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RESEARCH PAPER

Metallothioneins 2 and 3 contribute to the metal-adapted phenotype but are not directly linked to Zn accumulation in the metal hyperaccumulator, *Thlaspi caerulescens*

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Abstract

To study the role of metallothioneins (MTs) in Zn accumulation, the expression of *TcMT2a*, *TcMT2b*, and *TcMT3* was analysed in three accessions and 15 F_3 families of two inter-accession crosses of the Cd/Zn hyperaccumulator *Thlaspi caerulescens*, with different degrees of Zn accumulation. The highest expression levels were found in the shoots of a superior metal-accumulating calamine accession from St Laurent le Minier, with >10-fold *TcMT3* expression compared with another calamine accession and a non-metallicolous accession. Moreover, F_3 sibling lines from the inter-accession crosses that harboured the *MT2a* or *MT3* allele from St Laurent le Minier had higher expression levels. However, there was no co-segregation of *TcMT2a* or *TcMT3* expression and Zn accumulation. To examine the functions of TcMTs in plants, *TcMT2a* and *TcMT3* were ectopically expressed in *Arabidopsis*. The transformant lines had reduced root length in control medium but not at high metal concentrations, suggesting that the ectopically expressed proteins interfered with the physiological availability of essential metals under limited supply. The *Arabidopsis* transformant lines did not show increased tolerance to Cd, Cu, or Zn, nor increased Cd or Zn accumulation. Immunohistochemical analysis indicated that in roots, MT2 protein is localized in the epidermis and root hairs of both *T. caerulescens* and *Arabidopsis thaliana*. The results suggest that TcMT2a, TcMT2b, and TcMT3 are not primarily involved in Zn accumulation as such. However, the elevated expression levels in the metallicolous accessions suggests that they do contribute to the metal-adapted phenotype, possibly through improving Cu homeostasis at high Zn and Cd body burdens. Alternatively, they might function as hypostatic enhancers of Zn or Cd tolerance.

Key words: Cd, crosses, metallothionein, protein, quantitative real-time PCR, *Thlaspi caerulescens*, Zn.

Introduction

The molecular basis of metal hyperaccumulation in plants has gained increasing interest during the past few years. One of the best studied metal hyperaccumulator species is *Thlaspi caerulescens*, having accessions that differ in metal tolerance, root to shoot transport, and uptake. Metal accumulation in *T. caerulescens* is suggested to be a consequence of increased root to shoot transport and internal sequestration to storage organelles (Assunção *et al.*, 2003b). Besides metal transporters, metallothioneins (MTs) are often strongly expressed in hyperaccumulators (Roosens *et al.*, 2004; van de Mortel *et al.*, 2006), suggesting that they also play some role in the hyperaccumulation phenotype.

In *Arabidopsis thaliana*, MTs form a diverse family, with seven translated genes belonging to four types (Cobbett and Goldsbrough, 2002). At least three metallothionein genes, *MT1*, *MT2*, and *MT3*, have been isolated from *T. caerulescens*

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Abbreviations: FP, accession St Félix de Pallières; LC, accession La Calamine; LE, accession Lellingen; LM, accession St Laurent le Minier; MT, metallothionein.

(Roosens *et al.*, 2004, 2005; Hassinen *et al.*, 2007). The *T. caerulescens MT2* gene isolated from a superior Cd-accumulating calamine accession from St Félix de Pallières (FP), South-France, shows characteristics of both *MT2a*- and *MT2b*-type genes (Roosens *et al.*, 2005), whereas the *MT2* gene isolated from another calamine accession from La Calamine (LC), Belgium, is a close homologue of *AtMT2a* (Hassinen *et al.*, 2007). Compared with *Arabidopsis*, a *T. caerulescens* MT3, isolated from the FP accession, shows a modified cysteine arrangement in the C-terminal metal-binding domain, which has been suggested to result in altered metal-binding characteristics, including a higher affinity for Cu (Roosens *et al.*, 2004). Moreover, another isoform or allele of MT3 has been isolated from the *T. caerulescens* accession LC, in which the cysteine arrangement is the same as in *Arabidopsis* MT3 (Hassinen *et al.*, 2007). These differences in the amino acid sequence may reflect divergent selection resulting from different soil metal compositions at the sites of origin.

Even though plant MTs have been the subject of many studies, their functions are still not fully understood, partly because it is difficult to isolate the intact proteins from plant tissues. Evidence suggests that MTs may play a role in metal buffering or homeostasis by providing a pool of available micronutrients for the cells, or in the protection of cells from oxidative damage caused by free radicals (Mir *et al.*, 2004).

The expression of plant *MT* genes is primarily inducible by copper, suggesting that they are predominantly involved in Cu homeostasis or Cu tolerance (Zhou and Goldsbrough, 1994; Guo *et al.*, 2003). *MT2a* expression in roots appeared to be correlated with Cu tolerance among *Arabidopsis* ecotypes (Murphy and Taiz, 1995). Based on a detailed co-segregation analysis, Van Hoof *et al.* (2001) suggested that enhanced *MT2b* expression in roots may be essential, though not sufficient for high-level Cu tolerance in cupricolous *Silene vulgaris*. However, enhanced *MT2b* expression was also consistently found in accessions from calamine soils with normal Cu concentrations (Jack *et al.*, 2007). Therefore, MTs may also be involved in the detoxification or homeostasis of metals other than Cu. When the MT1 family was knocked-down in *Arabidopsis* by an RNA interference (RNAi) construct, the knock-down lines were all hypersensitive to Cd and accumulated several-fold lower levels of As, Cd, and Zn than did the wild type, while Cu and Fe levels were unaffected. The authors concluded that MT1 confers tolerance to Cd and can assist in Zn homeostasis (Zimeri *et al.*, 2005). *Arabidopsis MT2a* and *MT3* have been shown to increase Cd tolerance when expressed in *Vicia faba* guard cells, also suggesting that the MTs may play a role in Cd detoxification (Lee *et al.*, 2004). Ectopic expression of *Brassica juncea BjMT2* in *A. thaliana* resulted in increased tolerance to both Cu and Cd (Zhigang *et al.*, 2006).

