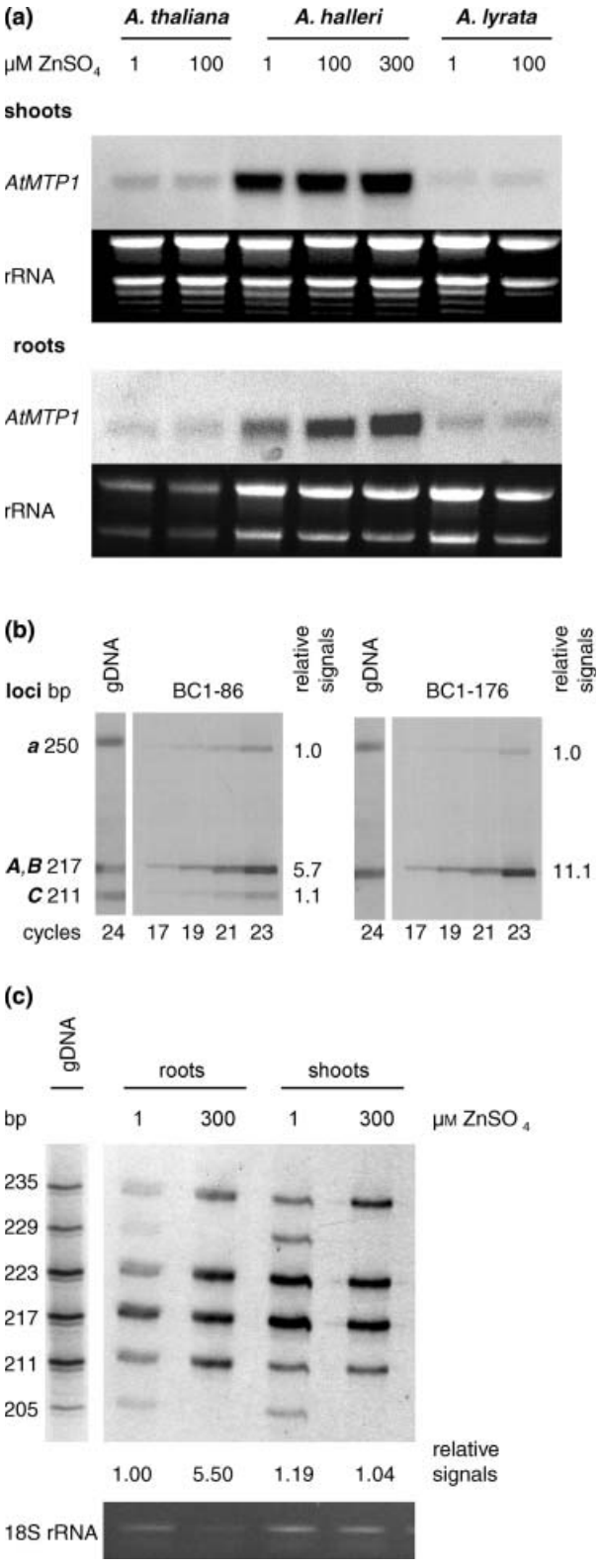


Figure 7. Analysis of steady-state levels of *MTP1* transcripts and their regulation by Zn in *A. halleri*, *A. thaliana* and *A. lyrata*.

(a) RNA gel blot analysis of *MTP1* expression in the three *Arabidopsis* species after growth in a hydroponic medium supplemented with different concentrations of added $ZnSO_4$, as indicated, for 4 days. Total RNA of shoots and roots was resolved electrophoretically, blotted onto a nylon membrane and probed with the full-length coding sequence of the *AtMTP1* cDNA. Ethidium bromide-stained ribosomal bands are shown as loading control.

(b) Semi-quantitative RT-PCR analysis of *MTP1* gene expression in shoots of BC1 individuals. The polymorphic region was amplified to differentiate between transcripts of different *MTP1* genes. PCR products were separated and visualized as described in Figure 6(a). The sizes of the PCR fragments and the corresponding loci are marked to the left of the panels. Each panel shows the results from one BC1 individual representative of three analysed BC1 individuals with equivalent *MTP1* allele configurations, respectively. Values next to the panels are mean relative signal intensities normalized to the signal intensity of the band at 250 bp (SD values were 0.48 for 217 bp and 0.19 for 211 bp in the left panel, and 1.21 for 217 bp in the right panel, $n = 3$ individuals of equivalent *MTP1* allele configuration), and values below the lanes indicate the number of amplification cycles. Plant BC1-86 represents three BC1 individuals carrying *A. halleri* loci *B* and *C* represented by PCR products of 217 and 211 bp, respectively, and *A. lyrata* alleles a_2 and a_3 (250 bp). Plant BC1-176 represents three BC1 individuals carrying *A. halleri* loci *A* and *B*, both represented by PCR products of 217 bp, and *A. lyrata* alleles a_1 or a_3 (250 bp).

