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ORIGINAL ARTICLE

Comparison of two ecotypes of the metal hyperaccumulator *Thlaspi caerulescens* (J. & C. PRESL) at the transcriptional level

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Abstract This paper investigates differences in gene expression among the two *Thlaspi caerulescens* ecotypes La Calamine (LC) and Lellingen (LE) that have been shown to differ in metal tolerance and metal uptake. LC originates from a metalliferous soil and tolerates higher metal concentrations than LE which originates from a non-metalliferous soil. The two ecotypes were treated with different levels of zinc in solution culture, and differences in gene expression were assessed through application of a cDNA microarray consisting of 1,700 root and 2,700 shoot cDNAs. Hybridisation of root and shoot cDNA from the two ecotypes revealed a total of 257 differentially expressed genes. The regulation of selected genes was verified by quantitative reverse transcriptase polymerase chain reaction. Comparison of the expression profiles of the two ecotypes suggests that LC has a higher capacity to cope with reactive oxygen species and to avoid the formation of peroxynitrite. Furthermore, increased transcripts for the genes encoding for water channel proteins could explain the higher Zn tolerance of LC compared to LE. The higher Zn tolerance of LC was reflected by a lower expression of the genes involved in disease and defence mechanisms. The results of this study provide a valuable set of data that may help to improve our understanding of the mechanisms employed by plants to tolerate toxic concentrations of metal in the soil.

Keywords Heavy metals · Hyperaccumulation · Phytoremediation · *Thlaspi caerulescens* · Transcripts · Zinc

Dedicated to Professor Cornelius Lütz on the occasion of his 65th birthday.

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Introduction

Hyperaccumulator plants can tolerate and accumulate much higher concentrations of heavy metals in their shoots than non-accumulator plants (Baker 1981). Among the hyperaccumulator plants, *Thlaspi caerulescens* is relatively well studied and is widely found in western Europe on various metalliferous soil types, including mine wastes or smelter sites; it also occurs on non-metalliferous soil types (Escarré et al. 2000; Reeves et al. 2001). *T. caerulescens* is a known metal hyperaccumulator of Zn, Cd and Ni and belongs to the Brassicaceae family

(Assunção et al. 2003a; Milner and Kochian 2008). Foliar metal concentrations of 30,000 μg Zn g^{-1} d. wt (Brown et al. 1995), 3,000 μg Cd g^{-1} d. wt (Lombi et al. 2000) or 4,700 μg Ni g^{-1} d. wt (Schat et al. 2000) have been reported in healthy plants growing in nutrient solution or on metalliferous soils.

Zn is one of the most essential micronutrients for the plant system. Zn plays a fundamental role in several critical cellular functions such as protein metabolism, gene expression, chromatin structure, photosynthetic carbon metabolism and indoleacetic acid metabolism (Vallee and Falchuk 1993; Marschner 1995; Prasad 1995; Cakmak and Braun 2001). Additionally, Zn is an important component of many vital enzymes in which it has a catalytic, co-catalytic or structural role, as well as being a structural stabiliser for proteins, membranes and DNA-binding proteins (Zn fingers; Vallee and Falchuk 1993), yet it is toxic at higher concentrations. In contrast to Zn, Cd is a nonessential, toxic element and does not have any metabolic significance (Marschner 1995). Ni is also a micronutrient that is required for the urease in plants to break down urea into usable nitrogen, for iron uptake and for seed germination (Klucas et al. 1983). At higher concentrations, Ni is also toxic.