Plant MTs have been shown to increase tolerance to metals in yeast, and are thus functional metal-binding proteins (Zhou and Goldsbrough, 1994; van Hoof *et al.*, 2001; Guo *et al.*, 2008). *Thlaspi caerulescens MT2* and *MT3* are capable of conferring Cd, Cu, or Zn tolerance (Roosens *et al.*, 2005) or Cd accumulation (Hassinen *et al.*, 2007) in yeast, although to levels not exceeding those of *Arabidopsis MT* genes, suggesting that the proteins from *Thlaspi* are not superior in their metal-binding capability compared with those of *Arabidopsis*. An exception is *TcMT3* from the FP accession, which confers increased Cu tolerance compared with *Arabidopsis MT3* in yeast (Roosens *et al.*, 2004).

The function of MTs in Zn accumulation has not been addressed previously. Two *MT* genes have been isolated from *T. caerulescens* (*TcMT2-LC* and *TcMT3-LC*) that showed higher expression in a Zn-adapted, calamine accession (LC) compared with an accession originating from uncontaminated soil [Lellingen (LE)] (Hassinen *et al.*, 2007). In the present study, another *MT2*-type gene was isolated and the expression of *TcMT2a*, *TcMT2b*, and *TcMT3* was analysed in *T. caerulescens* accessions and in lines from inter-accession crosses to test for possible co-segregation of MT expression and Zn accumulation capacity. Metal accumulation was analysed in transgenic *Arabidopsis* expressing *TcMT2a* or *TcMT3*. Moreover, MT2 protein localization in roots was analysed in *T. caerulescens* as well as in transgenic *Arabidopsis* using immunohistochemistry.

Materials and methods

Plant material

Three *T. caerulescens* accessions, differing in metal uptake, translocation from root to shoot, and tolerance, were used. The accession LC originates from soil contaminated with calamine ore waste (Zn, Cd, and Pb) near La Calamine, Belgium, and accession St Laurent le Minier (LM), formerly named 'Ganges', from a similar site in France (Zhao *et al.*, 2002). Accession LE is from a non-metalliferous soil near Lellingen, Luxembourg (Meerts and Van Isacker, 1997). The LC×LE cross is described in Assunção *et al.* (2003*b*), and the LC×LM (= GA) cross in Deniau *et al.* (2006). In this study, three low- and two high-accumulating F_3 lines of the LC×LE cross and five low- and five high-accumulating F_3 lines of the LC×LM cross were used. When grown at 10 μM Zn in the nutrient solution, the high-accumulating lines accumulated at least 10 times more Zn than the low-accumulating ones (data not given). The plants were grown as described by Assunção *et al.* (2003*a*). They were exposed to 0, 10, 100, and 1000 μM $ZnSO_4$ for 1 week, after which the shoots and roots were separately collected, frozen in liquid nitrogen, and stored at –80 °C until use.

Isolation of MT sequences

TcMT2a-LC and *TcMT3-LC* were previously isolated from a cDNA library prepared from *T. caerulescens* accession LC (Hassinen *et al.*, 2007). The *TcMT2a-LM* and *TcMT3-LM* alleles from the accession LM were amplified in the present study using primers designed from LC cDNA. The *TcMT2b* was isolated from the accessions LC and LM, using primers designed from a *Thlaspi* expressed sequence tag (EST; DN923775) homologous to *Arabidopsis MT2b*. The primer

sequences for *MT2b* were: 5′ ATGTCTTGCTGTGGAGGAAACT 3′ and 5′ TCATTTGCAGGTACAAGGGTT 3′.

Quantitative real-time PCR

Each sample consisted of shoots or roots pooled from three plants. Total RNA was isolated with an RNeasy extraction kit using DNase I on-column digestion (Qiagen). The cDNA was synthesized using an oligo(dT) primer from 1 μg of total RNA with a DyNAmo 2 step SYBR Green qPCR kit (Finnzymes). Quantitative real-time PCRs were done using a Dynamo HS SYBR Green kit (Finnzymes) in a 20 μl reaction volume with 0.5 μM gene-specific primers and 2 μl of diluted cDNA, derived from 2.5 ng of total RNA, as a template. *Thlaspi caerulescens* tubulin, a homologue to *Arabidopsis* α2 tubulin (At1g04820), was used as a reference gene. The amplicon lengths were 297 bp for tubulin, 115 bp for *MT2a*, 194 bp for *MT2b*, and 121 bp for *MT3*. The *TcMT2a* amplicon was from the 3′-untranslated region (UTR), while *TcMT2b* and *TcMT3* primers spanned an intron. All primers had full homology to both LM and LC alleles. The primer sequences were 5′ CCTACGCACCAGTCATCTCT 3′ and 5′ CGAGATCACCTCCTGGAACA 3′ for tubulin; 5′ GCAATAATGGCTGTAGCCTTGT 3′ and 5′ GAAGTACAAACGGGACCATCAA 3′ for *TcMT2a*; 5′ GTCTTGCTGTGGAGGAAACTGT 3′ and 5′ CATCATTCTCGGCAACGAAGGT 3′ for *TcMT2b*; and 5′ CCAGCTACACCTTGGACATGGT 3′ and 5′ TTGACGCAGCTGCAGGTAGA 3′ for *TcMT3*. The reactions were performed in an iCycler iQ Real-time PCR (Bio-Rad) in triplicate. The PCR was: 95 °C for 15 min, followed by 35 cycles of 95 °C for 15 s, 58 °C for 20 s, 72 °C for 20 s. After final annealing (72 °C, 5 min), and redenaturation (95 °C, 1 min), a melt curve analysis was done by decreasing the temperature from 95 °C to 60 °C at 0.5 °C intervals. The fold-change in gene expression was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001). The correlation coefficient of amplification, determined from serial dilutions, was 0.996 for *TcMT2a*, 0.998 for *TcMT2b* and *TcMT3*, and 0.999 for tubulin.

