The MTP1 promoters from Arabidopsis halleri reveal cis-regulating elements for the evolution of metal tolerance

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Summary

• In the hyperaccumulator Arabidopsis halleri, the zinc (Zn) vacuolar transporter MTP1 is a key component of hypertolerance. Because protein sequences and functions are highly conserved between A. halleri and Arabidopsis thaliana, Zn tolerance in A. halleri may reflect the constitutively higher MTP1 expression compared with A. thaliana, based on copy number expansion and different cis regulation.
• Three MTP1 promoters were characterized in A. halleri ecotype I16. The comparison with the A. thaliana MTP1 promoter revealed different expression profiles correlated with specific cis-acting regulatory elements.
• The MTP1 3’ untranslated region, highly conserved among A. thaliana, Arabidopsis lyrata and A. halleri, contains a dimer of MYB-binding motifs in the A. halleri promoters absent in the A. thaliana and A. lyrata sequences. Site-directed mutagenesis of these motifs revealed their role for expression in trichomes. A. thaliana mtp1 transgenic lines expressing AtMTP1 controlled by the native A. halleri promoter were more Zn-tolerant than lines carrying mutations on MYB-binding motifs. Differences in Zn tolerance were associated with different distribution of Zn among plant organs and in trichomes.
• The different cis-acting elements in the MTP1 promoters of A. halleri, particularly the MYB-binding sites, are probably involved in the evolution of Zn tolerance.

Introduction

Plants have evolved different adaptive strategies to extreme environmental conditions. Metal hypertolerance and hyperaccumulation (i.e. the ability to tolerate exceptionally high concentrations of metals in soil and to accumulate them in the above-ground tissues, respectively; Baker, 2002; Krämer, 2010), are particularly interesting because understanding the combination of underlying mechanisms provides useful insights into the metal homeostasis network and the evolution of adaptive traits. Furthermore, such knowledge could be used to develop phytoremediation and biofortification technologies (Verbruggen et al., 2013). Recent efforts to understand the genetic mechanisms of metal hyperaccumulation have focused mostly on the Brassicaceae family, in which the trait has evolved independently several times (Krämer, 2010). The zinc (Zn) and cadmium (Cd) hyperaccumulators Arabidopsis halleri and Noccaea caerulescens have attracted the most interest as a result of their close phylogenetic relationship with Arabidopsis thaliana, which provides a deeper insight into the molecular basis of hyperaccumulation.

Although it is evident that hyperaccumulation requires tolerance to high concentrations of metals in leaves, the two characters are mostly genetically independent, as indicated by the quantitative trait locus (QTL) analysis on F2 populations derived from interspecific crosses of A. halleri and the closely related Arabidopsis lyrata ssp. petraea, which is both nontolerant and a nonaccumulator (Macnair et al., 1999; Bert et al., 2003). Indeed, Zn tolerance has been found as associated with three major QTLs in a metallicolous population of A. halleri: the most promising candidates resulting from this analysis were the loci coding for the metal transporters AhHMA4, AhMTP1-A1/A2 and AhMTP1-B (Willems et al., 2007). The QTL containing AhHMA4, partially accounting for Zn tolerance, overlaps with QTLs responsible for Zn accumulation in both low and high Zn pollution levels (Frérot et al., 2010), as well as for Cd tolerance and accumulation (Courbot et al., 2007; Willems et al., 2010), suggesting a partial pleiotropic control of these traits (Verbruggen et al., 2009).

At the molecular level, both hypertolerance and hyperaccumulation reflect the up-regulation of several classes of metal transporters, chelators and proteins involved in stress responses. Genes into these categories are expressed at higher levels in hyperaccumulator species than in corresponding nonmetallophyte species, as highlighted by both transcriptomic analysis (Becher et al., 2004; Weber et al., 2004; Hammond et al., 2006; van de Mortel et al., 2013). The zinc (Zn) and cadmium (Cd) hyperaccumulators Arabidopsis halleri and Noccaea caerulescens have attracted the most interest as a result of their close phylogenetic relationship with Arabidopsis thaliana, which provides a deeper insight into the molecular basis of hyperaccumulation.
corresponding to four different loci were identified, three of which could not be assigned to MTP1 promoter A, B and C copies identified in the Auby population: the three novel sequences, pAhMTP1-\(\alpha\), pAhMTP1-\(\beta\), and pAhMTP1-\(\gamma\), share high homology in the first 800 bp upstream of the start codon with the promoter sequences of the Auby population and diverge in the upstream region, whereas pAhMTP1-D is conserved in both populations (and therefore the same nomenclature has been maintained).

Most importantly, the comparison of these promoter sequences with their A. thaliana and A. lyrata counterparts revealed MYB-binding sites that are present in A. halleri but not A. thaliana or A. lyrata. These MYB sites are required for MTP1 expression in the trichomes and support the hypotolerance phenotype of A. halleri.

Materials and Methods

Plant material and growth conditions

The following Brassicaceae species were used for promoter analysis: Arabidopsis thaliana (L.) O’Kane & Al-Shehbaz population I16 (Val del Riso, northern Italy, 45°5’34.40 N 9°52’34.94 E; Meyer et al., 2015); Noccaea praecox (Wuljen) F.K.Mey., Cardamine resedifolia L., Thlaspi arvense L., Cachlearia officinalis L., Sisymbrium officinale (L.) Scop. and Descurainia sophia (L.) Webb ex Prantl (wild populations from Trentino, northern Italy); and Noccaea caerulescens (J.Presl & C.Presl) F.K.Mey. ecotype Ganges (St Laurent le Minier, southern France; Lombi et al., 2002). Promoter sequences from Arabidopsis thaliana (L.) Heynh. accession Columbia (Col-0) and Arabidopsis lyrata (L.) O’Kane & Al-Shehbaz were obtained from the Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org; Lamesch et al., 2012). Arabidopsis thaliana Col-0 and mtp1 mutant were used for the stable transformation (see Supporting Information Methods S1); the mtp1 homozygous mutant (SALK_204398C) was obtained from NASC (http://arabidopsis.info; Scholl et al., 2000).

Plants were cultivated in soil in the glasshouse, with a 16 h photoperiod at 23°C (standard growth conditions). For in vitro cultivation, seeds were sterilized with 70% ethanol for 1 min, and then with 10% sodium hypochlorite containing 0.03% Triton X-100 for 15 min, before being rinsed three times with sterile water. Sterile seeds were sown on solid MS medium (Murashige & Skoog, 1962) supplemented with 30 g l\(^{-1}\) sucrose and vernalized for 2 d at 4°C. Plants were maintained in vitro with a 16 h photoperiod at 23°C.