(c) Semi-quantitative RT-PCR analysis of *MTP1* gene expression in the roots and shoots of clones of one *A. halleri* plant (accession Auby) following exposure to control (1 μM) and high (300 μM) Zn concentrations for 2 days (right panel), after 22 amplification cycles, performed as in (b). As a constitutive control, 18S rRNA was amplified using an identical PCR programme and a ratio of primers and competimers of 3:7 (bottom panel, see Methods). To show the genotype, genomic DNA extracted from the plant of origin of the clones was amplified under the same conditions (left lane). The numbers below the upper panel indicate the relative total *MTP1* transcript levels after normalization to the constitutive control.

bit higher levels of Zn tolerance than yeast cells transformed with *AtMTP1*-pFL61. This suggested that when expressed in yeast, there is no major functional difference between MTP1 proteins carrying stretches of HD repeats of differential lengths. We decided to focus here on a detailed analysis of the regulation of transcript levels of *MTP1* genes.

Expression of MTP1 *genes and differential transcriptional regulation of* MTP1 *gene copies in* A. halleri

RNA gel blot analysis indicated that shoot steady-state *MTP1* transcript levels were substantially higher in *A. halleri* than in *A. thaliana* or *A. lyrata* (Figure 7a). In microarrays hybridized with labelled shoot cRNA, normalized signal intensities for *ZAT/AtMTP1* were between 14- and 23-fold higher in *A. halleri* compared with *A. thaliana* (Becher *et al.*, 2004). Under control conditions (1 μM Zn in the hydroponic nutrient solution) root steady-state *MTP1* transcript levels in *A. halleri* were approximately equivalent to those in *A. thaliana*. After 4 days of exposure to 100 and 300 μM Zn in the hydroponic solution, however, root *MTP1* transcript abundance increased incrementally in *A. halleri*, but not in the two other *Arabidopsis* species. Compared with plants grown under control conditions there was no change in *MTP1* transcript levels following exposure to 10 μM Co, 30 μM Cd, 5 μM Cu or 10 μM H_2O_2 in either the roots or shoots of *A. halleri* (data not shown). This showed that in the roots of *A. halleri*, *MTP1* transcript abundance is upregulated specifically in response to high Zn concentrations.

In the BC1 population, *MTP1* loci *A* and *B*, but not *C*, from *A. halleri* exhibited a significant co-segregation with Zn tolerance (see Figure 5). By PCR analyses of the polymorphic region in the parent *A. halleri* individual (P1) and in the BC1 population (see Figure 6a,b) we established that *AhMTP1* loci *A* and *B* (and the derived cDNAs) correspond to PCR fragment lengths of 217 bp and locus *C* corresponds to a fragment length of 211 bp (data not shown). In order to separately analyse the expression of the *MTP1* loci in the BC1 generation, we performed semi-quantitative RT-PCR on RNA extracted from the shoots of selected BC1 individuals and visualized length polymorphisms on a denaturing polyacrylamide gel (Figure 7b). Equivalent amplification efficiencies were demonstrated for the different fragment lengths occurring in *A. halleri* (Figure S2). We first analysed three BC1 individuals harbouring *A. halleri MTP1* loci *B* and *C*, and *A. lyrata* alleles a_2 and a_3. When compared with the transcript levels of the *A. halleri MTP1* locus *C*, the transcript levels of the *A. halleri MTP*1 locus *B* were on average 5.7-fold higher (Figure 7b). The expression of locus *C* was approximately equivalent to the expression of the *MTP1* locus *a* from *A. lyrata*, represented by a PCR fragment of 250 bp in length. In BC1 individuals carrying *A. halleri MTP1* loci *A* and *B* and the *A. lyrata* allele a_1 or a_2, the transcripts of *A. halleri MTP1* loci *A* and *B* together were on average 11.1 times more abundant than the *A. lyrata MTP1* transcripts. This is in accordance with the co-segregation analysis of the BC1 population shown in Figure 5 and suggests that the *AhMTP1* locus *C*, which does not co-segregate with metal tolerance in the BC1, contributes only minor amounts of transcripts in the BC1. We concluded that the *AhMTP1* loci *A* and *B* are likely to account for the high transcript levels in the shoots of *A. halleri*, as found in the Northern blot analysis (see Figure 7a).

