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ORIGINAL ARTICLE

Isolation of Zn-responsive genes from two accessions of the hyperaccumulator plant *Thlaspi caerulescens*

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Abstract Several populations with different metal tolerance, uptake and root-to-shoot transport are known for the metal hyperaccumulator plant *Thlaspi caerulescens*. In this study, genes differentially expressed under various Zn exposures were identified from the shoots of two *T. caerulescens* accessions (calaminous and non-calaminous) using fluorescent differential display RT-PCR. cDNA fragments from 16 Zn-responsive genes, including those encoding metallothionein (MT) type 2 and type 3, MRP-like transporter, pectin methylesterase (PME) and Ole e 1-like gene as well as several unknown genes, were eventually isolated. The full-length *MT2* and *MT3* sequences differ from those previously isolated from other *Thlaspi* accessions, possibly representing new alleles or isoforms. Besides the differential expression in Zn exposures, the gene expression was dependent on the accession. *Thlaspi* homologues of ClpP protease and MRP transporter were induced at high Zn concentrations. MT2 and PME were expressed at higher levels in the calaminous accession. The MTs and MRP transporter expressed in transgenic yeasts were capable of conferring Cu and Cd tolerance, whereas the Ole e 1-like gene enhanced toxicity to these metals. The MTs increased yeast intracellular Cd content. As no significant differences were found between *Arabidopsis* and *Thlaspi* MTs, they apparently do not differ in their capacity to bind metals. However, the higher levels of MT2 in the calaminous accession may contribute to the Zn-adapted phenotype.

Keywords Cadmium · Differential display RT-PCR · Metal hyperaccumulation · *Thlaspi* · Zinc

Abbreviations

FDD	Fluorescent differential display
MT	Metallothionein
PME	Pectin methylesterase
RT-PCR	Reverse-transcription polymerase-chain reaction

Introduction

Zinc is an essential micronutrient required for a variety of functions in cellular metabolism, while being toxic at higher concentrations. Some plants have the ability to withstand, even accumulate, large quantities of Zn. In

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these hyperaccumulators, metals are extensively mobilized, taken up by the plant into the roots and loaded into the xylem. Once in the shoots, the metal excess is sequestered, e.g. in the mesophyll vacuoles and apoplast (Frey et al. 2000; Ma et al. 2005). Hyperaccumulation is thus a concerted action of several processes.

Thlaspi caerulescens is a Zn and Cd hyperaccumulator with considerable within and between population variability in the hyperaccumulation trait (Meerts and Van Isacker 1997; Assunção et al. 2001). Comparison of *T. caerulescens* accessions suggests that Zn tolerance, transport and accumulation are subject to independent variation (Assunção et al. 2003a, b). Moreover, the Zn accumulation capacity is governed by more than one gene (Assunção et al. 2006). Zinc tolerance is dependent on internal Zn sequestration capacity and, to a lesser extent, on reduced Zn accumulation (Assunção et al. 2003b).

At the molecular level, the most extensively studied genes from the hyperaccumulator plants are those encoding transmembrane metal transporters. The *ZNT1* gene, a member of the *ZIP* (ZRT/IRT-like proteins) transporter gene family, mediates high-affinity Zn uptake in *T. caerulescens* roots (Pence et al. 2000). The expression of *ZNT1* is higher in *T. caerulescens* than in the non-accumulator *Thlaspi arvense*, and is not down-regulated by elevated Zn, thus potentially resulting in the increased Zn uptake (Pence et al. 2000; Assunção et al. 2001).

The *T. caerulescens* P-type ATPase, TcHMA4, may participate in the root-to-shoot transport of Zn. The gene encoding TcHMA4 was originally isolated using functional complementation in yeast under Cd exposure (Bernard et al. 2004). The expression of *TcHMA4* is induced in the roots by elevated Zn and Cd as well as by Zn deficiency, and it is proposed to play a role in the xylem loading of Zn in plant roots (Papoyan and Kochian 2004).

A few genes suggested to play a role in the intracellular Zn sequestration have also been isolated. The *T. caerulescens ZTP1* (Assunção et al. 2001) is highly homologous to *Arabidopsis thaliana MTP1* (formerly *ZAT1*), a member of the cation diffusion facilitator family of transporters involved in vacuolar Zn sequestration (van der Zaal et al. 1999; Kobae et al. 2004). The *ZTP1* gene is more highly expressed in the roots and shoots of a Zn-tolerant *T. caerulescens* accession, suggesting that this gene may contribute to Zn tolerance (Assunção et al. 2001).

Metallothioneins (MTs) are suggested to contribute to metal buffering in *T. caerulescens* (Roosens et al. 2004, 2005). Other metal chelators such as organic acids or amino acids, particularly histidine and nicotianamine have also been connected to metal tolerance or hyperaccumulation in plants (Krämer et al. 1996; Sarret et al. 2002; Vacchina et al. 2003; Weber et al. 2004).

Despite recent advances the molecular basis of metal hyperaccumulation is still largely unresolved. With cross-species microarray (Weber et al. 2004; Becher et al. 2004; Hammond et al. 2006) there is a risk that important genes with low sequence identity will not be detected. Although a *T. caerulescens* specific cDNA array has been made (Plessl et al. 2005), the number of cDNA sequences is still far from that needed to make a "whole-genome" cDNA array (Rigola et al. 2006). Non-targeted approaches, such as differential display (DD), are viable alternatives. As demonstrated by Mandaokar et al. (2003), additional differentially expressed genes were found from *Arabidopsis* with DD compared to microarray. DD (Liang and Pardee 1992) has been applied for the isolation of genes involved in many processes in *Arabidopsis*, such as in the response to light (Dunaeva and Adamska 2001) and Cd (Suzuki et al. 2001), and in dormancy and germination (Toorop et al. 2005). In the present study, differentially expressed genes were identified by comparing shoot transcript patterns of two *T. caerulescens* accessions exposed to 0.2 or 500 μM Zn.