Promoter isolation and sequencing

Genomic DNA was extracted from the species listed earlier using the Qiagen Genomic DNA Extraction Kit. The promoter fragments were obtained by using the GenomeWalker Universal Kit (Clontech Laboratories Inc., Mountain View, CA, USA) according to the manufacturer’s instructions, combining the MTP1 coding sequence-specific primer MTP1_1 and its nested primer MTP1_2 (Table 1). Taq DNA polymerase was used to add
adenylate tails to the amplified promoter fragments for cloning in the pGEM-T vector (Promega). The positive clones were purified and sequenced with a 96-capillary 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Promoter analysis was performed as described in Methods S2.

Expression analysis of AhMTP1-a/b/c and D in A. halleri
Total RNA was extracted with TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA); three pools from leaves of 3-wk-old plants grown in control conditions were used as biological replicates. After DNase treatment, first-strand cDNA was synthesized using the Superscript III Reverse Transcriptase Kit (Thermo Fisher Scientific). Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed with a StepOnePlus Real-Time PCR System (Applied Biosystems) using KAPA SYBR FAST ABI Prism 2X qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA). Each reaction (40 amplification cycles) was carried out in triplicate and melting curve analysis was used to confirm the amplification of specific targets. Primers

### Table 1 Primer sequences

<table>
<thead>
<tr>
<th>Target sequence</th>
<th>Primer sequence</th>
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<tr>
<td>MTP1 promoter cloning by Genome Walking</td>
<td>MTP1_1: CAACGAAATGAGCTGGATAGACACAAG&lt;br&gt;MTP1_2: GATAGCAAAGGCAGCAACGTCAGAGA&lt;br&gt;Fw: GGTACCATGGTCCGTCCTGTAGAAACC&lt;br&gt;Rev: CTCGACATTATTTGTTGGCCTCCTGCTG&lt;br&gt;</td>
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<tr>
<td>GUS</td>
<td>GUS Fw: GGTACCATGGTCCGTCCTGTAGAAACC&lt;br&gt;Rev: CTCGACATTATTTGTTGGCCTCCTGCTG</td>
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<td>CDS of Arabidopsis thaliana MTP1 - AtMTP1 (At2g46800)</td>
<td>AtMTP1 Fw: GGTACCATGGAGTCTTCAAGTCCCCA&lt;br&gt;Rev: CTCGACATTATTTGTTGGCCTCCTGCTG&lt;br&gt;</td>
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<td>A. thaliana MTP1 promoter pAtMTP1</td>
<td>pAtMTP1 Fw: CCCGGGTGTTCGAAGTTTGGAAAGT&lt;br&gt;Rev: GGTACCCTGCATAAGAAAAAAAATAGAAGA&lt;br&gt;</td>
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<td>Arabidopsis halleri MTP1 promoters pAhMTP1-A1 and -A2</td>
<td>pAhMTP1-A1 Fw: ATAAGTTCAACATGTTTTCACGTA&lt;br&gt;Rev: CTGCATAGAAAAAGAAGAAAGTTA&lt;br&gt;</td>
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<td>A. halleri MTP1 promoter pAhMTP1-B</td>
<td>pAhMTP1-B Fw: TAATGTTTTAATGTTTTGACCAATG&lt;br&gt;Rev: CTGCATAGAAAAAGAAGAAAGTTA&lt;br&gt;</td>
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<tr>
<td>A. halleri MTP1 promoters pAhMTP1-C and pAhMTP1-D</td>
<td>pAhMTP1-C Fw: GATGGTGTAGTTTTGCCCGC&lt;br&gt;Rev: CTGCATAGAAAAAGAAGAAAGTTA&lt;br&gt;</td>
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<td>A. halleri MTP1 entire promoters pAhMTP1-a, -b and -c</td>
<td>pAhMTP1-a, -b and -c Fw: ATAAGTTCAACATGTTTTCACGTA&lt;br&gt;Rev: CTGCATAGAAAAAGAAGAAAGTTA&lt;br&gt;</td>
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<td>A. halleri MTP1 truncated promoter Δ810</td>
<td>pAhMTP1-Δ810 Fw: CCTCATGTTCCTCATCATTCC&lt;br&gt;Rev: CTGCATAGAAAAAGAAGAAAGTTA&lt;br&gt;</td>
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<tr>
<td>A. halleri MTP1 truncated promoter Δ362</td>
<td>pAhMTP1-Δ362 Fw: GCCCTCATTGTAACTAGTCGT&lt;br&gt;Rev: CTGCATAGAAAAAGAAGAAAGTTA&lt;br&gt;</td>
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<td>Mutagenesis of –125 bp position in A. halleri MTP1 promoter</td>
<td>Mutagenesis of –125 bp position in A. halleri MTP1 promoter Fw: GGGCCAGCCCCAGGGTC&lt;br&gt;Rev: AAGTGTGAAGAATCTACAAACC&lt;br&gt;</td>
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<td>Mutagenesis of –155 bp position in A. halleri MTP1 promoter</td>
<td>Mutagenesis of –155 bp position in A. halleri MTP1 promoter Fw: GGGCCAGCCCCAGGGTA&lt;br&gt;Rev: AAGTGTGAAGAATCTACAAACC&lt;br&gt;</td>
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<td>Mutagenesis of –303 bp position in A. halleri MTP1 promoter</td>
<td>Mutagenesis of –303 bp position in A. halleri MTP1 promoter Fw: ATACATGTCTTTGTCCTTTGAG&lt;br&gt;Rev: CAGAACTCGAAATCAACAAACG&lt;br&gt;</td>
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<td>Mutagenesis of –400 bp position in A. halleri MTP1 promoter</td>
<td>Mutagenesis of –400 bp position in A. halleri MTP1 promoter Fw: CTCGCTTAGGGGCCCAGC&lt;br&gt;Rev: CTAAAATATCGATTCAAGTTTG&lt;br&gt;</td>
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<td>AhMTP1-a for real-time RT-PCR on A. halleri MTP1 5'UTR</td>
<td>AhMTP1-a for real-time RT-PCR on A. halleri MTP1 5'UTR Fw: GGGCCAGCCCCAGGGTC&lt;br&gt;Rev: AAGTGTGAAGAATCTACAAACC&lt;br&gt;</td>
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<td>AhMTP1-b for real-time RT-PCR on A. halleri MTP1 5'UTR</td>
<td>AhMTP1-b for real-time RT-PCR on A. halleri MTP1 5'UTR Fw: GGGCCAGCCCCAGGGTA&lt;br&gt;Rev: AAGTGTGAAGAATCTACAAACC&lt;br&gt;</td>
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<td>AhMTP1-c for real-time RT-PCR on A. halleri MTP1 5'UTR</td>
<td>AhMTP1-c for real-time RT-PCR on A. halleri MTP1 5'UTR Fw: ATACATGTCTTTGTCCTTTGAG&lt;br&gt;Rev: CAGAACTCGAAATCAACAAACG&lt;br&gt;</td>
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<td>AhMTP1-D for real-time RT-PCR on A. halleri MTP1 5'UTR</td>
<td>AhMTP1-D for real-time RT-PCR on A. halleri MTP1 5'UTR Fw: CTCGCTTAGGGGCCCAGC&lt;br&gt;Rev: CTAAAATATCGATTCAAGTTTG&lt;br&gt;</td>
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<tr>
<td>GUS for real-time RT-PCR</td>
<td>GUS for real-time RT-PCR Fw: TACACCGACATGTGGAGTGA&lt;br&gt;Rev: CCATACCTGTTCCAGCACAG&lt;br&gt;</td>
</tr>
<tr>
<td>MTP1 for real-time RT-PCR</td>
<td>MTP1 for real-time RT-PCR Fw: GGAGAGTACACCCAGAGAGAT&lt;br&gt;Rev: GCCAAATGTAAGCTCATTAGA&lt;br&gt;</td>
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<tr>
<td>β-actin (At5g09810)</td>
<td>β-actin (At5g09810) Fw: GAGAAGAGAGAGAGAGAGAGAGATG&lt;br&gt;Rev: AGGAGGAGAGAGAGAGAGAGAGATG&lt;br&gt;</td>
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<tr>
<td>Ubiquitin 10 (At4g05320)</td>
<td>Ubiquitin 10 (At4g05320) Fw: GAGAAGAGAGAGAGAGAGAGAGATG&lt;br&gt;Rev: AGGAGGAGAGAGAGAGAGAGAGATG&lt;br&gt;</td>
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The underlined nucleotides indicate the restriction sites introduced by PCR. The underlined italics nucleotides indicate the mutagenized sequences. RT-PCR, reverse transcription polymerase chain reaction.
for this analysis were designed on discriminating sequences of the 5’ untranslated region (UTR) and are reported in Table 1. Data were normalized using the two endogenous reference genes β-actin (At5g09810) and ubiquitin 10 (At4g05320) and analyzed using the 2−ΔΔCT method (Livak & Schmittgen, 2001). The amplification efficiency of each primer pair (c. 2) was calculated using LinREGPCR v.7.5 software (Ramakers et al., 2003).