T. caerulescens as well as the metal hyperaccumulator *Arabidopsis halleri* have been intensively studied at the transcriptional level (Becher et al. 2004; Dräger et al. 2004). Large expression differences in genes for Zn homeostasis, stress response and lignin biosynthesis between *T. caerulescens* and the non-accumulator *Arabidopsis thaliana* have been reported (van de Mortel et al. 2006). The known coding sequences of *T. caerulescens* showed about 88.5% identity to the sequence of *A. thaliana* (Rigola et al. 2006). Similarly, great transcriptional differences were found between *T. caerulescens* and the non-hyperaccumulator *Thlaspi arvense* (Hammond et al. 2006). The comparison of contrasting ecotypes of *T. caerulescens*, adapted and non-adapted to heavy metal-contaminated soil, is aimed at understanding the genetics of heavy metal accumulation and tolerance. A higher Zn accumulation capacity and lower metal tolerance were consistently found in the non-metallicolous ecotypes of *T. caerulescens* compared to the metallicolous ecotypes (Meerts and Van Isacker 1997; Escarré et al. 2000; Assunção et al. 2003b). According to van de Mortel et al. (2006), metallothioneins that act as metal transporters are often strongly expressed in hyperaccumulators. An understanding of accumulation and tolerance mechanisms would also help to support the concept of phytoremediation: the use of green plants to remove pollutants from the environment or to render them harmless (Cunningham and Berti 1993). The removal of heavy metals from the soil by tolerant plants is also referred to as phytoextraction (Vassilev et al. 2004).

Recently, Zn-responsive genes from two accessions of *T. caerulescens* have been reported (Hassinen et al. 2007), and an expressed sequence tag (EST) collection from *T. caerulescens* zinc-exposed roots and shoots has been published (Rigola et al. 2006). Here, we report on the development of a microarray spotted with the *T. caerulescens* cDNAs out of this collection and present the results of the comparative transcript profiling of the roots and shoots from two different *T. caerulescens* ecotypes grown at different Zn exposures. As progress to a published microarray (Plessl et al. 2005), the new microarray includes both root and shoot ESTs. Thus, hybridisation of both tissues occurs simultaneously.

Materials and methods

Plant material

The two ecotypes of *T. caerulescens* used in this study were originally collected at two different sites: A non-metalliferous soil (Lellingen (LE), Luxembourg) and a soil highly contaminated with Zn, Cd and Pb (La Calamine (LC), Belgium). In the Zn-uptake experiments performed by Assunção et al. (2001), the LC plants maintained a normal growth and leaf pigmentation at 1,000 μM Zn in a nutrient solution. In contrast, LE plants already showed chlorosis at 100 μM Zn. However, the total plant Zn accumulation rate and foliar metal concentrations of LE were around 3- to 5-fold higher than in LC (Assunção et al. 2003b) considering the same level of Zn supply. Furthermore, LE displayed higher shoot to root concentration ratios for Zn. Apparently, the Zn tolerance in LC plants was associated with a decreased uptake and transport of Zn to the shoots compared to the uptake and translocation results to the non-adapted ecotype LE (Assunção et al. 2001).

Seeds from the two ecotypes were first grown on moist peat for 3 weeks, and then the plants were transferred to 1-l polyethylene pots (three seedlings per pot) filled with modified half-strength Hoagland's solution. The culture of the *T. caerulescens* plants and the conditions in the climate chambers were as described by Assunção et al. (2003b). After 1 week of growth, the plants were transferred to the same solution, albeit with different Zn concentrations (0, 2, 10, 100 and 1,000 μM $ZnSO_4$) for an additional 2 weeks (Assunção et al. 2003b). During harvest, the roots and shoots of each plant were carefully separated, frozen in liquid nitrogen and stored at −70°C.

Extraction of total RNA

Referring to both LC and LE, three batches of roots or shoots from three plants each, respectively, were homoge-

nised in liquid nitrogen. Total RNA was extracted from each batch using about 120 mg homogenised material with 1 ml TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's instructions with minor modifications: Both the complete chloroform step for phase separation and the complete RNA wash step were repeated once. The RNA concentration was measured using a NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). For quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), any remaining genomic DNA was digested with RNase-free DNase I (Promega, Mannheim, Germany). The extracted RNA was checked on a 1.5% (*w*/*v*) agarose gel after the DNase I digestion.