To confirm the differential expression of different *AhMTP1* loci in *A. halleri*, we determined transcript levels after the exposure of clones of a single *A. halleri* individual to 300 μM Zn for 2 days using semi-quantitative RT-PCR (Figure 7c). To allow for the best possible resolution between expression of different *MTP1* loci and alleles, an *A. halleri* plant was chosen that, in a PCR amplification of the polymorphic region of *MTP1* from genomic DNA, yielded the maximum number of six fragment lengths. Different from the *A. halleri* parent individual (P1), which was used to generate the BC1 population, each *MTP*1 locus of this *A. halleri* individual thus generates up to two different PCR fragment lengths (Figure 7c). Transcripts corresponding to two of the fragments (229 and 205 bp) were detected in lowest amounts in control plants and were downregulated in plants exposed to high Zn concentrations. Because of their coordinate regulation it is possible that these two fragments correspond to two alleles of a common locus and may thus represent *MTP1* locus *C* of this *A. halleri* individual. In contrast, the transcripts corresponding to fragments of 235 and 211 bp, and 223 and 217 bp appeared to be expressed at higher basal levels, were upregulated in roots and remained more or less unchanged in shoots in response to high Zn. These fragments may thus include the transcripts derived from the alleles of *MTP1* loci *A* and *B* in this *A. halleri* individual. These data confirm that expression of different *AhMTP1* loci is regulated differentially in *A. halleri*.

Discussion

A yeast complementation screen for metal tolerance genes of the metal hyperaccumulator plant *A. halleri* identified the *AhMTP1-3* cDNA (Figures 1 and 2). The encoded protein is homologous to *A. thaliana* ZAT/MTP1 of group III (Blaudez *et al.*, 2003) of the CDF family of metal transport proteins (Figure 2). Expression of *AhMTP1-3*, as well as of *AtMTP1*, complemented the Zn hypersensitivity of the *zrc1 cot1* double mutant of budding yeast. Other group III CDF proteins have previously been reported to complement a *zrc1* single mutant, namely TgMTP1 (Persans *et al.*, 2001) and PtdMTP1 (Blaudez *et al.*, 2003), which are 84 and 73% identical to AhMTP1-3, respectively. In our hands, a cDNA library screen for complementation of the *zrc1* single mutant was not stringent and identified cDNAs encoding Zn metalloproteins (Meyer *et al.*, unpublished data). Expression of the

AtMTP1 cDNA was previously reported to complement the Zn hypersensitivity of the *S. pombe zhf1* mutant (Bloss *et al.*, 2002). Interestingly, *zhf1* cells expressing *AtMTP1* were partially Cd-tolerant when compared with wild-type cells, suggesting a higher degree of specificity of the AtMTP1 protein for Zn over Cd or a different subcellular localization, when compared with SpZhf1p (Clemens *et al.*, 2002a). *In vitro*, the AtMTP1 protein reconstituted into liposomes transported Zn^{2+}, but not Co^{2+} or Cd^{2+} (Bloss *et al.*, 2002). This is in agreement with our findings that in *S. cerevisiae* cells AhMTP1-3 and AtMTP1 confer tolerance to Zn^{2+}, but not to the other metal ions tested.

When cloned identically in precisely the same vector and expressed in *zrc1 cot1* cells, *AhMTP1-3* or other length variants of *AhMTP1* did not confer enhanced Zn tolerance when compared with *AtMTP1*. An HD-repeat motif in the metal hyperaccumulator MTP1 protein corresponds to a $(CATGAT)_{3-8}$ hexanucleotide microsatellite at the nucleotide sequence level (Figure 6) and is not observed in AtMTP1 or AlMTP1 (Figure 2). Direct hexanucleotide repeat motifs are rarely observed within the coding sequence of cDNAs and large trinucleotide expansions are associated with human neurodegenerative diseases (Ross, 2002). A hexanucleotide repeat has been reported in the coding sequence of the tomato *HERO* gene, which confers broad-spectrum resistance against the potato cyst nematode (Ernst *et al.*, 2002). The encoded $(EV)_3E_4(EV)_3$ repeat motif is located in the leucine-rich repeat (LRR) domain of the HERO protein, but not in the LRR domains of closely related proteins encoded by other genes of the same gene cluster.

Zinc is known to be detoxified by storage inside mesophyll vacuoles of *A. halleri* (Küpper *et al.*, 2000). Therefore we hypothesized that AhMTP1-3 is involved in the transport of Zn into the vacuole. Consistent with this hypothesis, a GFP–AhMTP1-3 fusion protein was localized to the vacuolar membrane following transient expression in *A. thaliana* protoplasts (Figure 3). Other group III CDF proteins from *A. thaliana* have been localized to the ER and the plasma membrane using this approach (A.-G. Desbrosses-Fonrouge and U. Krämer, unpublished data). The subcellular localization has not previously been experimentally investigated for AtMTP1 or TgMTP1. A GFP fusion of PtdMTP1, which also belongs to group III of CDF proteins, has been localized to the tonoplast (Blaudez *et al.*, 2003), as well as a fusion to GFP of the group I ShMTP1 Mn transporter (Delhaize *et al.*, 2003).