Cloning and DNA manipulation

MTP1 promoter sequences were amplified using genomic DNA templates from A. thaliana (for pAtMTP1) and A. halleri population I16 (for pAhMTP1-α/β/γ and truncated forms Δ810 and Δ362). A PCR approach was followed in order to verify the existence in the I16 population of the AhMTP1-A1/A2/B/C/D promoters, found in the A. halleri collected in Auby (Shahzad et al., 2010). These reactions were performed with different primers designed on the available sequences corresponding to the four different copies present in Auby genotype (Table 1); different PCR conditions (both magnesium (Mg) stringency and annealing temperatures) were tested.

Platinum PfX DNA polymerase (Thermo Fisher Scientific) was used according to the manufacturer’s instructions with the primers listed in Table 1. For the generation of the mutant303/400, mut155 and mut125/155 sequences, site-directed mutagenesis was performed on the pGEM-pAhMTP1-γ plasmid using PfX polymerase (Promega) and the mutagenic primers listed in Table 1 (19 cycles of 95°C for 30 s, 55°C for 1 min and 68°C for 9 min). The parental DNA was digested with DpnI (Promega) and the mutagenesis product was used to transform Escherichia coli strain DH5α.

The β-glucuronidase (GUS) gene was amplified from the vector pENTR-GUS (Thermo Fisher Scientific). The AtMTP1 coding sequence was amplified from A. thaliana genomic DNA using the primers listed in Table 1 and cloned in the pGEM-T easy vector (Promega). The sequences pAtMTP1, pAhMTP1-α/β/γ, Δ810, Δ362, mut303/400, mut155 and mut125/155 were digested using the enzymes SphI and KpnI and fused to the GUS gene. The sequences pAhMTP1-α/γ and mut125/155 were also fused to the AtMTP1 coding sequence. All cassettes were cloned in vector pMD1 (Das et al., 2001) which was deprived of the CaMV 35S promoter by cutting with HindIII and XhoI.

Analysis of GUS expression

For the histochemical GUS assay, plant tissues (six 10-d-old plantlets and leaves of 3-wk-old plants for three lines for each construct) were incubated overnight at 37°C in GUS reaction buffer (100 mM sodium phosphate, pH 7.0, 1 mM EDTA, pH 8.0, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 1% Triton X-100, 500 mg L−1 5-bromo-4-chloro-3-indolyl β-d-glucuronide in dimethylformamide). Chlorophylls were removed by incubating the tissues in 70% ethanol at 70°C. Plants were examined using a Zoom stereomicroscope AZ100 (Nikon, Tokyo, Japan) and a Leica DM RB microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Fluorimetric GUS assays were carried on three pools of leaves collected from five plants for each transgenic line (three lines were considered for each constructs described). Plants were 3-wk-old and grown under standard growth conditions. GUS activity was detected as described by Cervera (2004). Fluorimetric measurements were performed using a Jasco FP-8200 spectrophotometer (Jasco Inc., Easton, MD, USA) at an excitation wavelength of 365 nm and an emission wavelength of 455 nm.

Analysis of Zn tolerance

Tolerance to Zn was tested in vitro on A. thaliana plants transformed with the empty pMD1 vector (control) and mp1 mutant plants, as references, and on three homozygous single-copy lines for both the pAhMTP1-γ::AtMTP1 and mut125/155::AtMTP1 constructs; each line displayed similar expression levels in real-time RT-PCR analysis (Methods S3). Eighteen plants representing each line were considered under each experimental condition. One-week-old plantlets were grown for 8 d in Gelrite-solidified MS medium containing different ZnSO4 concentrations: 30 μM (ZnSO4 concentration in standard MS medium), 300 and 500 μM. Zn tolerance was evaluated in terms of root length, biomass and Chl content. Chlorophylls were extracted in buffered 80% aqueous acetone and the total Chl content was measured as described by Porra et al. (1989).