Microarray preparation

For the *T. caerulescens* microarray, complete inserts from randomly picked and partially sequenced ESTs were used (Rigola et al. 2006). The cDNAs originated from the library that was made from the roots and shoots of the 3-week-old *T. caerulescens* plants (accession LC), which were raised in a hydroponics solution containing half-strength Hoagland's solution and 10 μM $ZnSO_4$ (Assunção et al. 2001). To annotate the transcripts, Basic Local Alignment Search Tool (National Center for Biotechnology Information) database searches were carried out. Gene codes used in this publication refer to the unique codes of the '*Arabidopsis* Genome Initiative' (AGI) for *A. thaliana*. The current array consists of about 1,700 root and 2,700 shoot cDNAs. All cDNAs were obtained stepwise from our cooperation partner at the Wageningen University. According to Rigola et al. (2006), these cDNAs represent 709 unigenes of *T. caerulescens*.

PCR products of the cDNA clones (200 μl) were purified using 96-well multi-screen filter plates (Millipore, Bedford, MA, USA) and were resuspended in 16 μl spotting solution (3× standard saline citrate (SSC) supplemented with 1.5 M betaine; SSC—0.15 M NaCl, 0.015 M Na_3-citrate, pH 7). The PCR products were amino-modified in order to facilitate linkage to silylated microscope slides (Genetix Aldehyde slides, Genetix GmbH, München, Germany) by standard attachment chemistries. The produced spotting solutions were arrayed from 384-well microtiter plates onto the slides using a DNA array robot (model GMS 417; BioRobotics, Cambridge, UK). About 4,400 spots (1,700 root ESTs + 2,700 shoot ESTs) were printed on one microscope slide without any replication. After an incubation time of at least 2 days, the printed arrays were blocked (Huang et al. 2002).

The fluorescent probes were made using the indirect aminoallyl labelling method (http://pga.tigr.org/sop/M004.pdf). Twenty-five micrograms of total RNA was separately reverse-transcribed with SuperScript III (Invitrogen GmbH, Karlsruhe, Germany; dUTP to dTTP ratio, 4:1). After dissolving Cy3- and Cy5-dUTP in 21 μl dimethyl sulfoxide, 2 μl of each solution was dispensed into Eppendorf microtubes and concentrated to dryness in a centrifugal vacuum concentrator. Dried aminoallyl-labelled cDNA was dissolved in 11 μl Na_2CO_3 buffer (50 mM) and transferred to the dry Cy dyes. After mixing and incubating for 2 h in the presence of Cy3-dUTP or Cy5-dUTP (Amersham Biosciences, Freiburg, Germany), the samples were finally purified according to the protocol cited above. For each experiment, a dye swap of Cy3-dUTP and Cy5-dUTP was performed.

Hybridisation and microarray analysis

Pre-hybridisation of slides and the necessary steps for hybridisation with the Cy3- and Cy5-labelled probes were performed as described by Huang et al. (2002). Labelled probes were dissolved in 50 μl hybridisation buffer and hybridised overnight under a 24×50-mm^2 glass cover slip in VersArray hybridisation chambers (Bio-Rad, München, Germany). The subsequent washing steps were performed as described by Huang et al. (2002).

The arrays were scanned using an AXON GenePix 4000 scanner (Axon Instruments, Union City, CA, USA) at a resolution of 10 μm per pixel. To identify differentially expressed genes, the GenePix Pro (v 6.0) and Acuity (v 4.0, Axon Instruments, Union City, CA, USA) software package were used. Background fluorescence was calculated as the median fluorescence signal of nontarget pixels around each gene spot. Spots showing less than 50% difference between background and signal were automatically excluded. For normalisation, only median intensity of all spots were used which were not marked with 'bad', 'absent' or 'not found'. The ratios of both fluorescence signals (635 and 532 nm) computed by the Acuity software were further processed with GEPAS (Herrero et al. 2003), which is intended to perform tasks such as scale transformation, replicate handling, missing value imputation, filtering and normalisation of patterns. The data preprocessed with this tool were further processed by the expression profiler software EPCLUST (European Bioinformatics Institute, Cambridge, UK) in order to perform hierarchical clustering separately for root and shoot cDNA. The presented data show the medians of six separate microarray experiments per treatment or ecotype. The six experiments were performed on three homogenised pools of three plants each with the particular addition of the complementary dye swap. Gene expression was considered as up- or downregulated if the transcript level showed a minimum of 2.0-fold change. Spot intensities represent the means of the three homogenised pools (LC is labelled with Cy5) not including the spot intensities of the dye swaps.