Compared with the non-tolerant *A. thaliana* or *A. lyrata*, steady-state transcript abundance of *MTP1* was constitutively higher in the shoots of *A. halleri* (Figure 7a). Expression for *AtMTP1* (At2g46800) was quantified in the Massively Parallel Signature Sequencing (MPSS) database at 269 and 144 ppm in the roots and leaves of untreated 21-day-old *A. thaliana* plants, respectively (http://mpss.udel.edu/at/java.html?). These values are comparable with expression levels reported in poplar, in which one EST in 7000 or 8 ESTs in 95,151 were reported to correspond to *PtdMTP1* in two different cDNA libraries, respectively (Blaudez *et al.*, 2003). Thus the expression of *MTP1* or related genes is constitutively high specifically in the shoots of metal tolerant metal hyperaccumulator plants (Assuncao *et al.*, 2001; Persans *et al.*, 2001).

In *A. halleri*, a 2- or 4-day exposure to high Zn concentration specifically induced an increase in root *MTP1* transcript levels. To our knowledge, metal-responsive transcriptional regulation has not been reported for any plant *MTP* gene. Under exposure to low Zn concentrations, however, root *MTP1* transcript levels were as low in *A. halleri* as in *A. thaliana*. Under these conditions, relatively low rates of Zn sequestration in the vacuoles of root cells of *A. halleri* may enable these plants to transport Zn from the roots into shoots, thus permitting metal hyperaccumulation in the shoot. At high Zn supply, enhanced Zn sequestration in the root cells of *A. halleri* may participate in protecting the shoot from accumulating excess Zn.

Previous transgenic approaches designed to increase metal tolerance and shoot accumulation of metals in plants have involved the expression of a metal transporter gene under the control of a strong constitutive promoter (Hirschi *et al.*, 2000; Lee *et al.*, 2003; Song *et al.*, 2003; Van der Zaal *et al.*, 1999). The regulation of *MTP1* expression, as observed in *A. halleri*, could thus serve as a model to improve the performance of transgenic plants in the phytoextraction of metals from contaminated soils.

In the genome of *A. halleri*, several *MTP1* genes are present, whereas *MTP1* is a single gene in the genomes of the closely related species *A. lyrata* and *A. thaliana* (Figure 4). Amplification of a metal tolerance gene in the genome may result in increased gene expression that could in turn enhance metal tolerance. In a copper tolerant ecotype of *Silene vulgaris*, a cluster of tandem copies of metallothionein type 2b (Sv *Mt2b*) genes was proposed to account for the high *MT2b* transcript levels, when compared with a non-tolerant ecotype, according to the total signal intensity on a Southern blot (Van Hoof *et al.*, 2001). Based on the segregation of *MT2b* signals and their expression in crosses between the copper-tolerant and the copper-sensitive ecotype of *S. vulgaris*, the authors excluded that *SvMt2b* is a major determinant in copper tolerance of *S. vulgaris* and proposed that the *Mt2b* allele of the copper-tolerant ecotype may act as a hypostatic enhancer of copper tolerance. Genomic organization, expression and the number of copies of metallothionein genes were not investigated in detail.

There are reports of clustering of multiple gene copies or closely related genes at loci conferring resistance against plant pathogens and parasites (Ernst *et al.*, 2002; Jones *et al.*, 1994; Martin *et al.*, 1993). Single genes within a cluster of closely related genes were finally identified as determinants of resistance, but it is not known whether there was differential expression among the genes in the cluster.

Curiously, the situation in *A. halleri* is somewhat different, because *MTP1* gene copies were found not to be clustered (Tables 1 and 2, Figure S1) and were genetically unlinked instead.

Naturally occurring plant metal tolerance has, in some cases, been explained by additive polygenic models and more often by models invoking a single or a very small number of major genes, the identities of which remain unknown (Macnair, 1993). For example, tolerance of *A. halleri* to Cd was attributed to between two and three major genes (Bert *et al.*, 2003). Based on the occurrence of leaf chlorosis upon growth of the F2 population of a cross between *A. halleri* and *A. lyrata* at 250 μM Zn for 4 days, Zn tolerance was proposed to be a dominant trait involving a single major locus and a small number of hypostatic genes (Macnair *et al.*, 1999). These results have to be seen as preliminary, however, because observed segregation ratios are highly dependent on the definition of tolerance and the types and conditions of tolerance assays.

In the BC1 generation of a cross between *A. halleri* and *A. lyrata*, two out of the three segregating *AhMTP1* loci, namely *A* and *B*, co-segregated with Zn tolerance, whereas the third locus, *C*, did not co-segregate with Zn tolerance (Figure 5). As a genetic map of *A. halleri* is not available, verification of these results using QTL mapping is not possible to date. Moreover, the breeding system of *A. halleri* and *A. lyrata* will complicate the process of fine mapping of QTLs. However, the fact that BC1 individuals harbouring both *AhMTP1* loci *A* and *B* were significantly more tolerant than plants which possessed only one of these two *MTP1* loci (Figure 5) provides circumstantial support for the involvement of *AhMTP1* loci *A* and *B* in Zn tolerance of *A. halleri*. Further support for this model is provided by fragment-length specific expression analysis of selected BC1 individuals, which harbour only a subset of *AhMTP1* loci (Figure 7b). This indicated that *AhMTP1* loci *A* and *B* are expressed at distinctly higher levels than *AlMTP1* or *AhMTP1* locus *C*, which did not co-segregate with Zn tolerance, in BC1 individuals (Figure 7b). Consistent with these results, differential regulation of expression of different *AhMTP1* gene copies was also observed in *A. halleri* (Figure 7c). Our data is thus consistent with the hypothesis that the two unlinked *AhMTP1* loci *A* and *B* identified here account largely for the enhanced expression of *MTP1* and are among the genetic determinants of metal tolerance in *A. halleri*. It is now possible to investigate the molecular basis of the differential regulation of *AhMTP1* genes.