Analysis of Zn accumulation

mp1 plants transformed with the pAhMTP1-γ::AtMTP1 and the mut125/155::AtMTP1 constructs, as well as control plants, were germinated in solid MS medium and then transferred in hydroponic culture in Hoagland solution (Hoagland & Arnon, 1950). Three-week-old plants were grown in 0.7 and 25 μM ZnSO4 for 7 d, where 0.7 μM is the ZnSO4 concentration in standard Hoagland solution. Leaves and roots were oven-dried separately at 60°C and subjected to microwave-assisted acid digestion (EPA 3052, 1996). The Zn content was determined by inductively coupled plasma MS (EPA 3051A 2007 and EPA 6010C 2007).

Environmental scanning electron microscopy coupled with energy-dispersive X-ray spectroscopy (ESEM-EDS)

Leaves from 3-wk-old plants grown in 25 μM ZnSO4 for 7 d were analyzed for Zn accumulation in single trichomes. Three biological replicates were analyzed for each A. thaliana line, two leaves for each line and 10 trichomes for each leaf. All experiments were conducted using an ESEM instrument Quanta 250 FEG (FEI, Hillsboro, OR, USA) equipped with an energy-dispersive spectrometer (EDS) for X-ray microanalysis (Bruker Nano GmbH, Berlin, Germany), operating in wet mode. The EDS has a QUANTAX XFlash 6 | 30 detector with energy resolution ≤126 eV full width at half maximum at MnKα. For further details on the analysis, see Methods S4.
Results

Amplification of MTP1 promoter sequences from A. halleri I16

An MTP1 promoter sequence (1194 bp) was isolated by genome walking from A. halleri population I16 and was used to design specific primers for the amplification of further MTP1 promoter sequences using the I16 genomic DNA as a template. PCR amplification yielded three amplicons (1904, 1698 and 1194 bp) representing different promoter copies designated as α, β and γ, respectively. The γ variant corresponded to the sequence identified by genome walking.

The aligned α, β and γ sequences shared high levels of identity, particularly in the distal and proximal regions (96.8% and 90.2%, respectively). Sequence divergence in the proximal region primarily reflected the presence of a 63 bp insertion in sequences α and β (light blue box in Fig. 1a; Fig. S1). The comparison of the α, β and γ sequences with the five paralogs in the Auby population and the MTP1 promoters from A. thaliana and A. lyrata revealed high conservation in the proximal 800 bp (Fig. 1a). The pAhMTP1-γ and pAhMTP1-A1/A2/B/C/D sequences shared >93% nucleotide identity (Table S1), suggesting that the γ promoter is probably the nearest to the parental sequence from which sequences α and β evolved in the I16 population, as indicated by the phylogenetic tree (Fig. 1b).

Primers based on the promoters of the AbMTP1-A1/A2/B/C/D loci of the Auby population were used to amplify A. halleri I16 genomic DNA to determine whether these sequences are also present in the I16 genome. A PCR product was obtained for the D copy and the sequence was included in the alignment in Fig. S1. No amplification resulted for pAhMTP1-A/B/C.

Real-time RT-PCR confirms that AbMTP1-α/β/γ/D are expressed in A. halleri I16, with AbMTP1-γ and AbMTP1-D having the lowest and highest expression levels, respectively (Fig. S2).

Expression profiles conferred by the A. thaliana and A. halleri MTP1 promoters

The spatial MTP1 expression profiles conferred by the α, β and γ promoter sequences identified in A. halleri I16 and a 2000 bp control sequence amplified from the A. thaliana MTP1 promoter (pAtMTP1) were determined by GUS assay. Under standard growth conditions, GUS expression driven by the A. thaliana promoter was weak and localized in the roots, hydathodes, leaf vascular system, guard cells and inflorescence, as previously reported (Desbrosses-Fonrouge et al., 2005; Kawachi et al., 2009). The pAhMTP1-α/β/γ promoters conferred identical GUS expression profiles, with intense GUS staining in the shoots and roots, also observed in floral organs, siliques and in the trichomes (Fig. 2a). Quantitative analysis of GUS expression confirmed that the pAhMTP1-α/β/γ promoters are two orders of magnitude stronger than the pAtMTP1 promoter, and approximately one order of magnitude stronger than the constitutive 35S CaMV promoter. Among the AhMTP1 promoters, the α and β sequences showed comparable expression levels, whereas a lower level was observed for the γ promoter (Fig. 2b,c).

Expression profiles conferred by truncated A. halleri MTP1 promoters

The pAhMTP1-γ promoter was used for the generation of truncated promoter forms in order to define regulatory regions essential for MTP1 expression in A. halleri. A 810-bp-long promoter (Δ810) included the entire proximal region of pAhMTP1, highly conserved among the A. halleri MTP1 promoter sequences, while the shortest truncated form (Δ362) includes part of the MTP1 5’UTR (Fig. 3a). Expression pattern and levels for the Δ810 promoter are approximately the same as in the pAhMTP1-γ entire promoter (Fig. 3b,c). Interestingly, GUS expression was completely abolished in the shoots of the Δ362::GUS plants but weak GUS staining was observed in the roots (Fig. 3b).

Identification of cis-acting elements in the A. thaliana, A. lyrata and A. halleri MTP1 promoters

pAhMTP1-γ, A. thaliana and A. lyrata MTP1 promoter sequences were analyzed using the Signal Scan Search tool on the PLACE database to identify annotated cis-acting elements. The complete list of the identified motifs is reported in Table S2. No TATA-box was found in any of the promoters, but an initiator element (Inr) was found 60, 55 and 54 bp downstream of the putative transcriptional start site in the A. thaliana, A. lyrata and A. halleri promoters, respectively.

PLACE analysis highlighted the presence of several cis-acting elements involved in cell/tissue-specific expression. Dof-binding motifs that conferguard cell-specific expression (Plesch et al., 2001) were found in all promoters; one tandem repeat is present in the pAhMTP1-γ sequence, whereas the A. thaliana and A. lyrata sequences contain five and four tandem repeats, respectively. Some root-specific motifs were also found in all promoters, such as ATATT motifs that possibly drive gene expression in the vasculature (Elmayan & Tepfer, 1995), and telo-box motifs that are reported as necessary for expression in root primordia (Tremousaygue et al., 1999; Manevski et al., 2000). Root hair-specific cis-acting elements (Kim et al., 2006; Won et al., 2009) were found only in pAtMTP1 and pAhMTP1-γ (Table S2).