Materials and methods

Plant materials

Two *T. caerulescens* accessions were used. Accession La Calamine (LC) originates from soil contaminated with calamine ore waste (Zn, Cd, Pb) near La Calamine, Belgium. Accession Lellingen (LE) is from non-metalliferous soil at Lellingen, Luxembourg (Meerts and Van Isacker 1997). LC is much more Zn-tolerant than LE, whereas Zn accumulation is higher in LE than in LC (Assunção et al. 2001). The seeds were germinated in a 1:1:1 mixture of B2 peat, sand and garden compost. Six-week-old plants were transferred to 6-l pots (20 plants pot^{-1}) filled with nutrient solution described by Shen et al. (1997; experiment 2, final treatment), which contained (mmol m^{-3}): 400 $Ca(NO_3)_2$, 200 $MgSO_4$, 200 K_2HPO_4, 9.2 H_3BO_3, 1.8 $MnSO_4$, 0.2 Na_2MoO_4, 0.32 $CuSO_4$, 10.8 Fe(III)EDTA and 0.2 $ZnSO_4$. The solutions were continuously aerated and changed twice a week. The experiment was performed in a climate chamber (20/18°C day/night; 150 μmol m^{-2} s^{-1}, 14 h day^{-1}). After 4 weeks, the plants were exposed to four different Zn concentrations (0.2, 25, 100 and 500 μM $ZnSO_4$). After 1-week exposure, the

roots and shoots were harvested, frozen in liquid nitrogen and stored at −80°C.

RNA isolation

Total RNA was isolated from frozen plant tissue (2 g) with the cetyltrimethylammoniumbromide method (Chang et al. 1993). For Northern analysis, the plant RNA isolation kit (Qiagen) was used.

Fluorescence DD RT-PCR

The DNase-treated total RNA (0.5 μg), isolated from the shoots of LC and LE grown at 0.2 or 500 μM $ZnSO_4$, was reverse transcribed into 12 cDNA pools using oligo-dT primers (Table 1). The cDNAs were amplified with the same oligo-dT primers in combination with four arbitrary and two family-targeted primers labelled with fluorochrome Cy5 (Amersham Biosciences). The MT-targeted primer (MtU) was designed based on the Cys-rich domain of *Arabidopsis MT2* genes, and the *ZIP*-targeted primer (ZnU) based on the transmembrane domain VII of *ZIP*-type genes (Grotz et al. 1998). The estimated cDNA fragment lengths were designed to cover less than 200 bp of the coding regions. The PCR program was: 4 min at 94°C, 4 cycles of 1 min at 94°C, 4 min at 40°C, 1 min at 72°C, then 25 cycles of 45 s at 94°C, 2 min at 60°C, 1 min at 68°C, and the final 6 min at 72°C. All PCR reactions were carried out in duplicate. The absence of DNA was confirmed by including RNA without reverse transcriptase in fluorescence differential display (FDD). No amplification was ever detected in these controls, indicating that the fragments isolated were amplified from the cDNAs. A 4-μl aliquot of the PCR reactions containing Cy5 labelled internal sizer (100 or 300 bp) was applied to automated sequencer (ALFexpress) and the data were analysed using ALFwin fragment analyser (Amersham Biosciences). Whenever differences were found in the cDNA patterns, the PCR samples were subjected to manual run in 6% polyacrylamide gel. The cDNA fragments were excised from the gels, precipitated and amplified using the same PCR conditions as in the DD RT-PCR. After TOPO-TA cloning (Invitrogen), the fragments were sequenced.

Table 1 Oligo-dT (T1–T12), arbitrary (U1–U4) and family-specific primers (ZnU and MtU) used in the DD

Primer name	Primer sequence
T1	$CGGAATTCGGT_{12}AA$
T2	$CGGAATTCGGT_{12}AC$
T3	$CGGAATTCGGT_{12}AG$
T4	$CGGAATTCGGT_{12}AT$
T5	$CGGAATTCGGT_{12}CA$
T6	$CGGAATTCGGT_{12}CC$
T7	$CGGAATTCGGT_{12}CG$
T8	$CGGAATTCGGT_{12}CT$
T9	$CGGAATTCGGT_{12}GA$
T10	$CGGAATTCGGT_{12}GC$
T11	$CGGAATTCGGT_{12}GG$
T12	$CGGAATTCGGT_{12}GT$
U1	ACGGGAATTCGGTACTAAGG
U2	GCCCGAATTCTCCATGACTC
U3	ACGGGAATTCGACCATTGCA
U4	GGCCGAATTCATGGTAGTCT
ZnU	GCGTGAATTCCTTCTAGCTG
MtU	AGTCGAATTCTGCAAGTGCG

Sequence analysis

The sequences were analysed using TAIR WU-BLAST2 with AGI Transcripts dataset (−introns, +UTRs) and BLASTn at NCBI. Matches were considered relevant when the *E* values were below 10^{-5}. Alignment of the sequences was done using ClustalW.

Macroarray and Northern analysis

Macroarray analysis was performed as described by Zhang et al. (1996). The amplified cDNA fragments from five to ten individual clones originating from the same DD band were denatured and applied to duplicate nylon membranes with bio-dot microfiltration apparatus (Bio-Rad Laboratories). Radioactively labelled cDNA probes were prepared from 10 μg of RNA isolated from the shoot samples of LC and LE (0.2 and 500 μM Zn). Equal ^{32}P counts of purified probes were used in the hybridizations. Two fragments expressed at equal intensities in all samples in the DD were used as controls to monitor equal labelling. For Northern analysis, 20 μg of total RNA were used. The RNA samples were size-separated and hybridized according to standard procedures using ^{32}P-dCTP labelled cDNA fragments as probes.

Microarray

For microarray analysis, a different set of Zn-exposed LC and LE plants was used (Plessl et al. 2005). The growth conditions were as described by Assunção et al. (2003a). Seedlings grown for 3 weeks in moist peat were transferred to hydroponic solution and, after 1 week of cultivation, were exposed to 0, 2, 10 or 100 μM $ZnSO_4$, LC also to 1,000 μM $ZnSO_4$ for additional 2 weeks. RNA extraction, microarray design and analysis are described by Plessl et al. (2005). The means of four separate experiments derived from a

homogenized pool of 9–11 plants per treatment and population were calculated.

Isolation of full-length cDNAs

Thlaspi caerulescens lambda cDNA library was made from the shoots of LC exposed to 10 μM $ZnSO_4$. Library construction and screening are described by Assunção et al. (2001). Full-length *MT2*, *MT3*, *Ole e 1*-like and *MRP* genes were isolated from the library using the cDNA fragments obtained from DD as probes. The probes were labelled with ^{32}P using random priming (Rad prime kit, Invitrogen). Membranes were washed under stringent conditions (twice with 0.1 × SSC; 1% SDS at 65°C).