Experimental procedures

Plant material and growth conditions

Seeds of *A. thaliana* (L.) Heynhold (accession Columbia, Col-0) were obtained from Lehle Seeds (Round Rock, TX, USA). Seeds of *A. halleri* ssp. *halleri* (L.) O'Kane & Al-Shehbaz (accessions Langelsheim, Germany and Auby, France), formerly termed *Cardaminopsis halleri* Hayek (L.), were collected at the sites. Seeds of *A. lyrata* ssp. *petraea* (L.) O'Kane & Al-Shehbaz originated from an uncontaminated site in the Czech Republic (Unhost, Central Bohemia; Macnair *et al.*, 1999). Unless indicated otherwise plants were grown in a potting mix consisting of 1:1 (v/v) standard soil (GS90) and vermiculite in a greenhouse (light and humidity conditions change according to the season, the temperature is kept above 5°C during the night in winter). Hydroponic plant culture was performed in a modified 0.25-strength Hoagland solution as described (Becher *et al.*, 2004) in a climate-controlled growth chamber (temperature: 20°C day/16°C night; relative humidity: 60% day/75% night; light: 16 h day/8 h night at 120 μE). *Arabidopsis thaliana*, *A. halleri* and *A. lyrata* plants were supported by floating polystyrene lids. After germination, solutions were exchanged weekly.

Cloning and DNA manipulations

All cloning and DNA manipulations were performed according to standard procedures (Sambrook and Russel, 2001) with chemicals from Roche (Basel, Switzerland) unless stated otherwise. All PCR reactions designated for cloning or sequencing purposes were performed using a proofreading polymerase (Pfu Turbo; Stratagene, La Jolla, CA, USA) and PCR conditions as recommended by the manufacturer. The *AhMTP1-3* cDNA was isolated as follows. A cDNA library, which was constructed in the pYES2 vector (Invitrogen, Carlsbad, CA, USA) from RNA of mixed root and shoot tissues of *A. halleri* plants exposed to different concentrations of Zn and Cd in a hydroponic nutrient solution, was obtained from Amplicon Express (Pullman, WA, USA). The open-reading frame of *AhMTP1-3* was subsequently amplified using the primers 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGAGTCTTCAAGTCACATTG-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAACGCTCGATTTGTATCGTAAC-3′ and cloned into the entry vector pDONR201 (Invitrogen) by *in vitro* site-directed recombination for later recombination into the vector pFL613. The plasmid pFL613 was constructed as follows. A fragment encoding a triple haemagglutinin (HA) tag was amplified from the vector pFA6a-His3MX6-pGAL1-3HA (Longtine *et al.*, 1998), which was kindly provided by Herman Edskes (University of Wageningen, the Netherlands) using the primers 5′-ATGCAAGCTTAGCGCTATGGGTTACCCATACGATGTTCCT-3′ and 5′-CAGTTCTAGACGATATCAGCGTAATCTGGAACGTCATATG-3′. The 129 bp long amplification product was digested with *Hin*dIII and *Xba*I and cloned into pBluescript SK (Stratagene). The resulting plasmid was cut with *Eco*RV, followed by the blunt insertion of the Gateway cassette rfA (Invitrogen). The resulting hypercassette was excised from this plasmid using *Hin*dIII and *Xba*I. After filling in the overhangs with the Klenow fragment of DNA polymerase I, the hypercassette was ligated into pFL61 (Minet *et al.*, 1992), which had been linearized with *Not*I and blunt-ended. For construction of the *AhMTP1-3* D90A mutant cDNA, linear amplification was performed of the *AhMTP1-3* entry clone in a PCR reaction using the mutagenic primers 5′-GCTCATTTGCTCTCTGCCGTTGCTGCCTTTGC-3′ and 5′-GCAAAGGCAGCAACGGCAGAGAGCAAATGAGC-3′ (10 cycles of 95°C for 30 sec, 55°C for 60 sec, 68°C for 12 min), followed by digestion of the parent DNA with *Dpn*I, which digests methylated and hemimethylated DNA, and subsequent transformation of *E. coli* for repair of the doubly nicked plasmid.