Three telo-box sequences were found in the A. thaliana and A. lyrata MTP1 promoters, whereas two were found in all MTP1 promoters in both the I16 and Auby populations (red bars in Fig. 1a). Despite the presence of the 63 bp insertion at position −340 bp in the pAhMTP1-α/β promoters, there was no relevant variation in the spacing between the two motifs in any of the
promoters. Indeed, the distal motif is located within the insertion in sequences a and b but upstream of the insertion site in sequence c and all the Auby promoters (Fig. 1a). The activity of telo- boxes has been reported to require other cis-acting elements, such as the tef- box, trap-40 elements and site II motifs (Tremousaygue et al., 1999; Manevski et al., 2000; Gaspin et al., 2010). The MTP1 promoters were therefore screened for these motifs but none were present. Notably, a tandem repeat of MYB-binding elements (CTGTTG; blue bars in Fig. 1a) with a putative role in trichome-specific gene expression (Wang et al., 2002) was identified in all the A. halleri MTP1 promoters (Fig. 1a). The motifs are located in the 5′UTR 125 and 155 bp upstream of the start codon. Despite the high degree of conservation among the A. thaliana, A. lyrata and A. halleri MTP1 promoters in the 5′UTR, the MYB-binding elements were not found in pAtMTP1 as a result of single point mutations, whereas only one site is conserved in pAlMTP1 (Fig. 4). The promoters of several A. thaliana genes involved in metal tolerance and accumulation (Table S3) were also screened for this motif but no paired MYB-binding sites were found in the 5′UTRs of any of these genes.

Site-directed mutagenesis of telo-boxes

The telo-box motifs (TTAGGGTT) were selected for further analysis as a result of their conservation in both sequence and position among the A. halleri, A. thaliana and A. lyrata promoters (Fig. 1a) and their putative role for expression in root primordia. Double site-directed mutagenesis on the pAhMTP1-γ promoter replaced the TTAGGGTT sequences at position −303 and −400 bp with a C8 substitute to preserve the relative spacing of
other cis-acting elements (Fig. 5a). GUS expression driven by the resulting mut303/400 promoter showed the same profile as the wild-type pAhMTP1-γ promoter, but was higher in both shoots and roots when measured by real-time RT-PCR (Fig. 5b,c), with a particularly marked difference in leaves, as indicated by arrows in Fig. 5(b).

Site-directed mutagenesis of MYB-binding sites

As shown in Fig. 2(a), pAhMTP1, but not pAtMTP1, drives GUS expression in leaf trichomes. MYB-binding sites have been proposed as correlated with trichome-specific expression (Wang et al., 2002). To confirm the ability of these motifs to drive expression
Fig. 3 β-Glucuronidase (GUS) reporter assay on the truncated forms of the Arabidopsis halleri MTP1 promoter. pAhMTP1-γ, entire A. halleri MTP1 promoter; Δ810, 810 bp-long truncated form; Δ362, 362 bp-long truncated form. (a) Schematic representation of the truncated forms of pAhMTP1-γ. (b) Qualitative GUS reporter assay in 10-d-old plantlets transformed with the entire and truncated A. halleri MTP1 promoters: (from left to right) pAhMTP1-γ::GUS, Δ810::GUS, Δ362::GUS, detail of the roots of the Δ362::GUS plants. (c) Quantitative GUS reporter assay. In (a) and (b), representative plants are shown for each construct, while (c) represents the results obtained considering three independent homozygous lines carrying single insertion of the indicated constructs. In (c), data are represented as means ± SD; lowercase letters indicate statistical significance, evaluated by Welch’s ANOVA followed by a Games–Howell post hoc test (P < 0.05).

Fig. 4 Alignment of the MTP1 promoters of Arabidopsis thaliana, Arabidopsis lyrata and Arabidopsis halleri in the region of the tandem MYB-binding sites (in green letters). The equivalent A. thaliana and A. lyrata sites are indicated in red letters. pAtMTP1, A. thaliana MTP1 promoter; pAlMTP1, A. lyrata MTP1 promoter; pAhMTP1, A. halleri MTP1 promoters. Asterisks under the alignment indicate nucleotide conservation in all the sequences considered.

Involvement of MYB-binding motifs in Zn tolerance and accumulation

The necessity of MYB-binding sites for the specific expression of MTP1 in A. halleri trichomes prompted us to investigate their involvement in Zn tolerance. A. thaliana mtp1 mutant plants transformed with the constructs pAhMTP1-γ::AtMTP1 and mut125/155::AtMTP1 were tested in vitro in the presence of 300 and 500 μM ZnSO₄ (Fig. 7a). In terms of root length (Fig. 7b), biomass (Fig. 7c) and Chl content (Fig. 7d), transformation with pAhMTP1-γ::AtMTP1 was able to restore Zn tolerance in the mtp1 background. On the other hand, mtp1 plants harboring the mut125/155::AtMTP1 construct were significantly more sensitive to Zn excess, similar to what was observed for mtp1 mutants.

Since MTP1 overexpression induces a different Zn distribution in A. thaliana (van der Zaal et al., 1999), Zn accumulation was also investigated in control plants and mtp1 transformed with the pAhMTP1::AtMTP1 and mut125/155::AtMTP1 vectors after exposure to 0.7 μM ZnSO₄ (standard conditions) and
25 μM ZnSO₄ (excess Zn conditions). Under Zn excess, the leaves of the mut125/155::AtMTP1 transgenic plants accumulated more Zn (Fig. 8a), whereas in roots, Zn concentrations were c. 50% higher in pAhMTP1-γ::AtMTP1 plants than in all other genotypes (Fig. 8b). Significant differences were also observed in roots under control conditions, with mut125/155::AtMTP1 plants accumulating lower metal concentrations (Fig. 8b).

Zn accumulation in trichomes was analyzed by ESEM-EDS in plants grown in 0.7 μM (standard conditions) and 25 μM ZnSO₄. Zn was not detected along the entire body of the trichome but only in a ring at the trichome base (Fig. 9a), as previously reported for A. halleri (Zhao et al., 2000; Sarret et al., 2009) and A. thaliana (Ager et al., 2003; Isaire et al., 2006). Zn accumulating in this region was therefore used for a semiquantitative comparison among the lines. Zn was not detectable in the trichomes of plants grown under standard conditions; in the presence of 25 μM ZnSO₄, the Zn content of the trichomes of mut125/155::AtMTP1 plants was significantly lower than that of both control and pAhMTP1::AtMTP1 plants (Fig. 9b).