Yeast complementation analysis

Full-length *MT2*, *MT3*, *Ole e 1*-like and *MRP* from *T. caerulescens* accession LC, and *MT2a* and *MT3* from *Arabidopsis* were cloned into yeast shuttle vector pAJ401. For double transformation *TcOle e 1* was also cloned into vector pRS415. The constructs were transferred into a common laboratory yeast strain *Saccharomyces cerevisiae* DBY746 (MATα, his3-del1, leu2-3, leu2-112, ura3-52, trp1-289, Cyhr, cir+) for Cu and Cd tolerance analysis, and into the *ycf1* mutant yeast strain JWY53 (MATα, leu2-3, -112, ura3-52, his3-Δ 200, trp1-Δ 901, lys2-801, suc2-Δ 9, Mel-, ycf1-Δ1::hisG) for the determination of intracellular Cd content. Transformation was done according to Gietz et al. (1992).

For metal tolerance assay, 100 μl of overnight culture was mixed with top agar (5 ml YPAD, 1.5% agarose) and poured on YPAD plates. Metal solutions (0.1 M $CuSO_4$ or 2 mM $CdSO_4$) were applied on filter discs placed on the surface of the top agar. After 1-day incubation at 30°C, the inhibition zones around the filter discs were measured.

Intracellular Cd content was analysed according to Bernard et al. (2004) after 24-h exposure of the yeasts to 5 μM $CdSO_4$ by using atomic absorption spectrophotometer (Perkin-Elmer AAS 5100).

Results

Fluorescent DD was used for searching differentially expressed genes from the shoots of two *T. caerulescens* accessions (LC, LE) exposed to two different Zn concentrations (0.2 or 500 μM) in hydroponics for 1 week. These comparators were chosen to cover at once differences between the *Thlaspi* accessions and their response to Zn exposure. Twelve oligo-dT primers were used in combination with four arbitrary and two family-targeted primers (Table 1). The family-targeted primers were designed to amplify sequences related to the metal-binding domain of MT or the transmembrane domain of ZIP-like transporter in order to increase the chances of finding genes homologous to those already suggested to contribute to metal tolerance and transport. Altogether 576 reactions plus negative controls were run, including all primer combinations in all plant samples in duplicate.

Altogether 35 differentially expressed cDNA fragments were isolated and cloned. Sequence analysis indicated that four fragments (2, 9, *MT2*, *MT3*; Table 2) were isolated at least twice, usually with different primer combinations. This multiple priming is a known phenomenon (Suzuki et al. 2001). Eventually 16 cDNA fragments that generated signals in subsequent expression analyses were arranged (Table 2). The annotated *Thlaspi* cDNAs share 87–90% (88% on average) sequence identity with *Arabidopsis* genes in coding regions, which is in line with previous studies (Peer et al. 2003; Rigola et al. 2006). In FDD, amplification is based on poly-A signal and thus the cDNA fragments contain the 3′-UTR, which is usually more variable than the coding sequence. The length of the 3′-UTR was 250 bp on average, while the average length of the cDNAs was 375 bp. The sequence identity decreased to 58–90% (76% on average) when the UTR regions were taken into consideration.

In the database search, homology was found for 13 cDNAs (Table 2). Three fragments (2, 34, 37) had no significant homology in the *Arabidopsis* database or nucleotide or EST databases at NCBI. All three fragments were less than 200 bp in length, most likely representing 3′-UTRs. Two fragments (9, 21) showed highest homology to *Arabidopsis* intergenic regions where no cDNAs are annotated. Interestingly, the genes corresponding to these five sequences (2, 9, 21, 34, 37) were all expressed in *Thlaspi*.

Expression of the isolated cDNA fragments was studied with macroarray to confirm that correct cDNAs corresponding to the DD bands of interest were cloned. This was done by blotting the cDNA fragments onto duplicate membranes and hybridizing with 0.2 and 500 μM Zn-exposed shoot samples of the *Thlaspi* accession from which the fragments were originally isolated. Table 3 exemplifies the differential expression of genes originating from LE exposed to 0.2 μM Zn. The expression of selected genes in the shoots was subsequently studied with Northern analysis (Fig. 1). The fragments were also included in microarray and hybridized to a different set of Zn-exposed root samples of both accessions (Table 4).

Table 2 List of differentially expressed fragments isolated from *T. caerulescens* and comparison to *Arabidopsis* genes of which the best hit is presented

No	Acc	Size (bp)	Primers	Best homology	ID number	*E*-value
Isolated from Zn-exposed samples						
2	LC	123	T1/U2	No homology found		
9	LE	460	T1/MtU	*A. thaliana* chloroplast	NC_000931	1.4e−74
12	LC	265	T2/U3	*AtMT2a*	At3g09390	1.9e−09
14	LC	390	T2/ZnU	Pectinesterase	At3g59010	8.5e−26
19	LC	391	T3/MtU	*AtMT3*	At3g15353	7.4e−30
22	LE	465	T3/ZnU	*A. thaliana* expressed protein	At2g15960	2.1e−41
40	LC	1,050	T11/ZnU	*AtMRP10*	At3g62700	5.4e−178
Isolated from non-exposed samples						
1	LE	219	T1/U1	Ole e 1 allergen and extensin-family protein	At1g78040	2.3e−07
4	LE	300	T1/U2	Respiratory burst oxidase protein D	At5g47910	1.5e−11
13	LC	525	T2/U4	Regulator of chromosome condensation (RCC1) family protein	At3g26100	1.0e−60
20	LE	208	T3/ZnU	Nuclear RNA binding protein (RGGA)	At4g16830	7.4e−13
21	LE	205	T3/ZnU	*A. thaliana* hypothetical protein, genomic	At5g26730	1.3e−10
24	LE	553	T5/U3	ATP-dependent Clp protease subunit	Atcg00670	7.5e−12
26	LC	456	T6/U4	*A. thaliana* expressed protein	At3g15310	2.4e−06
34	LE	194	T9/ZnU	No homology found		
37	LE	193	T10/ZnU	No homology found		

Cut-off value for homology is $E = 1.0e{-}5$ (10^{-5})

No fragment number, *Acc* accession, *LE* Lellingen, *LC* La Calamine

Table 3 Macroarray hybridization of the fragments isolated from LE at 0.2 μM Zn

Fragment (number)	LE 0.2/LE 500
Respiratory burst oxidase D	4.9
RNA binding protein RGGA	3.4
Hypothetical protein (*21*)	11.9
Clp protease	3.2
Unknown (*34*)	2.4
Unknown (*37*)	4.5