The open-reading frame of *AtMTP1* was amplified by PCR from the intron-free coding sequence of the gene using genomic DNA from *A. thaliana* as a template and the primers 5′-ATGGAGTCTTCAAGTCCCCAC-3′ and 5′-TTAGCGCTCGATTTGTATCGTG-3′. The PCR product was cloned into the vector pCRII-TOPO

(Invitrogen) according to the manufacturer's instructions. *AtMTP1* was then excised with *Eco*RI and subcloned into pYES2. The open-reading frame of the *ScZRC1* gene was amplified by colony-PCR from *S. cerevisiae* using the primers 5′-GTCATGATCACCGGTAA-AGAA-3′ and 5′-ATTACAGGCAATTGGAAGTATTG-3′. The product was cloned into pCRII-TOPO, excised with *Eco*RI and *Xho*I and thus subcloned into pYES2. All constructs were verified by sequencing.

For isolation of the coding sequences of the *AlMTP1* cDNA, RNA extracted from the shoot of *A. lyrata* was reverse transcribed using the SuperScript™ First Strand Synthesis System (Invitrogen). First strand cDNA products were amplified using primers based on the open-reading frame of the *A. thaliana MTP1* sequence (see above). PCR products were cloned into the pCR2.1-TOPO cloning vector (Invitrogen) and sequenced.

Fragments encompassing the polymorphic region, which corresponds to the cytoplasmic loop between membrane spanning domains IV and V of MTP1 proteins, were amplified from genomic DNA of *A. thaliana*, *A. halleri* and *A. lyrata* individuals using primers *loop-1* 5′-CTAGTGGTGAACATCATAATGGC-3′ and *loop-2* 5′-CTGCGACTTGAGTCTTCG-3′ in the following PCR programme: 2 min at 92°C, followed by 30 cycles of 30 sec at 92°C, 30 sec at 58°C, 40 sec at 72°C and a final extension step of 5 min at 72°C. Prior to the PCR reaction, the forward primer was radio-labelled at the 5′ end as follows: 200 ng of the *loop-1* primer were mixed with 40 μCi of [γ^{33}P]-ATP (Hartmann; Analytics, Braunschweig, Germany) and 4 U of T4 polynucleotide kinase (Roche, Mannheim, Germany) in a 20-μl reaction volume. The reaction was kept at 37°C for 60 min. After the PCR reaction, PCR products were separated on a denaturing 6% (v/v) polyacrylamide gel and visualized by autoradiography. Single fragments were excised from the gel, eluted, PCR-amplified as described above and sequenced directly using the primers as described for the initial amplification.

Yeast experiments

A *S. cerevisiae zrc1 cot1* mutant (*Mat a*, *zrc1::natMX3*, *cot1::*kan-MX4, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*) and its parental wild-type strain BY4741 (*Mat a*, *his3Δ1*,*leu2Δ0*, *met15Δ0*, *ura3Δ0*) were transformed with pYES2, pFL61 or pYES2 or pFL613 containing *AtMTP1*, *AhMTP1-3* or *AhMTP1*-3(D90A) as indicated using the polyethylene glycol method (Brachmann *et al.*, 1998; Dohmen *et al.*, 1991; Goldstein and McCusker, 1999). Transformants were selected, maintained and experimented with on-media lacking uracil (Sherman *et al.*, 1986), and with D-glucose or D-galactose as a carbon source for experiments with pFL61 and pYES2, respectively. For complementation assays, transformed yeast strains were grown overnight in 5 ml liquid minimal medium (SC) to early stationary phase (OD_{600} approximately 1.0). Yeast cells from overnight cultures were washed three times with ultrapure H_2O and then used to inoculate experimental media at a starting OD_{600} of 0.003. Complementation experiments were carried out in a total volume of 4 ml of a modified low sulphate/phosphate (LSP) medium (Conklin *et al.*, 1992), which contained 80 mM NH_4Cl, 0.5 mM KH_2PO_4, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 2 mM NaCl, 10 mM KCl, trace elements, vitamins and supplements as in SC, 2% (w/v) carbon source. LSP without added Zn was supplemented with various concentrations of $ZnSO_4$ (1.4 μM for controls and between 50 μM and 2.5 mM for experimental treatments). All yeast cells were grown at 30°C, with continuous mixing of liquid cultures in a culture wheel.

For Western blotting, protein extracts were prepared from cells grown in LSP-URA as described (Adams *et al.*, 1997), separated on a denaturing 10% (v/v) polyacrylamide gel (Lämmli, 1970) and blotted on a PVDF membrane (Immobilon™-P; Millipore, Bedford, MA, USA) as described (Towbin *et al.*, 1979). Detection was carried out using a monoclonal anti-HA antibody (Covance Research Products, Santa Clara, CA, USA) and chemiluminescent detection according to the manufacturer's instructions (ECL kit; Amersham Biosciences, Little Chalfont, UK).