Comparison of MTP1 promoter sequences in the Brassicaceae family

To investigate the role of cis-regulation in the MTP1 promoter in more detail, a variety of Brassicaceae species were compared, including both metallophyte (A. halleri I16, C. resedifolia, N. caerulescens, N. pratensis) and nonmetallophyte species (A. thaliana, A. lyrata, C. officinalis, S. officinale, D. sophia, T. arvense). MTP1 promoter sequences were obtained by genome walking, with the exception of the A. thaliana and A. lyrata sequences, which were retrieved from TAIR. The sequences ranged in length from c. 600 bp (C. officinalis) to c. 2000 bp (C. resedifolia). The first 600 bp of each sequence were only moderately conserved, with a mean pairwise identity of 37%. Multiple Em for Motif Elicitation (MEME) analysis to identify conserved motifs among the different Brassicaceae MTP1 promoters (Fig. S4; Table S4) revealed no statistically significant association between motif occurrence and the metallophyte status of the species considered, indicating that the identified motifs are probably not involved in the evolution of heavy metal hypertolerance. The most significantly conserved motif was the telo-box (Table S4), whose involvement in MTP1 regulation has been investigated in this work.

Discussion

Although copy number expansion and transcriptional up-regulation have been proposed to contribute to the higher expression levels observed for hypertolerance/hyperaccumulation determinants, as MTP1 in hyperaccumulator species (Shahzad et al., 2010), there is no characterization of the differential regulation of MTP1 in metallophyte hyperaccumulators and nonmetallophyte species. We chose to perform our study on the metallicolicous A. halleri I16, which is characterized by both hypertolerance and hyperaccumulation of Zn. Similarly to A. halleri Auby, this population grows in soils highly contaminated with Zn, Cd and lead (Pb) (Meyer et al., 2015) and displays very high Zn accumulation levels (Decombeix, 2011).

The three novel MTP1 promoter sequences amplified from A. halleri I16 share high sequence identity in the first 800 bp upstream of the start codon with the five promoters found in the Auby ecotype (Shahzad et al., 2010). Remarkable sequence conservation was observed in the 5’UTR compared with the
corresponding *A. thaliana* and *A. lyrata* sequences, as reported previously for the Auby population (Shahzad et al., 2010). The tentative amplification of ecotype I16 promoter sequences equivalent to *AhMTP1-A1/A2/B1/C1/D* in the *A. halleri* Auby population, performed with discriminating primers designed upstream the 800-bp-long conserved region of the promoters, provided positive results only for the D copy. It is therefore plausible that the geographically distant populations I16 and Auby, belonging to different phylogeographic units that have evolved independently since the Last Glacial Maximum (Pauwels et al., 2012), have diverged in the distal region of the *MTP1* promoters. The novel promoters identified herein have acquired different transcription abundances. Owing to the lack of genomic information data and the limit imposed by the genome walking technique, the genomic context of each copy remains unknown, and therefore it cannot be determined whether the four sequences identified are allelic variants or distinct loci.

As *AhMTP1-D* has been discussed in previous reports (Shahzad et al., 2010), we focused on the three novel *MTP1* promoters. All four *AhMTP1* sequences identified were actively transcribed in *A. halleri* I16, as highlighted by real-time RT-PCR. By quantitative GUS assay, the *A. halleri* *MTP1-α/β/γ* promoters were more active than the *A. thaliana* *MTP1* promoter, suggesting that the higher *MTP1* expression levels in *A. halleri* reflect a combination of copy number expansion and transcriptional regulation. The 800 bp proximal region, including the 5'UTR of the *MTP1* mRNA, was highly conserved among all *AhMTP1* promoter sequences, and is thus probably the functional core of the *A. halleri* *MTP1* promoter, as confirmed by the comparable expression levels conferred by the *AhMTP1-γ* promoter and the
Fig. 7 Analysis of zinc (Zn) tolerance in control (empty vector), mtp1 mutant, and mtp1 pAhMTP1-γ::AtMTP1 and mut125/155::AtMTP1 plantlets grown in standard MS medium (30 μM ZnSO₄) and excess Zn (300 and 500 μM ZnSO₄). (a) Genotypes after the different Zn treatments. (b) Root length; (c) shoot biomass; (d) total Chl content. pAhMTP1-γ, wild-type Arabidopsis halleri MTP1 promoter; mut125/155, A. halleri MTP1 promoter lacking the two MYB-binding sites. Statistical comparisons refer to the different genotypes under the same Zn condition. Data are represented as means ± SD; lowercase letters indicate statistical significance, evaluated by Welch’s ANOVA followed by a Games–Howell post hoc test (P < 0.05).
corresponding Δ810 truncated form. Essential regulatory elements are situated between 362 and 810 bp upstream of the start codon because the Δ362 truncated promoter showed only minimal activity. Indeed, the transcriptional start site is predicted to lie 546 bp upstream of the start codon in the pAhMTP1-c sequence, as inferred by alignment with the A. thaliana promoter. The A. thaliana, A. lyrata and A. halleri MTP1 promoters lack a TATA box, which is not unexpected because this element is only found in 25% of A. thaliana promoters (Yamamoto et al., 2009). Interestingly, an initiator element previously identified in the light-induced tobacco gene PsaDb (Nakamura et al., 2002) was found close to the transcriptional start site in all the promoters, suggesting that this element controls the transcriptional initiation of the MTP1 gene.