The ratio of the normalized intensity of hybridization signals between LE shoots exposed to 0.2 μM versus 500 μM Zn is given

Metallothioneins

Two cDNAs with high homology to plant *MT*s were found (*TcMT2a-LC*, *TcMT3-LC*). Fragment 12 (265 bp) has high homology to *Arabidopsis MT2*. The deduced translation product contains an eight amino acid sequence (KCDPCTCK) identical to the Cys-rich C-terminal domain of AtMT2a, with one amino acid difference to AtMT2b (KCNPCTCK). The deduced amino acid sequence of the full-length cDNA, isolated from *Thlaspi* cDNA library, is also highly homologous to that of AtMT2a (90% sequence identity), most of the differences being present in the spacer region between the Cys-rich domains (Fig. 2). The sequence identity of TcMT2a-LC to AtMT2b is lower (80%) with differences also in the N-terminal Cys-rich region. We thus propose to call this gene *TcMT2a-LC* according to the accession from which this allele was isolated. Interestingly, *TcMT2a-LC* derived amino acid sequence differs considerably from the predicted TcMT2 sequence of *T. caerulescens* accession St Félix-de-Pallières (TcMT2-FP; Roosens et al. 2005; Fig. 2).

Because of rather high sequence similarity between *TcMT2a-LC* and the hypothetical *TcMT2b* (if present in *Thlaspi* and homologous to the corresponding *Arabidopsis* gene), fragment 12 instead of the full-length cDNA was used as a probe in subsequent expression studies. Most of this fragment consists of 3′-UTR which would probably be more variable between *MT2a* and hypothetical *MT2b*. In line with FDD, Northern analysis indicated higher expression in LC shoots at elevated Zn (25, 100, 500 μM), while in LE the expression was stronger at low (0.2 μM) or at high (500 μM) Zn exposures (Fig. 1). Overall, the transcript levels were higher in LC than in LE. Also in the roots the expression was 3.7–6.4-fold in LC compared to LE at 0, 2, and 10 μM Zn (Table 4). At high Zn concentrations, no induction was apparent in LC roots. In LE roots, the expression was up-regulated by Zn deficiency and, more pronounced, at 100 μM compared to 2 μM Zn (Table 4), indicating a similar tendency to that in the shoots.

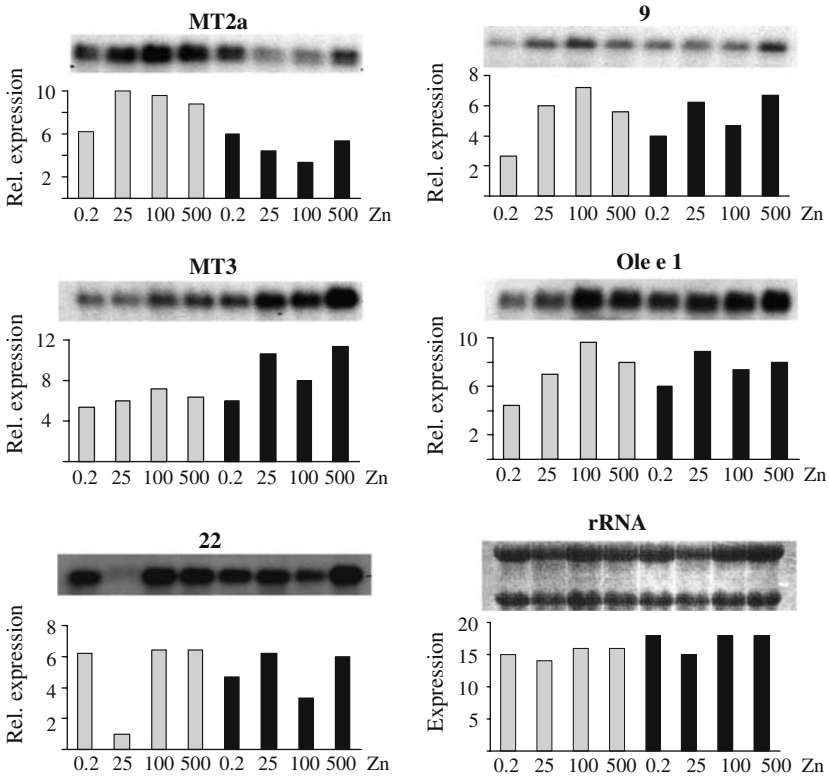

Fig. 1 Northern blots of shoot tissues from Zn-exposed (0.2–500 μM $ZnSO_4$) *T. caerulescens* accessions LC (*light grey columns*) and LE (*dark grey columns*) probed with *MT2a*, *MT3*, *Ole e 1*-like gene and fragments 9 and 22. The *columns* show relative expression of each gene normalized to 18 S RNA

Table 4 Microarray analysis of selected cDNA fragments hybridized with transcripts isolated from the roots of LC and LE exposed to various Zn concentrations, e.g. LE2 is Lellingen population exposed to 2 μM Zn

Fragment	LC0/LE0	SD	LC2/LE2	SD	LC10/LE10	SD	LC100/LE100	SD
Accessions								
Clp protease	0.5	*0.2*	0.4	*0.2*	0.5	*0.1*	0.3	*0.1*
TcMRP10	1.5	*1.1*	3.2	*2.7*	0.7	*0.1*	0.1	*0.1*
TcMT2a	3.7	*1.0*	6.4	*2.3*	4.6	*2.1*	1.6	*0.6*
TcMT3	2.0	*0.7*	1.4	*0.5*	1.2	*0.2*	1.3	*0.1*
	LE0/LE2	SD	LE100/LE2	SD	LC100/LC2	SD	LC1000/LC10	SD
Zn exposures								
Clp protease	0.6	*0.2*	1.2	*0.6*	0.6	*0.2*	3.4	*0.7*
TcMRP10	1.8	*0.5*	2.8	*2.5*	0.1	*0.0*	4.1	*1.3*
TcMT2a	2.2	*1.1*	2.8	*0.4*	0.4	*0.3*	0.4	*0.1*
TcMT3	1.0	*0.4*	1.1	*0.3*	1.2	*0.4*	0.6	*0.1*

The ratios of expression between accessions and Zn exposures are given. The cut-off values used for the ratios were: down-regulation: ≤0.5; up-regulation: ≥2.0

SD standard deviation

Fragment 19 from Zn-exposed LC was multiply primed, and a homologous fragment was isolated from LE. The cDNA fragment as well as the deduced amino acid sequence of the full-length cDNA has the highest homology to *T. caerulescens* TcMT3 from St Félix-de-Pallières (TcMT3-FP) and *Arabidopsis* AtMT3 (Fig. 2). As there is only a four amino acid difference from TcMT3-FP we refer our cDNA to *TcMT3-LC*. The most important differences in the amino acid sequences are present in two Cys residues (Cys_{11} for Ala_{11}, Gly_{62} for Cys), the LC and *Arabidopsis* sequences being identical.