Determination of MT alleles in the crosses

The allelic distribution of *MT* genes was analysed from shoots of 12 plants of the parental accessions LM and LC, and the five low- and high-accumulating F_3 sibling lines from the corresponding inter-accession cross. *TcMT2a* was amplified from the 3′ UTR using a 5′ fluorescently labelled reverse primer (5′ GCAATAATGGCTGTAGCCTTGT 3′ and 5′ FAM-GAAGTACAAACGGGACCATCAA 3′). The length of the *TcMT2a-LM* amplicon was 118 bp, which is 3 bp longer than the *TcMT2a-LC* amplicon. *TcMT3* was amplified from genomic DNA with a 5′ FAM-labelled forward primer (5′ FAM-ACAAGACCCAGTGCGTGTAAGT 3′; 5′ TACTCTTCTTGCTGCGGTCACA 3′). The primers span the first intron which is 16 bp longer in LM, resulting to amplicon lengths of 386 bp for the LM allele and 370 bp for the LC allele. The *TcMT2b* gene was amplified from genomic DNA (primers 5′ TAATCTGTTCACGTCCTCGGTT 3′ and 5′ FAM-CATCATTCTCGGCAACGAAGGT 3′), with amplicon lengths of 313 bp and 302 bp for the LC and the LM allele, respectively, due to an 11 bp longer intron in the LC allele. After purification of the PCR products, the samples were applied to an automated sequencer (MegaBACE 750 DNA Analysis System, Amersham). The data were analysed using fragment analysis software (Fragment Profiler, Amersham).

Antibody production and immunofluorescence staining

Antibodies were developed against two synthetic biotinylated peptides (P1, GGCKRNPDLGYSGE; and P2, VLGVAPAMKNQYEASGE), designed from the spacer region between the *T. caerulescens* TcMT2a-LC cysteine-rich domains (Fig. 1). A rabbit was immunized with 100 μg of both P1 and P2 three times at 4-week intervals, using avidin-conjugated P1 and P2 in the first and second immunizations. Raising of antibodies was monitored using an enzyme-linked immunosorbent assay (ELISA) with serum taken before injection as a reference. Serum was collected 6 weeks after the last injection. The antibodies were purified by affinity chromatography, first with streptavidin–Sepharose followed by P1 and P2 coupled to streptavidin–Sepharose (Amersham Biosciences). Antibodies were tested with ELISA using streptavidin–P1- and –P2-coated plates.

For whole-mount immunofluorescence staining, *T. caerulescens* accessions LC, LE, and LM, as well as *A. thaliana* were germinated in Petri dishes on filter paper soaked in 0.5

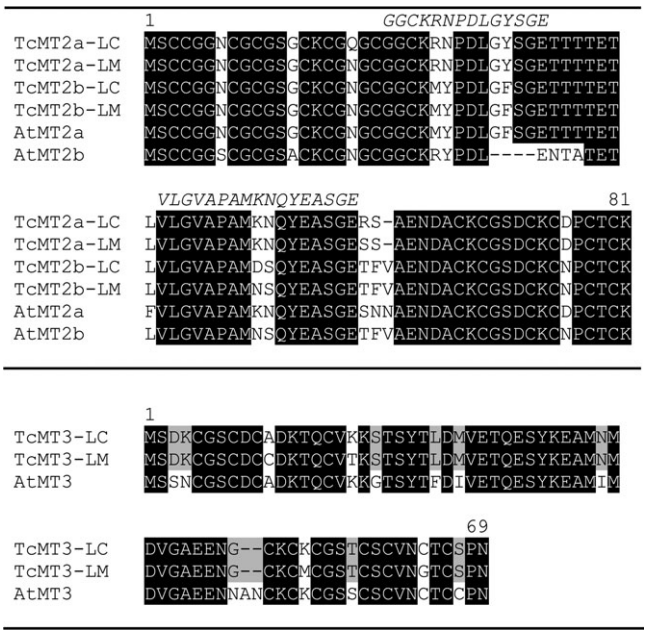

Fig. 1. Deduced TcMT2a, TcMT2b, and TcMT3 amino acid sequences from *T. caerulescens* accessions LC (La Calamine, Hassinen *et al.*, 2007) and LM (St Laurent le Minier). *Arabidopsis thaliana* AtMT2a (At3g09390), AtMT2b (At5g02380), and AtMT3 (At3g15353) are included for comparison. Peptides used for antibody production are shown in italics and cover the amino acids 21–34 and 42–58 of *T. caerulescens* MT2a-LC.

MS (Murashige–Skoog) medium. The 4-d-old *Arabidopsis* and 7-d-old *T. caerulescens* seedlings were fixed in 4% paraformaldehyde at 4 °C for 2 h and washed with phosphate-buffered saline (PBS; three times 5 min). The cell walls were digested with 4% cellulase 'Onuzuka R-10' (Duchefa) and 3% macerozyme R-10 (Duchefa) at 25 °C for 2 h, and washed with PBS. Before incubating with the primary antibody, the samples were blocked with Image-iT Signal Enhancer (Invitrogen) for 30 min and with blocking solution (2% milk powder, 0.5% bovine serum albumin) for 1.5 h. The plants were incubated with the affinity-purified MT2 antibody (1.7 μg ml^{-1}) at 4 °C overnight. After washing, the samples were incubated in 1:200 diluted secondary antibody (goat anti-rabbit IgG Alexa Fluor 488, Invitrogen) for 1.5 h. The samples were counterstained with 10 μg ml^{-1} propidium iodide (Invitrogen). After washing, the seedlings were transferred on microscope slides, mounted with PVA-DABCO antifading mounting medium (Sigma-Aldrich), and inspected with a confocal laser scanning microscope Eclipse-TE300 (Nikon Corporation, Japan)/Ultra VIEW (Perkin-Elmer, UK). As a negative control, purified MT2 antibody pre-adsorbed on the peptides used for immunization was included in each experiment. The images were processed using ImageJ (Abramoff *et al.*, 2004) and Adobe Photoshop softwares.

Arabidopsis *transformation*

The *TcMT2a-LC* and *TcMT3-LC* cDNAs were cloned into the pCAMBIA2301 vector under the cauliflower mosaic virus (CaMV) 35S promoter. Recombinant *Agrobacterium tumefaciens* (LBA4404) clones were selected in a medium containing kanamycin, rifampicin, and streptomycin. *Arabidopsis thaliana* (Col-0) was transformed using the floral dip method (Clough and Bent, 1998) and T_0 seeds were germinated on 0.5 MS agar plates under kanamycin selection. The presence of the transgene was confirmed by PCR with pCAMBIA2301-specific primers. Homozygous T_3 lines were further selected by segregation for kanamycin resistance.

Analysis of growth and metal content

Seeds of transgenic (T_3) and wild-type *Arabidopsis* plants were sown on 0.5 MS agar plates (control), or on plates supplemented with 400 μM $ZnSO_4$, 40 μM $CuSO_4$, or 15 μM $CdSO_4$. The plates were incubated at 22 °C with 16 h light/8 h darkness horizontally for 3 d, and then turned to the vertical position. The root length was determined using ImageJ software (Abramoff *et al.*, 2004) after 7 d of growth.