The expression profile conferred by the A. thaliana and A. halleri MTP1 promoters showed marked differences as revealed by GUS reporter assays. The stronger expression driven by the three AhMTP1 promoters is consistent with the key role of MTP1 in vacuolar Zn transport in the hyperaccumulator A. halleri. Whereas pAhMTP1 resulted in GUS staining in the whole plant, the activity of the A. thaliana promoter in the leaves was restricted to the vascular tissues, hydathodes and guard cells. The expression in the latter, common to both A. thaliana and A. halleri, is associated with the presence of clusters of
Dof-binding sites in the promoters (Plesch et al., 2001; Galbiati et al., 2008; Cominelli et al., 2011). Indeed, the stomatal expression previously reported for the A. thaliana MTP1 promoter (Kawachi et al., 2009) has also been observed for other transporters involved in metal accumulation, such as A. thaliana HMA3 (Morel et al., 2009) and MTP11 (Peiter et al., 2007) and N. caerulescens ZNT1 (Küpper et al., 2007). As also proposed for HMA3 (Morel et al., 2009), MTP1 expression in the guard cells of both nonaccumulator and hyperaccumulator species may be necessary to prevent metal toxicity and the subsequent impairment of stomatal functions. The prevalence of this motif in the promoters of nonmetallogryte species probably ensures the functionality of guard cells, whereas hyperaccumulators rely on the combined effect of diverse metal tolerance mechanisms, as also suggested by the low metal concentrations in the stomatal complexes of several hyperaccumulator species (Frey et al., 2000; Psaras et al., 2000; Küpper et al., 2001). Several root-specific cis-acting sites were identified in all the MTP1 promoters, but the telo-box is the most interesting, as it is conserved in MTP1 promoters from several hyperaccumulation and nonaccumulator Brassicaceae species. The telo-box has been found in genes encoding components of the translational machinery and reduct balance system (Regad et al., 1995; Tremousaygue et al., 1999; Gaspin et al., 2010) and is necessary for gene expression in root primordia (Tremousaygue et al., 1999; Manipolski et al., 2000). In the A. thaliana, A. lyrata and A. halleri MTP1 promoters, the telo-box elements are located in the 5’UTR as previously reported for genes involved in translation (Tremousaygue et al., 1999). Site-directed mutagenesis of these motifs in the A. halleri promoter (mut303/400) enhanced GUS expression in the shoots and roots without affecting the spatial expression pattern. Although this result contrasts with previous reports (Tremousaygue et al., 1999; Manipolski et al., 2000), a similar phenotype was reported following the deletion of a telo-box in the A. thaliana EF-1α gene (Curie et al., 1993). Furthermore, the consequences of site-directed mutagenesis in the A. halleri promoter are consistent with the presence of an additional telo-box motif in the A. thaliana and A. lyrata promoters, suggesting that this element may be involved in the negative regulation of the MTP1 promoter. The lack of motifs generally associated with telo-boxes in the A. thaliana, A. lyrata and A. halleri promoter sequences indicates that other cis-acting elements probably cooperate with telo-boxes in the MTP1 promoters to confer expression profiles other than the root meristem-specific pattern previously described.

In addition to telo-boxes, root-specific ATATT motifs possibly involved in root-specific expression in the vasculature (Elmawy & Tepfer, 1995) were found in the promoters of all species considered, and root hair-specific cis-acting elements (Kim et al., 2006; Won et al., 2009) were found in pAtMTP1 and pHMTP1. As the A362 truncated A. halleri promoter induced weak GUS expression in roots, some other unknown root-specific elements must be present in the most proximal region of the promoter, as neither ATATT nor root hair-specific cis-acting elements are located in this portion of pHMTP1.

In contrast to the A. thaliana sequence, the A. halleri MTP1 promoters identified herein conferred strong expression in the leaf mesophyll and trichomes. High levels of MTP1 in mesophyll cells may account for the high Zn tolerance observed in A. halleri compared with A. thaliana. No cis-acting elements for the induction of mesophyll-specific expression were identified by in silico analysis, and although several light-response elements were found, these cannot be responsible for the higher expression level in A. halleri because they were also present in the A. thaliana promoter. Therefore, pHMTP1-driven mesophyll expression is possibly a result of as yet unknown elements or of specific combinations of known motifs.

A pair of MYB-binding motifs was found to be highly conserved in all A. halleri MTP1 promoters analyzed, but these are absent in the A. thaliana and A. lyrata promoters as a result of single nucleotide substitutions, despite the generally high conservation in this region. MYB-binding sites were found involved in the expression regulation of some trichome-specific genes (Szymanski et al., 1998; Wang et al., 2004; Pesch & Hülskamp, 2011). In detail, the MYB-binding motif CTGTTG was proposed to drive trichome-specific expression in the tobacco CYP71D16 promoter (Wang et al., 2002) and is able to bind the TT2 (TRANSPARENT TESTA GLABRA 2) A Myb123 transcription factor, one of the partners of the TTB1 (TRANSPARENT TESTA 1) WD40-repeat protein, involved in seed coat pigmentation, trichome initiation and development (Thévenin et al., 2012). Site-directed mutagenesis of one (mut155) or both CTGTTG motifs to mimic the A. thaliana promoter (mut125/155) abolished GUS expression in trichomes, thus confirming the role of these MYB-binding motifs in MTP1 expression in trichomes. A search for the paired CTGTTG sequences in the 5’UTR of A. thaliana genes, whose orthologues are involved in metal tolerance and accumulation in the hyperaccumulator species A. halleri and N. caerulescens, revealed their absence. This result is consistent with a putative role of this motif in the evolution of the A. halleri hypertolerance phenotype. Such a hypothesis is also supported by Zn tolerance experiments, in which the mut125/155::AtMTP1 transgenic plants were less tolerant than the plants transformed with the cassette containing the wild-type A. halleri promoter. MEME analysis of the MTP1 promoter in different Brassicaceae species did not reveal any association between this motif and hypertolerant/hyperaccumulator species; indeed the paired MYB-binding sites were unique to the A. halleri promoter. The lack of this cis-acting element in the MTP1 promoters of Noccaea species may correlate with the absence of trichomes on their leaves (Al-Shehbaz et al., 2006). Genome walking along MTP1 promoters from the Brassicaceae species considered did not identify any specific feature correlating with the hypertolerance/hyperaccumulation trait. By contrast, promoter sequence homology reflected the phylogenetic proximity of the species – regardless of their status as metallophyte or not – consistently with the hypothesis that hypertolerance/hyperaccumulation has evolved independently several times in the Brassicaceae lineage (Krämer et al., 2007; Krämer, 2010). In this context, species derived from different evolutionary events are likely to have selected unrelated cis-acting modifications to increase MTP1 expression levels as
well as different strategies to cope with excess metals, for example MTP1 expression in trichomes in the case of A. hallieri.