The MT3 transcript levels in the shoots of both populations increased at elevated Zn exposures, as judged from Northern analysis, but the overall expression was higher in LE (Fig. 1). Based on microarray analysis, expression in the roots was twofold in Zn-deficient LC compared to LE (Table 4) but the standard deviation (SD) was high. At other Zn concentrations, the expression was relatively constant in both populations.

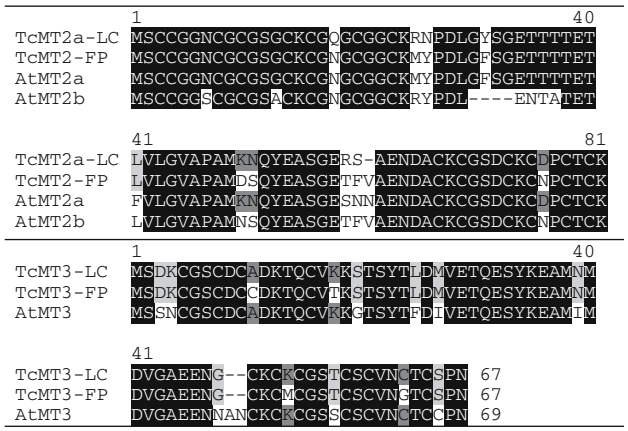

Fig. 2 Comparison of deduced amino acid sequences of TcMT2a-LC (*upper panel*) with TcMT2-FP (*T. caerulescens*, St Félix-de-Pallières accession, 665515), AtMT2a (*A. thaliana*, At3g09390) and AtMT2b (*A. thaliana*, At3g09390); and comparison of TcMT3-LC (*lower panel*) with TcMT3-FP (*T. caerulescens*, St Félix-de-Pallières accession, AAS99234) and AtMT3 (*A. thaliana*, At3g15353). Residues common to the proteins in each panel are shaded in *black*, residues common to TcMT2/3-LC and TcMT2/3-FP in *light grey* and residues common to TcMT2/3-LC and AtMT2/3 in *dark grey*. AtMT2b is included for comparison

When transferred into normal laboratory yeast, both *TcMT* genes were able to complement Cu and Cd sensitivity (Fig. 3a). *MT2* allowed the yeast to grow at higher metal concentrations than did the *MT3*s, with no significant difference between the *Arabidopsis* and *Thlaspi* genes. The same tendency was seen when *TcMT*s were introduced into *cup1* or *ycf1* mutant yeast strains. The increased Cd tolerance in the *ycf1* mutant strain is not related to a decreased Cd uptake. On the contrary, the Cd content of the transformed yeast was higher (Fig. 3b), suggesting that *Thlaspi* MTs bind Cd. There were no significant differences in the Cd content between *Arabidopsis* and *Thlaspi* MTs or between type 2 and type 3 MTs. However, in line with the tolerance tests, yeasts expressing MT2s had slightly higher Cd contents than did the MT3-transformed yeasts.

MRP transporter

Fragment 40, from Zn-exposed LC was the longest fragment isolated from FDD (ca. 1,000 bp). It is highly homologous to *A. thaliana MRP10* (At3g62700) and *MRP4* (At2g47800; Fig. 4). At deduced amino acid sequence level, identities are 87 and 82%, respectively. The almost full-length sequence isolated from *Thlaspi* cDNA library showed a similar level of identity to *AtMRP10* and *AtMRP4*. We call this gene *TcMRP10*. The protein has, similarly to those encoded by other *MRP* genes, an ABC (ATP-binding cassette) protein signature as well as Walker A and B motifs (Klein et al. 2006).

In Northern analysis TcMRP10 levels in the shoots were somewhat higher in LC than in LE, and slightly induced by Zn in both populations (Fig. 5). The expression was below the detection limit in the roots. In microarray, the expression in roots was higher in LC than in LE (Table 4) and induction by Zn was evident in both populations.

The nearly full-length *TcMRP10* isolated from *T. caerulescens* cDNA library lacked five 5′ codons compared to the *Arabidopsis* homologue *AtMRP10*. These amino acids were introduced by using primers designed based on the *Arabidopsis* sequence before introducing this reconstituted "full-length" cDNA into the yeasts. In addition, the truncated version, added with the start codon, was transferred into the yeasts. Both *TcMRP10*s provided the yeast a similar capacity to grow at higher Cu and Cd concentrations, even though the effect was less pronounced than with the *MT* genes (Fig. 3a). *TcMRP* had no effect on yeast Cd content (Fig. 3b).

Pectin methylesterase

A fragment highly homologous to *A. thaliana* pectin methylesterase (PME) (At3g59010) was isolated from Zn-exposed LC. Macroarray analysis confirmed that the expression was higher in LC shoots at 500 μM Zn compared to 0.2 μM Zn. Zn deficiency (0 μM Zn) increased the expression in LE compared to that in 2 μM Zn (4.3-fold, SD 2.1; microarray). In Zn-deficiency LC roots showed ca. threefold expression compared to LE which has been verified in a second microarray also for LC shoots (data not shown). Higher (two- to threefold) expression was found in LC roots compared to LE roots also at 2, 10 and 100 μM Zn exposure in this or in the confirmatory microarray study.

ATP-dependent Clp protease

Fragment 24, from LE exposed to 0.2 μM Zn shows similarity to *A. thaliana* plastid-encoded ATP-dependent ClpP protease subunit, which is part of the endopeptidase complex located in the thylakoid membrane. The expression in LE was higher in the shoots exposed to 0.2 than to 500 μM Zn (Table 3). Even though the greatest abundance of Clp proteases is found in the chloroplast, both mRNA and protein can be found in the roots as well. In *Thlaspi*, expression in the roots was higher in LE than in LC at all Zn exposures (Table 4). In LC roots the level increased at very high Zn exposures, which was confirmed in a second microarray experiment (data not shown).