Quantitative RT-PCR

To validate the microarray data, the expression of nine genes was quantified by RT-PCR relative to the 60S ribosomal protein L13 gene (BBC1 protein, which has 92% similarity to *A. thaliana* (At3g49010)) as a constitutively expressed control gene. Five micrograms of total RNA was reverse-transcribed using SuperScript III (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's instructions. To remove RNA, RNase H (USB, Cleveland, USA) and RNase A (Promega, Mannheim, Germany) were added, and the reaction was incubated at 37°C for 20 min. The cDNA concentration was measured with the fluorescent nucleic acid stain reagent RiboGreen® for RNA (Invitrogen GmbH, Karlsruhe, Germany). Primer design was performed with the Primer Express software (v 2.0, Applied Biosystems, Darmstadt, Germany). The primer search was based on the cDNA sequence data. Each PCR contained 12.5 µl of ABsolute QPCR SYBR® Green ROX Mix (ABgene, Epsom, UK), 0.5 ng cDNA and 120 nM of each gene-specific primer in a final volume of 25 µl. PCRs were performed in a 96-well plate with the 7500 Real Time PCR System (Applied Biosystems, Darmstadt, Germany) using SYBR Green to monitor cDNA amplification. The quantitative PCR conditions were 50°C (2 min) followed by 95°C (15 min) for one cycle, followed by 40 cycles at 95°C (15 s) and 60°C (1 min).

The data were analysed using the 7500 System SDS Software (v 1.2.2, Applied Biosystems). The absence of genomic DNA was confirmed by an analysis of the dissociation curves. Calculation of the relative gene expression was performed by using the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). According to that method, target gene expression was normalised to the control gene expression by subtracting the average threshold cycle (C_T) value of the 60S ribosomal protein L13 from the C_T of the target gene for the respective template. Between different cDNAs, the mean C_T for this control gene varied by less than 2.2% (±0.5 amplification cycles; mean C_T value 23.0). The presented qRT-PCR data are mean values calculated from three technical replicates from two separate biological experiments.

Results

The effects of Zn deficiency on gene expression were determined by omitting zinc, while concentrations of 2 µM $ZnSO_4$ were considered as a low metal supply medium (≈0.8 µM Zn^{2+} as in Weber et al. 2004). According to Assunção et al. (2001), the highest Zn exposure for LE was limited to 100 µM $ZnSO_4$ due to the development of leaf chlorosis. In LC, the first chlorosis became visible at 1,000 µM $ZnSO_4$.

After performing hierarchical clustering of the microarray experiments separately for root and shoot cDNA, genes could be identified that generally showed an obviously higher (ratio>2.0) or lower expression (ratio<0.5) by comparison at the given Zn concentrations. Hybridisation with the labelled cDNA of roots disclosed a higher expression of 3.0% (± 0.3) and a lower expression of 4.1% (± 1.7) of 4,072 (± 167) perceivable root and shoot genes in LC on the established microarray. When using cDNA from the shoots of both *T. caerulescens* ecotypes, 1.8% (±0.7) of the genes were higher, and 1.5% (±0.5) of the genes were clearly lower expressed in LC referring to 4,238 (±64) perceivable genes. As displayed in a Venn diagram (Fig. 1), hybridisation with both root and shoot cDNA of LC and LE reveals a differentially expression of 24 identical Zn-responsive genes (ratio>2.0 or <0.5) in at least two of the three different Zn concentrations. Consequently 144 Zn-responsive genes were exclusively differentially expressed by root cDNA. No annotation was available for around 13.7% of these 168 regulated genes. Simultaneously, only 89 extra genes were significantly regulated by using shoot cDNA for hybridisation, and a fraction of 11.5% of unknown genes was enclosed in this shoot setup.

The complete list of all the genes is available as a Supplementary Table. After creating a ranking order of these different expression profiles, a selection was made, and the functions of the selected genes that could be deduced are further discussed.