MTP1 expression in A. hallieri trichomes is remarkable because metal accumulation in trichomes has been reported in many hyperaccumulator species, as A. hallieri (Küpper et al., 2000; Zhao et al., 2000; Sarret et al., 2002), Alyssum murale (Broadhurst et al., 2004) and Astragalus bisulcatus (Freeman et al., 2006). However, other hyperaccumulators rely on different cell types for metal storage (Cosio et al., 2005; Freeman et al., 2006). On the other hand, metal storage in trichomes is not exclusive to hyperaccumulators, given that this process also occurs in A. thaliana (Ager et al., 2003; Isaure et al., 2006) and A. lyrata (Sarret et al., 2009). In A. hallieri, trichomes can accumulate substantially higher concentrations of metal than surrounding cells, but they constitute only a minor portion of the whole leaf biomass and therefore cannot be considered a major metal storage site (Küpper et al., 2000; Sarret et al., 2002, 2009; Huguet et al., 2012). Increasing metal concentrations (Küpper et al., 2000) or longer metal exposure times (Huguet et al., 2012) progressively saturate trichomes, whereas metal concentrations continue to increase in the mesophyll cells. These data support the hypothesis that trichomes are involved in short-term tolerance rather than long-term metal hyperaccumulation. Therefore, the role of MTP1 expression in trichomes was investigated in more detail in A. thaliana.

The mtpl plants transformed with the pAhMTP1-γ::AtMTP1 cassette tolerated excess Zn well and were mostly insensitive to the high metal concentrations under the conditions tested. By contrast, excess Zn had a significant impact on plant growth in the mut125/155::AtMTP1 lines, showing hypersensitivity similar to the mtpl mutant. Overall, these results indicate a role of the MYB-binding motifs in metal tolerance in A. thaliana, supporting their involvement in the evolution of Zn hypertolerance in A. hallieri.

Zinc accumulation was also considered in the A. thaliana mtpl transgenic lines containing the pAhMTP1-γ::AtMTP1 and mtpl/::AtMTP1 cassettes. When exposed to Zn excess, plants transformed with the mut125/155::AtMTP1 construct accumulated lower concentrations of Zn in trichomes than did control and pAhMTP1-γ::AtMTP1 transgenic lines, supporting the role of the MYB-binding motifs studied here in directing MTP1 expression in trichomes. Moreover, in these growth conditions, pAhMTP1-γ::AtMTP1 plants accumulated the highest concentrations of Zn in the roots, as observed previously for plants overexpressing AtMTP1 (van der Zaal et al., 1999); on the other hand, plants expressing mut125/155::AtMTP1 accumulated more Zn in the shoot. This different distribution observed between the two transgenic genotypes was unexpected, as, by GUS assay, MYB-binding sites seemed linked only with expression in trichomes; however, the higher root Zn accumulation in the pAhMTP1-γ::AtMTP1 lines suggests an increased Zn retention in roots owing to a possible control of these cis elements on MTP1 expression in roots as well. In fact, an eventual difference in GUS expression in specific root tissues may be masked by the high global root staining observed in GUS assay. In fact, the different metal distribution probably explains the noteworthy phenotype observed in the tolerance analysis: metal compartmentalization in root cells is a common mechanism for metal tolerance, known as the excluder strategy (Baker, 1981; Krämer, 2010). Indeed, the model system A. thaliana, used as a background for transformation, lacks the up-regulation of other determinants for hypertolerance and hyperaccumulation, as HMA4 (Willems et al., 2007; Frérot et al., 2010; Meyer et al., 2016), and relies on the excluder strategy to cope with metal stress (Becher et al., 2004). In this view, the higher Zn translocation to shoots observed in mut125/155::AtMTP1 plants combines with the reduced storage in the trichomes, resulting in the Zn-sensitive phenotype observed in this genotype. It is evident that Zn tolerance does not depend solely on Zn storage in the trichomes; however, the results of this study indicate that the role of this cell type in the evolution of hypertolerance is not negligible. Globally, these results underline the essential role of MTP1 expression regulation in the response to excess Zn, supporting the importance of this vacuolar transporter in the evolution of the hypertolerance trait in metallocolous populations of A. hallieri (Dräger et al., 2004; Willems et al., 2007; Meyer et al., 2016).

In conclusion, the differential cis-regulation of MTP1 expression in A. thaliana and A. hallieri, combined with the MTP1 copy number expansion in the latter, led to the overexpression of this transporter in A. hallieri. Interestingly, among the cis-acting elements identified in the promoters we analyzed, the MYB-binding sites located in the 5′UTR of the A. hallieri MTP1 gene mark the divergent evolution between the hyperaccumulator species A. hallieri and the nonaccumulators A. thaliana and A. lyrata. In addition, our data indicate that these motifs play a notable role in metal tolerance, demonstrating the importance of efficient expression regulation of genetic determinants for the evolution of a complex extremophile trait such as Zn hypertolerance.

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Author contributions

E.F. performed most of the experiments and wrote the article with contribution of A.F. and G.D. C.V. and M.L. amplified the Brassicaceae promoter sequences and helped with the bioinformatic analysis. G.V. and M.M. analyzed the zinc content in trichomes and contributed with photography. Funding for E.F.’s PhD was from MIUR (the Italian Ministry of University and Research).

References


Supporting Information

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**Fig. S1** CLUSTALW alignment of the *Arabidopsis halleri* MTP1 promoters amplified from the ecotype I16 (in red), compared to the promoters of the population Auby as reported by Shahzad et al. (2010) (in black).

**Fig. S2** Expression levels of the four MTP1 sequences analysed by real-time RT-PCR on leaves of *Arabidopsis halleri* I16 plants grown in standard conditions.

**Fig. S3** Qualitative GUS assay on leaf trichomes of 3-wk-old *Arabidopsis thaliana* plants transformed with the wild-type pAhMTP1-γ promoter and the mut155 promoter mutated in one MYB-binding site.

**Fig. S4** Position of conserved motifs found by MEME analysis in the MTP1 promoters of the different Brassicaceae species.

**Table S1** Conservation among MTP1 promoter sequences α, β and γ amplified from *Arabidopsis halleri* I16 and the MTP1 promoters from *Arabidopsis thaliana*, *Arabidopsis lyrata* and *A. halleri* Auby, resulting from pairwise alignments

**Table S2** List of the main *cis*-regulatory elements found in the *Arabidopsis thaliana*, *Arabidopsis lyrata* and *Arabidopsis halleri* MTP1 promoters by PLACE analysis

**Table S3** List of genes involved in metal tolerance and accumulation, whose promoter sequences were screened for MYB-binding motifs (CTGTTG)

**Table S4** List of significant motifs found by MEME analysis in the MTP1 promoters of different Brassicaceae species and their annotation in the PLACE database

**Methods S1** *Arabidopsis thaliana* transformation.

**Methods S2** Bioinformatic analysis of MTP1 promoters.

**Methods S3** Analysis of GUS and MTP1 expression in transgenic lines.

**Methods S4** Environmental scanning electron microscopy coupled with energy-dispersive X-ray spectroscopy (ESEM-EDS).

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