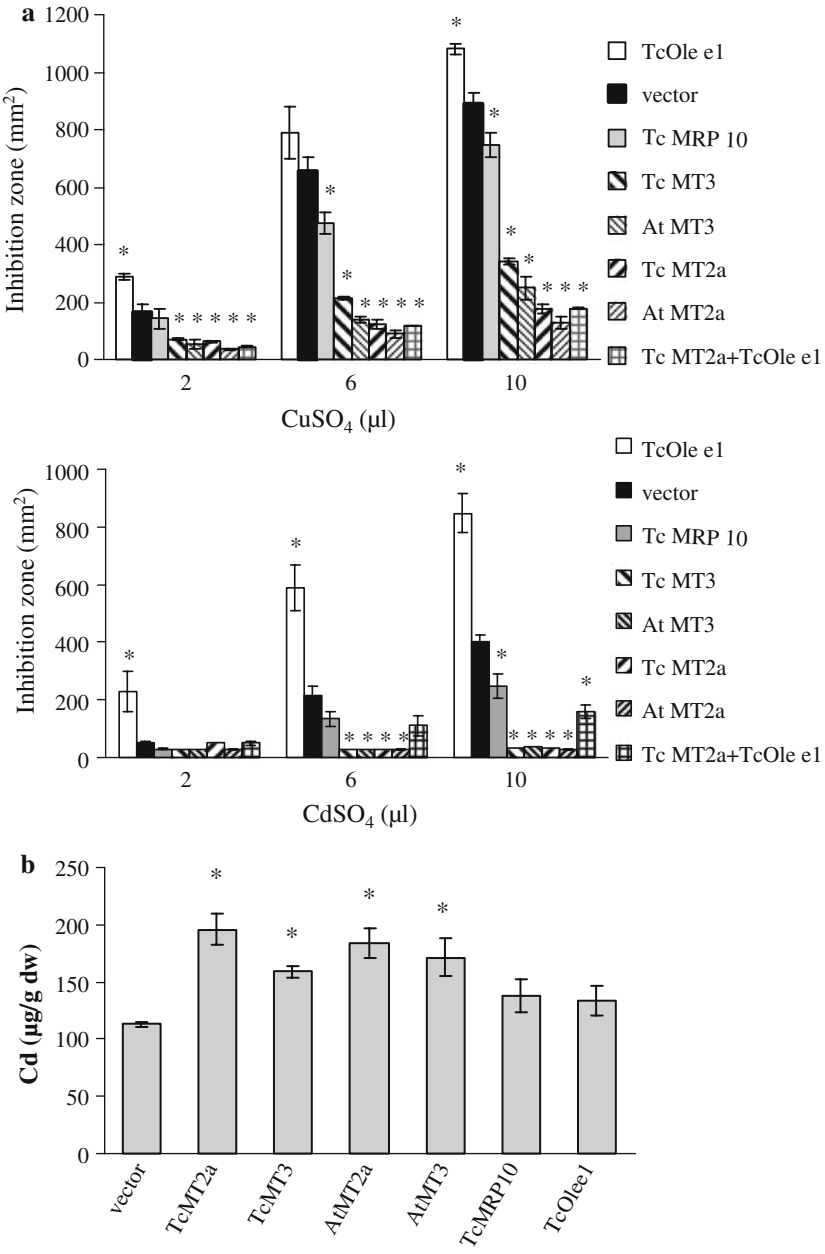

Fig. 3 **a** Cu (*upper*) and Cd (*lower*) tolerance, seen as smaller growth inhibition zones compared to vector control, of a common laboratory yeast strain (DBY746) expressing *Thlaspi* MT2a, MT3, MRP10 (*truncated version*) or Ole e 1-like or *Arabidopsis* MT2a or MT3. **b** Cd content of Δ*ycf1* yeast strain expressing *Thlaspi* or *Arabidopsis* MTs, *Thlaspi MRP10* or *Ole e 1*-like gene. Each tolerance test was done at least three times. *Error bars* indicate ±SD. *Means significantly different from vector control at $P<0.05$ using one-way ANOVA

Other isolated fragments

Fragment 2 with no significant homology in the *Arabidopsis* database was isolated from Zn-exposed LC. In FDD it showed an interesting expression pattern, being up-regulated in LC and down-regulated in LE shoots by high Zn concentrations. In LE roots, the expression was 2.4-fold (SD 0.6) in Zn deficiency compared to 2 µM Zn.

Fragments 9 and 22 were isolated from Zn-exposed LE. Fragment 9 has no homologues in the *Arabidopsis* cDNA database but shares a high sequence identity (91%) with an *A. thaliana* chloroplast genomic sequence. In *Arabidopsis*, no cDNAs are annotated to this region. Northern analysis showed, however, that the gene was up-regulated in both LC and LE shoots by Zn (Fig. 1). Fragment 22 represented a full-length cDNA. It shows homology to *A. thaliana* expressed protein (At2g15960) of 77 amino acids, which has no transmembrane regions or any annotated domains. Based on Northern analysis, the gene was slightly up-regulated in LE but not in LC shoots by Zn, but the expression patterns were complex (Fig. 1).

Fragments 13 and 26 had highest expression in LC exposed to 0.2 µM Zn. Fragment 13 is homologous to *A. thaliana* regulator of chromosome condensation (RCC1) family protein, whereas fragment 26 has homology to *A. thaliana* expressed protein. Based on