Homologous recombination was carried out using a yeast transformation protocol for gap repair (Elble, 1992). The double mutant strain described above was transformed adding the linearized, gapped plasmid (pFL613 containing *AhMTP1-3*) and gel-purified insert DNA fragments in equal amounts. The plasmid was linearized using *Nhe*I and *Mlu*NI (Roche) as restriction enzymes. Insert fragments of 434 and 458 bp were amplified from *AhMTP1* genes cloned into the pCR2.1-TOPO vector (Invitrogen) using forward primer 5′-CCGATGCAGCTCATTTGCTCTCTG-3′ and reverse primer 5′-TGTCACTGAGCAACTGTTGGAC-3′ in a standard protocol for colony PCR (von Stein *et al.*, 1997). Plasmid DNA was isolated from recombinant colonies, amplified in *E. coli* and sequenced.

Transient expression of chimeric GFP fusion proteins and confocal microscopy

To construct a cDNA encoding a chimeric fusion protein of an N-terminal RSsm-GFP (Davis and Vierstra, 1998; Haseloff *et al.*, 1997) and AhMTP1-3, the *AhMTP1-3* cDNA was subcloned by *in vitro* site-directed recombination (Invitrogen) into the plant transient expression vector pA7-p35S-N-GFP (Katrin Czempinsky, University of Potsdam, Germany) adapted for Gateway cloning (Ben Trevaskis, CSIRO Plant Industry, Canberra, Australia). For protoplast transformation, cell walls of an *A. thaliana* Col-0 suspension culture (Sébastien Thomine; CNRS, Gîf-sur-Yvette, France) were digested in Gamborg's B5 medium supplemented with 0.17 M glucose, 0.17 M mannitol, 1% cellulase 'Onozuka RS' and 0.2% macerozyme R-10 (Duchefa Biochemie BV, Haarlem, the Netherlands). The protoplasts were purified by flotation in Gamborg's B5 medium supplemented with 0.28 M sucrose. For transformation, 0.2×10^6 cells were mixed with 5 μg of plasmid DNA in a solution containing 25% polyethylene glycol (PEG) 6000 (Merck KGaA, Darmstadt, Germany), 0.45 M mannitol, 0.1 M $Ca(NO_3)_2$ (pH 9) and incubated in the dark for 20 min. Subsequently, protoplasts were washed twice with 0.275 M $Ca(NO_3)_2$, and then resuspended in Gamborg's B5 medium supplemented with 0.17 M glucose and 0.17 M mannitol, in which they were maintained until microscopic observation between 24 and 48 h after transformation. Fluorescence was imaged by confocal microscopy (Leica TCS SP2; Leica Microsystem, Wetzlar, Germany) with excitation at 488 nm, and the GFP fluorescence emission signals were detected between 505 and 520 nm.

DNA gel blots and generation and phenotyping of crosses

Genomic DNA of single plants was isolated as described (Dellaporta, 1983) and modified (Saumitou-Laprade *et al.*, 1999). Ten micrograms of genomic DNA was digested with 40 U *Eco*RI or *Nco*I (Roche), respectively, overnight at 37°C and separated on a 1% TAE-agarose gel, and the gel and blot processed according to standard procedures (Sambrook and Russel, 2001). The DNA was transferred onto an uncharged nylon membrane (Hybond N; Amersham Biosciences, Piscataway, NJ, USA) and cross-linked with UV light at 120 mJ (UV Stratalinker 1800; Stratagene). The hybridization buffer was composed as follows: 0.25 M sodium phosphate buffer (pH 7.2), 1 mM NaEDTA, 6.7% (w/v) SDS, 1% (w/v) BSA. The radio-labelled probes were generated by random priming using α^{32}-PdCTP (Hartmann Analytics, Braunschweig, Germany) and the open-reading frame of the *AtMTP1* cDNA according to the manufacturer's

instructions (ReadyPrime™ labelling kit; Amersham Biosciences). Blots were washed at high stringency (Sambrook and Russel, 2001). X-ray films were exposed for 1–5 days. The generation and phenotyping of crosses between *A. halleri* ssp. *halleri* (ecotype Auby) and *A. lyrata* ssp. *petraea* were as described (Bert *et al.*, 2003). The BC1 population was established by crossing *A. halleri* (P1, grandfather, accession Auby) with *A. lyrata* (individual 1, P2, grandmother). The reciprocal cross did not yield any progeny. Of the resulting F1 population, one individual (P3, father) was backcrossed to an *A. lyrata* individual distinct from P2 (individual 2, P4, mother) to obtain the BC1 population. Preliminary mapping of a limited number of markers demonstrated the occurrence of recombination between homoeologous chromosomes of *A. lyrata* and *A. halleri* (Saumitou-Laprade *et al.*, unpublished data).