For the analysis of metal uptake, the seeds of wild-type and transgenic (T_3) *Arabidopsis* were sown in a peat/soil/perlite/sand mixture (20/20/30/30; v/v/v/v). Three-week-old plants were transferred to modified half-strength Hoagland solution (Schat *et al.*, 1996): 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 1 μM KCl, 25 μM H_3BO_3, 2 μM $ZnSO_4$, 2 μM $MnSO_4$, 0.1 μM $CuSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, 20 μM Fe(Na)EDTA, 2 mM MES. The pH was set to 5.5 using KOH. After 2 weeks, the plants were transferred to the same nutrient solution (control) or to a similar solution supplemented with 10, 25, or 100 μM $ZnSO_4$ or 1 μM or 10 μM $CdSO_4$. The solutions were aerated continuously and changed twice a week. The hydroponic culture was done in a climate chamber with 20/15 °C (day/night), 65% relative humidity, 150 μmol m^{-2} s^{-1} light 12 h/day.

After 1 week of exposure, the roots were desorbed with ice-cold 5 mM $PbNO_3$ for 1 h. After washing with water, the shoot and root samples were dried at 65 °C for 40 h. The samples were decomposed with suprapur HNO_3 by microwave digestion, and the Cd and Zn contents of the samples were analysed using a flame atomic absorption spectrophotometer (Perkin Elmer AAS 5100).

Results

MT2a, MT2b, *and* MT3 *genes of* Thlaspi caerulescens

MT2a and *MT3* cDNAs which had been isolated from *T. caerulescens* accession LC (Hassinen *et al.*, 2007) differ from the *TcMT2* and *TcMT3* isolated from accession FP from the Ganges region (Roosens *et al.*, 2004, 2005) at the amino acid sequence level. These different MT alleles or isoforms in different *Thlaspi* accessions prompted the further amplification and sequencing of *TcMT2a*, *TcMT2b*, and *TcMT3* genes from a non-metallicolous and from the two calaminous accessions. The presence of both *MT2* types, i.e. *MT2a* and *MT2b*, in *T. caerulescens* was confirmed.

The *MT* genes from *Thlaspi* have generally longer introns than those of their *Arabidopsis* homologues. For example, the size of *MT2a* intron is 376 bp in *Thlaspi* and 206 bp in *Arabidopsis*, and those for *MT2b* are 363 bp and 176 bp, respectively. For *MT3* the lengths of the first intron are 343 bp and 208 bp in *Thlaspi* and *Arabidopsis*, respectively, whereas the second intron is about of equal size (78/84 bp).

The isolation of *MT2a* and *MT2b* cDNAs from the same plants indicates that there are two translated MT2 isoforms also in *T. caerulescens* (Fig. 1). As *TcMT2b-LC* has ~88% and 83% identity with *Arabidopsis AtMT2b* and *AtMT2a*, respectively, it is considered to be an *MT2b*. *Thlaspi caerulescens MT2a* and *MT2b* show higher sequence identity to each other than do the *Arabidopsis* type 2a and 2b genes.

The *MT*s of accession LM show some differences from the ones in the other *Thlaspi* accessions. The *MT2a* genes from LE, LC, or the serpentine accession Monte Prinzera are identical to each other but different from *MT2a-LM*. *MT2a-LM* also differs from the *TcMT2-FP* which originates from the accession FP from the Ganges region (Roosens *et al.*, 2005), the latter being identical to *TcMT2b-LC*. On the other hand, the two accessions from the Ganges region have identical MT3 proteins (Fig. 1) that differ from the other accessions analysed in the present study.

Expression of MT2a, MT2b, *and* MT3 *in* T. caerulescens *accessions and* F_3 *sibling lines*

The relative expression of the *MT* genes, calculated as fold-change normalized to the endogenous reference gene

α-tubulin, was analysed in the roots and shoots of three *T. caerulescens* parental accessions and in low and high Zn-accumulating F_3 sibling lines from two inter-accession crosses at deficient, control, and excess Zn supply using quantitative real-time PCR.

The *TcMT2a* expression was higher in the roots and shoots of both calamine accessions (LC and LM), compared with the non-metallicolous accession LE (Fig. 2a). The highest expression was found in Zn-exposed LM shoots. In a cross between the calamine accession LC and the non-metallicolous accession LE, the expression of *TcMT2a* was higher in the low-accumulating F_3 lines than in the high-accumulating lines (Fig. 2b). However, among the F_3 lines of the cross between the calamine accessions LC and LM, there was no correlation between *TcMT2a* expression and Zn accumulation capacity (Fig. 2c).

The expression of *MT2b* was rather constant in the different accessions. As for *MT2a*, the highest expression of *MT2b* was found in LM shoots, but the differences were small (Fig. 3). There was no apparent correlation between the expression of *MT2b* and the Zn uptake capacity among the LC×LM F_3 lines (data not shown).

The greatest differences in expression between the accessions were found in *TcMT3*. The expression of *TcMT3* was >10-fold in the shoots of LM than in those of the other accessions (Fig. 4). In the roots of LM grown at Zn deficiency, the expression of *TcMT3* was ~3-fold compared with LC and 4-fold compared with LE. However, there was