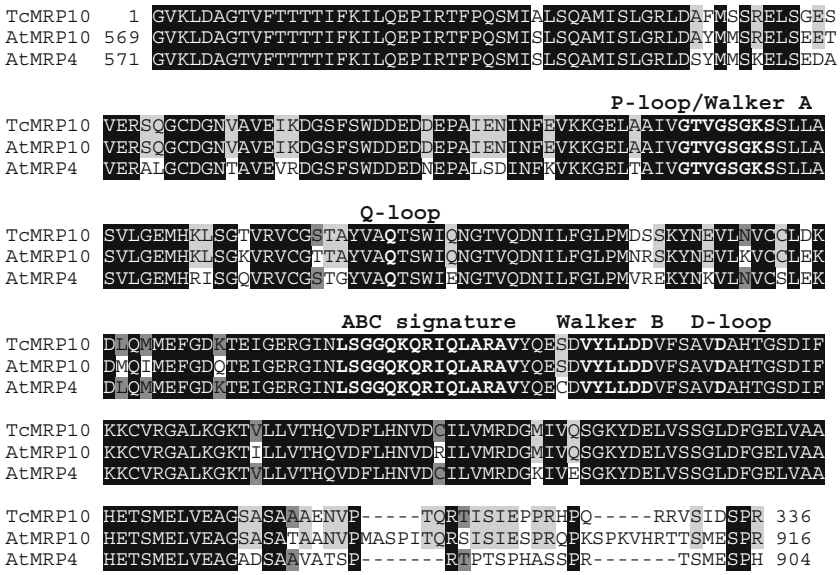

Fig. 4 Comparison of deduced amino acid sequence of TcMRP10 (fragment 40) with *A. thaliana* glutathione-conjugate transporter AtMRP10 (At3g62700) and AtMRP4 (At2g47800). Residues common to all three proteins are shaded in *black*, to TcMRP10 and AtMRP10 in *light grey* and to TcMRP10 and AtMRP4 in *dark grey*. Conserved ABC transporter domains are written in *boldface* type

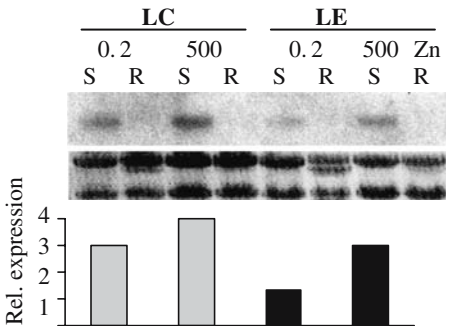

Fig. 5 Northern analysis of TcMRP10 (fragment 40) in LC and LE accessions after 1-week exposure to Zn. The *columns* show relative expression in shoots at 0.2 and 500 μM $ZnSO_4$ normalized to 18 S RNA. *S* shoot, *R* root

microarray analysis, both fragments were down-regulated in LC roots by 100 μM Zn compared to 2 μM Zn, being 0.1-fold (SD 0.0) and 0.5-fold (SD 0.3), respectively. Fragment 26 showed differential expression between the populations, with higher transcript level in LC than in LE roots at 10 μM Zn (3.7; SD 0.6).

Several fragments were isolated from LE exposed to 0.2 μM Zn. Fragment 1, which has homology to *A. thaliana* Ole e1 allergen and an extensin-family protein, was named *TcOle e 1*-like gene. The deduced amino acid sequence of the full-length cDNA has 77% identity to the *Arabidopsis* homologue. The protein has Ole e1 (IPR006041) and TonB-dependent receptor protein signatures (IPR010916), a combination normally not found in plant proteins according to Prosite database. The expression was up-regulated by Zn in the shoots of LC and, to a lesser extent, in LE (Fig. 1). In LE roots, the gene was up-regulated by 2.7-fold (SD 0.7) in Zn deficiency compared to 2 μM Zn. It is of particular interest that *TcOle e 1* made transgenic yeast more sensitive to Cu and especially to Cd (Fig. 3a). The increased Cd sensitivity was not related to an increased Cd uptake in the *ycf1* mutant strain (Fig. 3b) or in the common laboratory yeast strain. In a double transformant expressing both *TcOle e 1* and *TcMT2*, the toxic effects of Cu or Cd were alleviated and the yeast was able to grow at higher metal concentrations.

Fragment 4, homologous to *A. thaliana* respiratory burst oxidase, showed 3.1-fold (SD 0.9) expression in LE roots in Zn deficiency compared to 2 μM Zn. Additional fragments (20, 21, 34, 37) were also isolated from LE shoots exposed to 0.2 μM Zn (Table 2). The expression of the corresponding genes was confirmed with macroarray analysis (Table 3). These genes were not investigated any further.

Discussion

The aim of the present study was to identify and isolate genes that might play a role in the hyperaccumulation mechanism of the Zn hyperaccumulator plant *T. caerulescens*. Two different Zn exposures and two *Thlaspi* accessions known to differ in their Zn tolerance, root-to-shoot transport and accumulation were chosen for this experiment. The LE accession accumulates approximately fourfold Zn and has a higher root-to-shoot accumulation ratio for Zn compared to LC accession (Assunção et al. 2003a). On the other hand, LC has much higher tolerance to Zn than does LE.

Two Zn-responsive *MT* genes, *TcMT2a* and *TcMT3*, apparently involved in the intracellular metal binding, were identified. No other *MT* genes were found despite

the use of MT domain specific primers, suggesting that these genes encode MTs particularly relevant under Zn exposure. It has been proposed that the phloem-expressed, Cu-inducible AtMT1a and AtMT2b are involved in the distribution of Cu via phloem, whereas the chaperone-type MT2a and MT3 would protect the plant from Cu excess in the root tips and young leaves (Guo et al. 2003).

The *TcMT2a-LC* sequence of LC accession was different from the previously isolated *MT2* gene from *T. caerulescens* accession St Félix-de-Pallières from Ganges region (Roosens et al. 2005). The latter sequence has characteristics from both MT2a and MT2b types. At this point it is not clear if these sequences represent different genes or different alleles. Expression of *MT2* in the shoots of *T. caerulescens* (St Félix-de-Pallières) was reported to be fourfold compared to *A. thaliana*. Interestingly, in the present study differences in *MT2* expression were observed between the two *T. caerulescens* accessions, higher expression being observed in the shoots at high Zn supply of the Zn-tolerant, calaminous LC. The expression was also higher in the roots of LC than in those of the Zn-sensitive LE accession. Based on Southern analysis, the differences in the expression between the two accessions are not due to differences in gene copy number (data not shown). The St Félix-de-Pallières and LC accessions are equally tolerant to Zn (Assunção et al. 2003a), implying that higher levels of MT2 correlate with Zn tolerance. Zn treatment increases the expression of MT2 in the shoots of LC, and also of St Félix-de-Pallières (Roosens et al. 2005) as well as in *Arabidopsis* seedlings (Zhou and Goldsbrough 1994). The MT2 expression at low Zn concentrations (0.2 μM) in the shoots was equally high in the sensitive LE and in the tolerant LC accessions. Since metal accumulation in LE shoots is high already at low external Zn (Assunção et al. 2003a), it is possible that the higher expression reflects an increased requirement/ability for metal binding.