RNA gel blots

Approximately 25 plants were grown hydroponically in 3 l nutrient solution in each culture vessel. After 6 weeks of growth, the hydroponic solutions were supplemented with different concentrations of $ZnSO_4$ (controls no additions, 100 and 300 μM $ZnSO_4$) for 4 days. Roots and leaves were harvested separately. All plant tissues used for RNA extractions were frozen immediately in liquid nitrogen and subsequently stored at −80°C. Total RNA was isolated using Trizol reagent (Invitrogen) according to the instructions of the manufacturer. Five micrograms of total RNA were loaded on a formaldehyde/MOPS 1% (w/v) agarose formaldehyde gel following a standard protocol (Sambrook and Russel, 2001). Transfer of nucleic acids, labelling of the probe, hybridization and washing procedures were performed as described above. Two independent replicate experiments were performed.

Semi-quantitative RT-PCR

For semi-quantitative RT-PCR experiments *A. halleri* plants (accession Auby) were propagated vegetatively. Four cuttings, each of 6 cm in length, were made from the apices of shoot branches of one plant individual growing on soil in the greenhouse. The clones were allowed to root in 400 mL of a hydroponic solution for 6–7 weeks. For Zn exposure the hydroponic solution of was supplemented with 1.0 μM $ZnSO_4$ (controls) or 300 μM $ZnSO_4$ for 2 days with a daily exchange of solutions. Roots and shoots of two clones per treatment were harvested separately. Total RNA was extracted (RNeasy kit; Qiagen, Hilden, Germany) and genomic DNA was removed using DNase 1 (Qiagen). Total RNA was quantified spectrophotometrically. Equal amounts (1 μg of total RNA) were used as a template for first strand cDNA synthesis, using a SuperScript™ First Strand cDNA Synthesis kit (Invitrogen) and random decamer oligonucleotide primers supplied in the Ambion RETROscript™ kit (Ambion Europe Ltd, Huntingdon, UK) according to the instructions from the manufacturer. Three microlitres of a 1 : 10 dilution of first strand cDNA were then used for PCR in a 10 μl reaction volume. The PCR primers *loop-1* and *loop-2* (see above) were used to universally amplify fragments of the polymorphic region of *MTP1* cDNAs of between 205 and 241 bp in length. The amplification reaction was performed using the following PCR programme: 2 min at 92°C, followed by cycles of 30 sec at 92°C, 30 sec at 58°C, 40 sec at 72°C and a final extension step of 5 min at 72°C. All amplification reactions were carried out on a PTC-200 Thermal Cycler (MJ Research, Inc., Watertown, MA, USA) using *Taq* DNA polymerase (Invitrogen). The reaction mix was composed of buffer as recommended by the manufacturer, 0.2 mM of each dNTP (Invitrogen), 0.2 μM of each forward and reverse primers and 2.5 U of *Taq* polymerase. To verify the absence of contaminating genomic DNA, PCR reactions were performed using primers that span one intron of the *AtAKT1* gene (forward primer 5′-ATCGGATAACAATGGCAGAACAC-3′, reverse primer 5′-TAGTGAACCCAACATCCGTCCTGC-3′) for 30 cycles as described above using an annealing temperature of 60°C and an extension time of 1 min (data not shown). For the *AhMTP1* fragments, the optimum cycle number within the centre of the linear phase of amplification was determined by resolving PCR products on a 2% agarose gel after 20, 22, 24 and 26 amplification cycles. Subsequently, the PCR was repeated and run for 22 cycles unless indicated otherwise, with a radiolabelled forward primer (see above) and an unlabelled reverse primer. The cDNA derived from 18S rRNA was amplified with Ambion's Universal 18S Quantum-RNA primers and competimers at a ratio of 3 (primers) : 7 (competimers) as a constitutive control. PCR products were resolved and visualized as described above. For RT-PCR analysis of BC1 genotypes, leaf tissues were harvested from individuals growing on soil and processed as described above, except that cDNA synthesis was primed using oligo-dT (Ambion).

Signal intensities were quantified densitometrically using ImageJ software (ImageJ version 1.29; http://rsb.info.nih.gov/ij/; National Institute of Health, Bethesda, MD, USA). As a control for equal efficiency of amplification of fragments of different sizes within one amplification reaction, fragments of each size were amplified separately after excision from a polyacrylamide gel, checked for purity, quantified, mixed in equal proportions and amplified alongside cDNAs.

Sequence and statistical analysis

Sequence alignments were performed using the program Vector NTI 8 (Informax, Inc., Bethesda, MD, USA) and manually edited where necessary. Transmembrane topology was predicted using the program TMpred (Hofmann and Stoffel, 1993) at http://www.ch.embnet.org/software/TMPRED_form.html. Side-by-side boxplots and pair-wise comparison of means were carried out using SYSTAT version 10.2 (Systat Software UK Limited, London, UK).

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2143/TPJ2143sm.htm

Figure S1. RFLP patterns observed for *MTP1* genes in the parents and in the back-cross 1 (BC1) population.

Figure S2. All *AhMTP1* fragments are amplified with equivalent efficiencies in a PCR reaction.

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