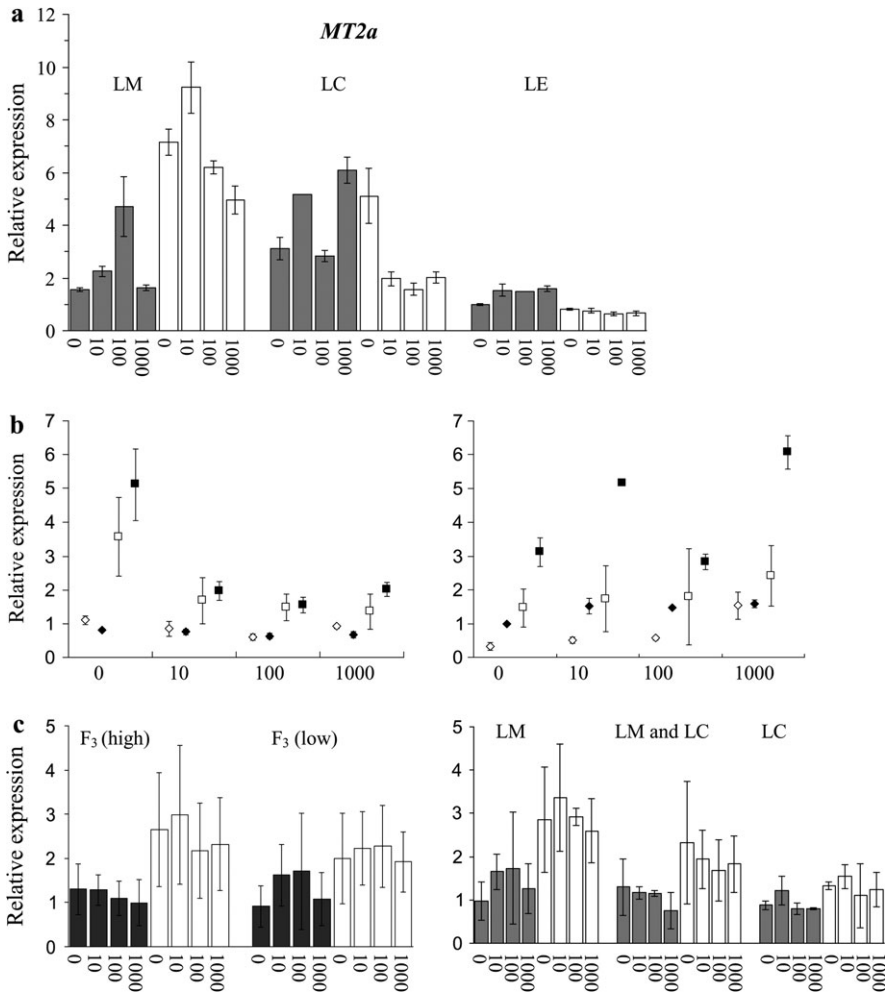

Fig. 2. *TcMT2a* expression in *T. caerulescens* at 0, 10, 100, and 1000 μM $ZnSO_4$ in (a) roots (grey) and shoots (white) of the calaminous accessions St Laurent le Minier (LM; high accumulation, high tolerance) and La Calamine (LC; low accumulation, high tolerance), and in the non-metallicolous accession Lellingen (LE; high accumulation, low tolerance); (b) in F_3 sibling lines of an LC×LE cross, two high or three low Zn-accumulating lines (open diamonds and squares, respectively), and in LC (filled squares) and LE (filled diamonds) shoots (left) and roots (right); (c) in the roots (dark grey) and in the shoots (white) of five low- and five high-accumulating F_3 sibling lines of an LC×LM cross, grouped according to high or low Zn accumulation (left) or according to the distribution of LM and LC alleles: five lines with exclusively the *MT2a-LM* allele, two lines with exclusively the *MT2a-LC* allele, and three lines containing both alleles (right). The data indicate the relative expression compared with the expression in LE roots at Zn deficiency (value 1), and represent means of three pooled roots or shoots. Error bars, ±SD.

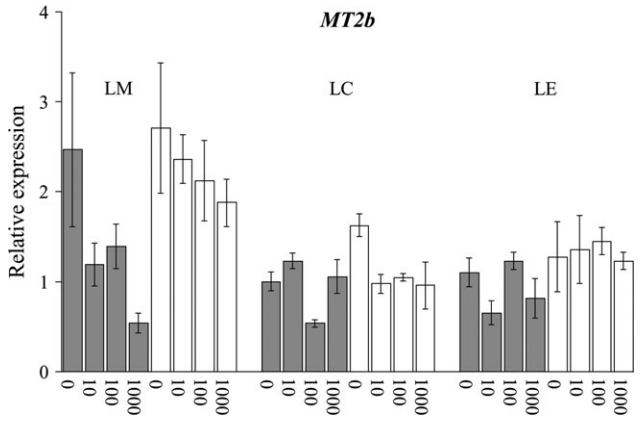

Fig. 3. *TcMT2b* expression in roots (grey) and shoots (white) of accessions St Laurent le Minier (LM), La Calamine (LC), and Lellingen (LE) at 0, 10, 100, and 1000 μM $ZnSO_4$. The data are the relative expression of samples consisting of three pooled roots or shoots compared with the expression in LC roots at Zn deficiency (value 1). Error bars, ±SD.

no correlation of Zn accumulation capacity and *MT3* expression among the F_3 lines.

Allele origins of MT2 *and* MT3 *in the F_3 lines*

As the *MT2a* and *MT3* alleles of LM and LC differ in their sequence, it was possible to establish the allele origins of the LC×LM F_3 lines. In addition to the sequence differences in the coding region, the *MT2a* alleles from LC and LM differ in the 3′-UTR, the LM allele being 3 bp longer than the LC allele, which was used to differentiate between the alleles. Among the LC×LM lines, five contained exclusively the *MT2a-LM* allele (three high- and two low-accumulating lines), two lines contained exclusively the *MT2a-LC* allele (both low-accumulating lines), and three lines contained both alleles (one low- and two high-accumulating lines). Among the F_3 families, high expression in the shoot was clearly associated with the *MT2a-LM* allele (Fig. 2c).

MT3-LM and *MT3-LC* alleles were identified from genomic DNA, in which the first intron of the LM allele is 16 bp longer than that in the LC allele. None of the 10 F_3 lines analysed contained exclusively the *MT3-LC* allele, and four contained exclusively the *MT3-LM* allele, of which two were high-accumulating and two were low-accumulating lines (data not shown). High expression in the shoot was clearly associated with the *MT3-LM* allele (Fig. 4c).