The *TcMT3* alleles isolated from LC and LE accessions were highly homologous in the translated regions. A different *MT3* allele was isolated from the St Félix-de-Pallières accession (Roosens et al. 2004) that has higher Cd tolerance and accumulation than LC and LE. The deduced amino acid sequence of the Cys-domain was shown to differ from that of *Arabidopsis*, possibly resulting in a larger cavity particularly for Cu and Cd chelation. The deduced amino acid sequences of the *TcMT3*s isolated in the present study do not share the same Cys-domain modification found in the St Félix-de-Pallières accession. This has been confirmed from a full-length cDNA isolated from LC cDNA library, and further from the genomic sequence of the *MT3* alleles from LC, LE and a serpentine population Monte Prinzera (data not shown). In yeast complementation studies, no significant differences were found between *Thlaspi* and *Arabidopsis* MT3s in Cd or Cu exposure, in contrast to the findings of Roosens et al. (2004) who reported that the ability of *TcMT3* (St Félix-de-Pallières) to increase yeast Cu tolerance was better than that of *AtMT3*. Differences in the *MT3* sequences and thus possibly in the function might reflect physiological adaptations to different soil types at the original location of the *T. caerulescens* accessions. The expression of *TcMT3* was up-regulated by Zn in the *Thlaspi* shoots, higher levels being present in LE than in LC, particularly at higher Zn exposures. Since LE is known to accumulate higher concentrations of Zn in the shoots (Assunção et al. 2003a), the *TcMT3* levels appear to reflect the shoot Zn levels and may thus have a function in metal homeostasis under Zn exposure.

Two genes with possible roles in metal sequestration were isolated in the present study, namely those encoding MRP transporter and PME. The *MRP* was highly homologous to *Arabidopsis MRP10* and *MRP4* genes. AtMRPs belong to a family of membrane-associated glutathione-conjugate transporters, which forms part of a superfamily called ABC transporters. In the yeast, ABC transporters are involved in the vacuolar sequestration of Cd (Ortiz et al. 1992; Wemmie et al. 1994). Bovet et al. (2003) studied the expression of several *AtMRP* genes in Cd-exposed *A. thaliana*. The expression of four genes, including *AtMRP4* and *AtMRP10*, was up-regulated exclusively in the roots where Cd is retained, but expression was also found in the shoots. In addition, Cu increased the expression of *AtMRP3* to the same degree as did Cd, whereas only a slight induction was detected with Zn. In our study, *TcMRP10* was up-regulated by high Zn concentrations in the roots and shoots of both LC and LE, the transcript levels being higher in the more Zn-tolerant LC. Expression of TcMRP in the yeast was able to complement Cd and Cu tolerance to some degree. Similarly, complementation of Cd sensitivity has been observed previously by at least AtMRP3 and AtMRP4 (Tommasini et al. 1998; Klein et al. 2006). In our study the yeast Cd content was not altered, suggesting that MRP was not able to sequester Cd to the vacuole. This might be due to the degradation or inappropriate targeting of the protein in the yeast. For example, targeting of a vacuolar transporter to outer membrane could result in metal efflux and hence increase the tolerance without increasing the Cd content. Neither can it be ruled out that by introducing the first five amino acids from *Arabidopsis*

sequence the functionality of the TcMRP10 protein may have changed. Recent findings indicate that MRP transporters have also other roles besides detoxification, and the vacuolar location has been questioned. In *Arabidopsis*, AtMRP4 has a function in stomatal opening (Klein et al. 2004). The possible role of *TcMRP10* in metal sequestration thus remains open.

Pectin methylesterase transcript levels were different in the two accessions, higher levels being present in the more Zn-tolerant LC than in LE. In *T. caerulescens*, Zn is mainly stored in the vacuoles of the leaf cells (Küpper et al. 1999) but the apoplast is also a major storage compartment (Frey et al. 2000). PME may have a role in the apoplastic storage of Zn. PMEs modify the properties of cell walls by demethylation of pectin residues, thus creating free carboxylic groups for interaction with divalent cations. Elevated expression of PME has been reported in *Arabidopsis* in long-term Hg exposure (Heidenreich et al. 2001). PME modulates Al sensitivity, since the free carboxylic groups are the major apoplastic binding sites for Al (Schmohl et al. 2000). A genetically modified potato cultivar over-expressing PME had higher Al content and was more sensitive to Al than was the wild type. It was also shown recently that the cell wall pectin has an important role in Cu accumulation in the fern *Lygodium japonicum* (Konno et al. 2005). Higher expression of PME in LC accession may thus result in an increased number of free carboxylic groups in the pectin matrix and thus increase the accumulation of Zn in the apoplast.

One of the genes isolated in the present study was *Ole e 1*-like gene. The expression was up-regulated by Zn in the shoots of both LC and LE, and by Zn deficiency in the LE roots. *Thlaspi Ole e 1*-like gene, as well as the *Arabidopsis* homologue, contains two domains, i.e. the Ole e1 allergen (extensin-family of proteins) and short N-terminal TonB signature. The bacterial TonB-dependent receptor proteins are involved in the energized transport of siderophore–metal complexes through the membranes (Postle and Kadner 2003). Interestingly, when expressed in yeast, the TcOle e 1 enhanced the toxic effects of Cu and, even more pronounced, of Cd. However, the Cd content was not altered significantly compared to the control yeasts, suggesting that Thlaspi Ole e 1 is not directly involved in Cd uptake or intracellular binding. The protein may contribute in a complex way to the maintenance of metal homeostasis or to the energy or metabolite balance during metal excess, as suggested by its domain.

One of the isolated genes with strong expression at high external Zn had a homology to ClpP1 protease. Clp belongs to a family of plastid proteases, which in *Arabidopsis* are constitutive under various stresses such as high salt (Zheng et al. 2002) and in senescence (Lin and Wu 2004). However, in *E. coli* a *clp* mutant was found to be more sensitive to environmental stresses, possibly due to a reduced ability to degrade misfolded proteins (Thomsen et al. 2002). If ClpP1 is related to stress responses, its induction by Zn in LC roots may imply an increased need for protein turnover at very high external Zn concentrations.

In conclusion, this study is amongst the first attempts to characterize the differences between *T. caerulescens* Zn hyperaccumulator populations at the non-targeted molecular level. Even though a small selection of genes was identified, genes with implications in metal adaptation were isolated. The function of the isolated genes *in planta* is currently underway to elucidate their possible involvement in Zn tolerance or accumulation.

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