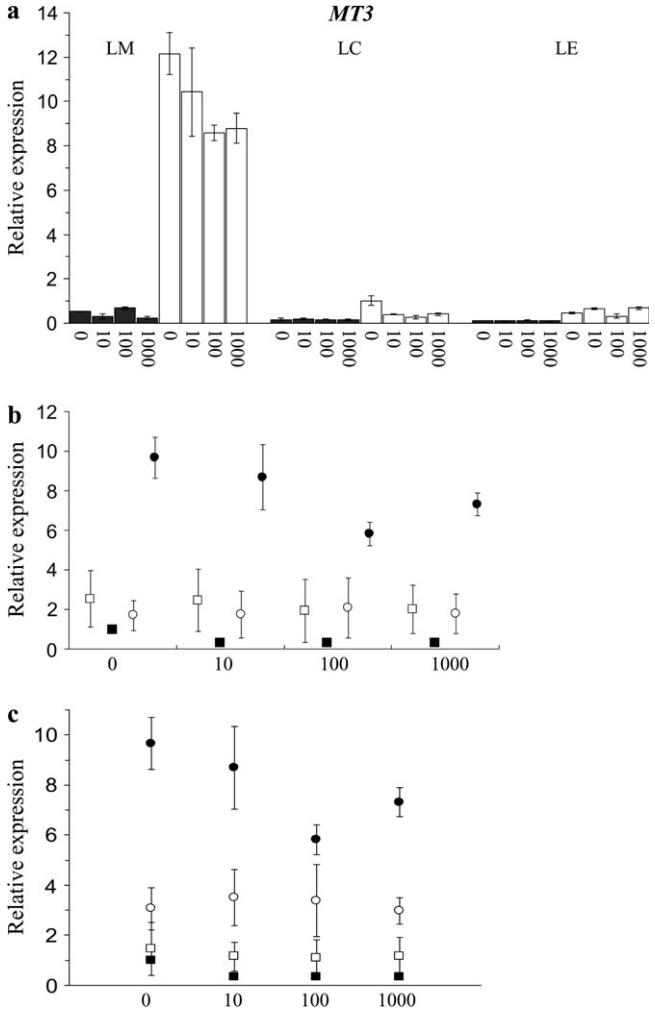

Fig. 4. Relative *TcMT3* expression in 0, 10, 100, and 1000 μM $ZnSO_4$ (a) in shoots (white) and in roots (black) of the parent calaminous accessions LM (high tolerance and accumulation) and LC (high tolerance, low accumulation), and in non-metallicolous LE (low tolerance, high accumulation); in shoots of five high and low Zn-accumulating F_3 lines from an LC×LM cross; (b) selected for low or high accumulation (open squares and circles, respectively) and in parent accessions LC (filled squares) and LM (filled circles); (c) grouped according to allelic distribution of the F_3 lines. Means and SDs of four lines containing exclusively the *TcMT3-LM* allele (open circles) and six lines containing both the *TcMT3-LM* and *TcMT3-LC* alleles (open squares) are shown. The data are the mean relative expression in samples consisting of three roots or shoots compared with the expression in LC shoots at Zn deficiency (value 1). Error bars, ±SD.

Localization of the MT2 protein

The TcMT2 protein was localized using whole-mount immunohistochemistry from the roots of *T. caerulescens* and from wild-type *Arabidopsis* as well as transgenic *Arabidopsis* lines expressing TcMT2 by using a TcMT2a-targeted anti-peptide antibody coupled to fluorescently labelled secondary antibody. In the roots, MT2 protein is localized mainly in the epidermal cells and in root hairs, both in *T. caerulescens* and in *A. thaliana* (Fig. 5), with relatively high abundance at the root tip. In transgenic *Arabidopsis* expressing *TcMT2a*, a strong fluorescence signal was observed especially at the root tip compared with the wild-type plants, indicating that the transformants produce TcMT2 protein.

The TcMT2a antibody was made against the spacer region between the cysteine-rich domains, with two and three amino acid mismatches between the two peptides and TcMT2b protein. As two MT2 isoforms are present in *Thlaspi*, it cannot be ruled out that the antibody binds to both MT2b and MT2a. However, the localization observed here is in line

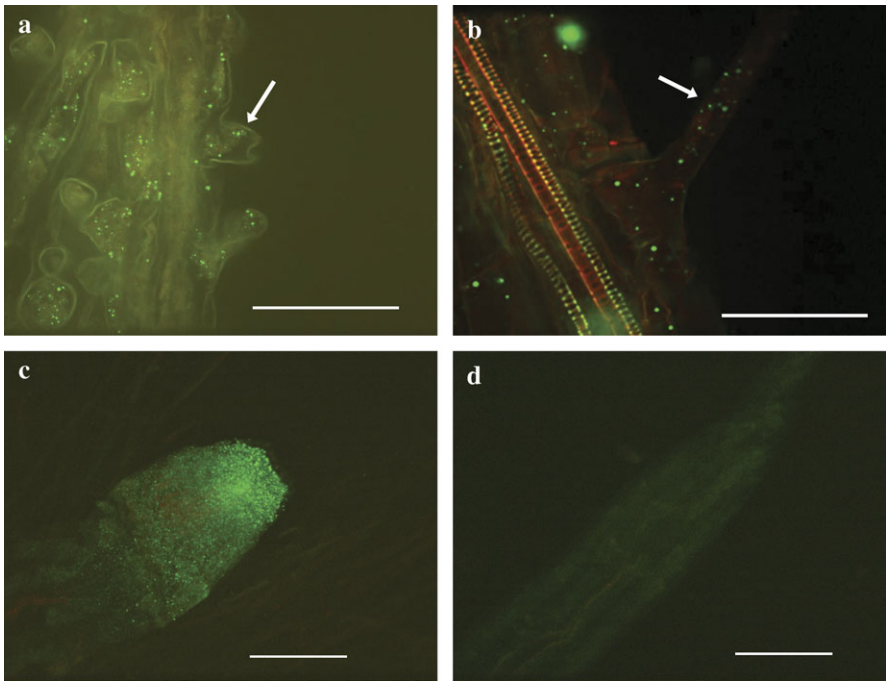

Fig. 5. Localization of MT2 protein (arrows) in root epidermal cells and root hairs in (a) 7-d-old *T. caerulescens*; (b) 4-d-old *Arabidopsis*. The root tip of 4-d-old *Arabidopsis* (c) transformed with *TcMT2* under the 35S promoter shows a bright fluorescence compared with (d) the wild type (Col-0). Immunocytochemical analysis was performed using primary antibodies raised against TcMT2. The secondary antibody was conjugated with Alexa Fluor 488, and fluorescent staining was imaged by laser-scanning confocal microscopy. The samples were stained with propidium iodide. Bar = 100 μM (c, d) or 50 μM (a, b).

with MT2a, with no indication of localization in the phloem which has been observed for MT2b (Guo *et al.*, 2003). The antibody is also able to detect *Arabidopsis* MT2, as the first peptide has a three amino acid mismatch with AtMT2a while the second peptide is identical to AtMT2a.

Expression of TcMT2a *and* TcMT3 *in* Arabidopsis

Arabidopsis seedlings were exposed to Cu, Cd, or Zn, and the root lengths were analysed from the vertically grown plants. In transgenic *Arabidopsis* lines expressing *TcMT2a* or *TcMT3*, the root length was reduced compared with wild-type *Arabidopsis* in control medium (Fig. 6). However, at 15 μM Cd, 40 μM Cu, or 400 μM Zn, the root lengths of the *TcMT2a* and *TcMT3* transformant lines were not significantly different from those in wild-type *Arabidopsis*, on average, and none of the individual transformant lines performed significantly better than the wild type. The same results were found in replicate experiments with other metal concentrations, i.e. 300 μM or 500 μM $ZnSO_4$, 20, 30, or 35 μM $CuSO_4$, or 5 μM or 10 μM $CdSO_4$ (data not shown). There were no differences in germination or in shoot growth in the transformant lines compared with the wild-type plants.

The *MT2* and *MT3* transformant lines did not show any differences in the concentrations of Cd and Zn in the roots or shoots compared with the wild-type *Arabidopsis* (Fig. 7). Also the root Cu concentration in the control solution was similar in the wild-type and the transformant lines (data not shown).

Discussion

The simultaneous isolation of both *MT2a*- and *MT2b*-type genes in this study shows that *T. caerulescens* has two *MT2* isoforms just like *Arabidopsis* does. The previously isolated *MT2* sequences are, in fact, examples of the two isoforms rather than of different alleles of the same locus. The *MT2* isolated from St Félix de Pallières (Roosens *et al.*, 2005) can now be classified as a 2b-type gene, whereas the isoform previously isolated from La Calamine (Hassinen *et al.*, 2007) is a 2a-type gene. The TcMT3s isolated have sequence differences in the N- and C-terminal domains, with the sequence from LC resembling the *Arabidopsis* sequence (Ala11 and Cys62) and that from LM being similar to a previously isolated sequence (Cys11 and Gly62) from St Félix de Pallières also originating from the Ganges region (Roosens *et al.*, 2004).

Differences in *MT* expression levels between the *Thlaspi* accessions under study were obvious. In general, the lowest expression was observed in the non-metallicolous accession LE, whereas the highest expression was observed in the accession LM, which is capable of accumulating high concentrations of Cd and Zn in the shoots. Especially striking was the high *MT3* expression in LM compared with the calaminous LC or non-metallicolous LE, which is in line with the higher expression in the FP from the Ganges region compared with another calamine and a serpentine accession (Roosens *et al.*, 2004). However, there is no correlation among the accessions in MT expression and Zn accumulation capacity (Assunção *et al.*, 2003a): LM has high MT expression and accumulation, LE has low MT

expression but high accumulation, whereas LC has generally higher MT expression but much lower accumulation capacity than does LE.

Zn accumulation capacity does not co-segregate with high MT expression in the F_3 sibling lines either. Even though there were some positive indications for *TcMT2a* expression with Zn accumulation in the LC×LE crosses, this was not evident in the F_3 lines from LC×LM crosses. However, the results show a correlation between high MT expression and the LM allele among the progeny of the cross. Among the F_3 lines, the expression of *MT2a* and *MT3* was higher in the lines having the LM allele exclusively. The variation in MT expression among F_3 lines is thus determined by allele origin, which could be due to differential *cis*-regulation of the *MT* genes.

When *Arabidopsis* plants were transformed with *TcMT2a* and *TcMT3*, no change in Zn content was evident compared with the wild-type plants, implying that overexpression of *TcMT*s in *Arabidopsis* does not enhance Zn accumulation. Moreover, the expression of *MT2a*, *MT2b*, or *MT3* was not induced upon Zn exposure. Taken together, the data imply that the differential MT expression between the accessions is not a direct cause or consequence of the variation in Zn accumulation capacity among the accessions, and the properties are clearly under independent genetic control. This has also been shown in yeast, where no direct involvement in Zn immobilization of yeast CUP-1 Cu-thionein or Zn-Cu thionein Crs5 is evident either (Pagani *et al.*, 2007).

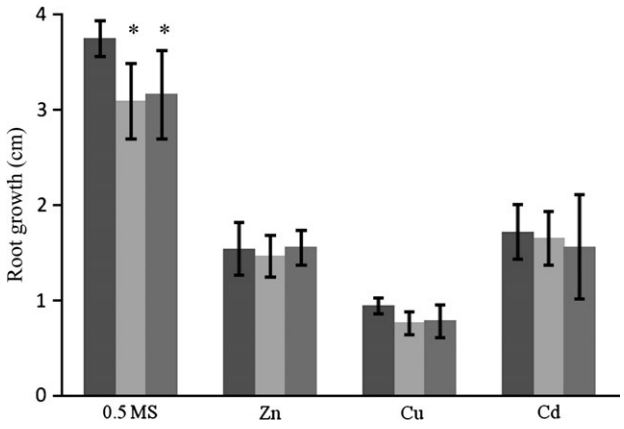

Fig. 6. Root length of wild-type (black), *TcMT2a* (light grey), or *TcMT3* (dark grey) transformed *Arabidopsis* seedlings grown in 0.5 MS (control) or in 0.5 MS spiked with 400 μM $ZnSO_4$, 40 μM $CuSO_4$, or 15 μM $CdSO_4$ for 11 d. The data are means of three plates (each containing 10 plants) of three independent *TcMT2a* or *TcMT3* transformants. Error bars indicate ±SD. *Means significantly different from wild-type (two-way ANOVA, followed by *a posteriori* testing using the minimum significant range, MSR).

The reason for the variation in MT expression between the accessions remains open. Since the homeostatic networks of different metals are strongly interconnected in yeast (Eide *et al.*, 2005), as well as in plants (van de Mortel *et al.*, 2006), MTs could be involved in the homeostasis or accumulation of metals other than Zn. Moreover, it has been shown that plant MT type 2 from *Quercus suber* can form complexes with either Cd, Cu, or Zn *in vivo* (Domenech *et al.*, 2006, 2007).

One hypothesis would be that MTs are related to Cd accumulation. In the LC×LM cross, Cd and Zn accumulation capacities were correlated (Deniau *et al.*, 2006), suggesting that the F_3 lines that are high accumulating for Zn are most probably also high accumulating for Cd, possibly due to Zn uptake via a highly Cd-preferential uptake system present in LM (Zha *et al.*, 2004). However, overexpression of MTs in *Arabidopsis* did not enhance Cd accumulation either. Additionally, MT expression and Cd accumulation are not correlated among accessions, since LE has higher Cd accumulation capacity, but lower expression than does LC (Assuncao *et al.*, 2003*a*). Moreover, MTs of type 2 and 3 are usually not significantly induced by Cd (Zhou and Goldsbrough, 1994; Roosens *et al.*, 2005). Therefore, it is unlikely that differential MT expression would be a direct cause or consequence of differential Cd accumulation capacity either.

Another hypothesis is that MTs have a role in Cd or Zn tolerance, rather than in the accumulation of these metals. The expression levels of MTs, i.e. LM>LC>LE, follow the order of Cd and Zn tolerance (Assuncao *et al.*, 2003*a*; Zha *et al.*, 2004). Tolerance-correlated enhanced MT2b

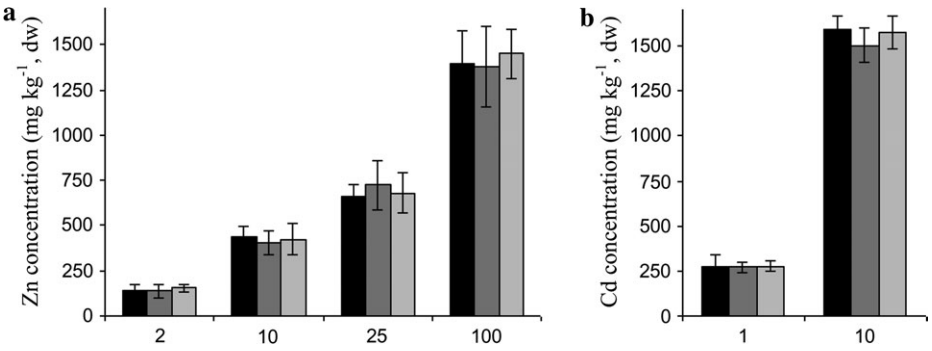

Fig. 7. Shoot (a) Zn concentration in control solution (2 μM Zn) or in solution spiked with 10, 25, or 100 μM $ZnSO_4$ and (b) Cd concentration in 1 μM or 10 μM $CdSO_4$ exposure in wild-type (black), *TcMT2* (dark grey), or *TcMT3* (light grey) transformed *Arabidopsis* seedlings. The data are means of three independent *TcMT2* or *TcMT3* transformants, with two samples (containing three pooled roots or shoots) each. Error bars, ±SD.

expression has also been found in calamine populations of non-hyperaccumulating metallophytes, e.g *Silene vulgaris* (Jack *et al.*, 2007). MTs seem to act as hypostatic factors in metal tolerance, enhancing the tolerance level when some other epistatic tolerance genes are also more highly expressed (van Hoof *et al.*, 2001). The TcMT proteins themselves bind and potentially provide tolerance to metals, as they can complement Zn and Cd tolerance in yeast (Hassinen *et al.*, 2007). Moreover, expression of *Brassica juncea BjMT2* increased tolerance against Cd when expressed in *A. thaliana*, as evidenced by increased chlorophyll content and shoot growth (Zhigang *et al.*, 2006). However, in *Arabidopsis*, the TcMT overexpression did not enhance tolerance to Zn, Cd, or Cu, as determined from root growth. No change in shoot growth was evident either. This might be due to the absence of some epistatic factor from the *Arabidopsis* genetic background, comparable with the situation in non-metallicolous *S. vulgaris* (Van Hoof *et al.*, 2001). Alternatively, these results might be explained by incorrect tissue specificity, due to the 35S promoter. These differences may also reflect the differential metal-binding capacities of MT proteins from different plant species, as shown for differential Cu tolerance in yeast mutants provided by MT3 from *Thlaspi* and *Arabidopsis* (Roosens *et al.*, 2004), due to differences in amino acid sequence. Moreover, it has been demonstrated that the sequences between the cysteine-rich domains, so-called spacer regions, also have a role in the functioning of MT proteins (Domènech *et al.*, 2006, 2007).

Another possible role for MTs could be in metal, especially Cu, homeostasis. When expressed in yeast, the *MT3-FP* from Ganges conferred higher Cu tolerance than did *Arabidopsis MT3* (Roosens *et al.*, 2004), whereas *MT3-LC* was not effective (Hassinen *et al.*, 2007). Also the *MT3-FP* expression is constitutive, not inducible by Cu excess, as was the case in a calamine and a serpentine accession (Roosens *et al.*, 2004), suggesting that the very high MT3 expression in FP or LM serves to guarantee Cu homeostasis in a cellular environment with high Cd content. Moreover, *A. thaliana* expressing *BjMT2* had reduced root growth in the absence of Cu exposure, which was proposed to be due to disturbances in metal homeostasis (Zhigang *et al.*, 2006). The reduced root growth at control conditions was also seen in *Arabidopsis* expressing TcMTs. At higher metal concentrations, the root growth was similar to that in the wild-type plants, perhaps reflecting the attainment of a balance between the overexpressed MT protein and metal availability. If the MTs are involved in Cu homeostasis, the addition of excess metals might displace Cu from TcMT and restore the homeostasis.

The localization of the MT2 protein also supports the role of MTs in metal buffering rather than in accumulation. In the roots of *T. caerulescens* and *A. thaliana*, the MT2 protein localized in the epidermal cells and root hairs, with a strong fluorescence at the root tip. The localization is in line with the previous findings where *Arabidopsis MT2* mRNA co-localized with the MT2 protein in the root tip (Murphy *et al.*, 1997). Localization at the root tip has been reported for the *Arabidopsis MT2a*; it was concluded that MT2a acts as a chaperone protecting the root apex, the first tissue to absorb excess metals (Guo *et al.*, 2003).

The results imply that MT2 and MT3 are not the primary determinants of Zn accumulation, as Zn accumulation and *MT* expression did not co-segregate. Moreover, ectopic expression of *TcMT2a* and *TcMT3* did not result in increased metal accumulation or metal tolerance. However, the higher expression of *MT*s in the superior metal-accumulating accession suggests that the MTs do contribute to the metal-adapted phenotype, possibly through enhancing either Cd or Zn tolerance, albeit as hypostatic factors, or Cu homeostasis in a high Cd/Zn environment.

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