Zn-responsive genes in the two ecotypes LC and LE after hybridisation with root cDNA

Many of the selected Zn-responsive genes can be grouped into functional categories like oxidative stress, transport processes and energy supply. First, the expression levels of

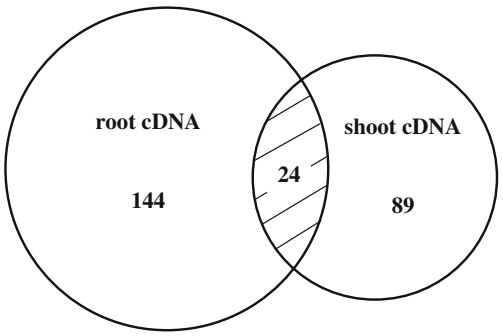

Fig. 1 Venn diagram of regulated Zn-responsive genes extracted by comparing cDNA of LC (metalliferous soil) with cDNA of LE (non-metalliferous soil). The diagram shows the number of overlapping and nonoverlapping genes after hybridisation with either root or shoot cDNA. The numbers represent gene ratios of >2.0 or <0.5 in at least two of the three tested Zn concentrations (0, 2 and 100 µM $ZnSO_4$)

the genes dealing with the responses to oxidative stress are shown. For example, the expression of the gene for arginyl-tRNA synthetase, which catalyses the formation of L-arginyl-$tRNA^{Arg}$ from L-arginine and $tRNA^{Arg}$ for protein synthesis, was strongly increased in LC compared to LE at all Zn concentrations (Table 1). Another selected gene belongs to the thioredoxin family. Transcript levels in LC were slightly higher at 2 μM $ZnSO_4$ and clearly higher when Zn was omitted. At 100 μM $ZnSO_4$, the thioredoxin-related transcripts existed at 11-fold more in LC than in LE. Also, the transcripts encoding enzymes like peroxidase or catalase 3 that function in the protection against oxidative stress in the form of H_2O_2 were increasingly assembled in LC. In contrast, a gene for an iron superoxide dismutase (FSD1) was less expressed in LC. Considering the responses to general stress, the gene for sucrose non-fermenting (SNF)-related protein kinase, which plays a role in intracellular signalling, was more expressed in LC at higher Zn concentrations. However, genes of a nitrate reductase (NR) and glutamine synthetase that play a role in N metabolism had reduced expression in LC. The class 1 non-symbiotic haemoglobin (class-1 ns-Hb) transcript levels of LC were diminished in all microarray experiments. This contrasted with previous results (Plessl et al. 2005) because different class-1 ns-Hb genes were analysed.

The genes involved in transport processes, like a gene that encodes a zinc transporter (cation diffusion facilitator (CDF) family), were overexpressed in LC, but the ratios between both ecotypes converged with increasing Zn concentrations. However, the genes for a metallothionein-like protein and for a nicotianamine synthase 1 (*Tc-NAS*1) were less expressed in LC. Regarding energy pathways and other physiological processes, transcript levels of a light-harvesting chlorophyll a/b binding protein (cab) was increased in LC at all Zn concentrations, as well as the levels of a copper ion binding gene (*SKS5*; Table 1).

Zn-responsive genes in the two ecotypes LC and LE after hybridisation with shoot cDNA

Similar to the classification using root cDNA, differentially expressed genes revealed by shoot cDNA are related to oxidative stress and transport processes. Additionally, genes were found that are involved in disease and defence mechanisms or in metabolic processes. Moreover, many, but not all, of the ESTs could be aligned to sequences provided by diverse databases. The expression of an unknown gene with no similarity to *A. thaliana* was strongly reduced in LC compared to LE (Table 2). In the Supplementary Table, other unknown genes with different expression levels are described.

Focussing on responses to oxidative stress, transcription levels of an arginyl-tRNA synthetase gene were strongly increased in LC than in LE, especially at 100 μM $ZnSO_4$ (Table 2). Using root cDNA for hybridisation already revealed this arginyl-tRNA synthetase gene that was highly expressed in LC (see above). A higher expression was also detected for a catalase 2 gene that protects against oxidative stress, mainly at higher Zn concentrations. However, the transcription levels of a gene encoding a glutathione transferase, which is also involved in detoxification, were diminished.

Hybridisation with the shoot RNA extracted many genes that are related to transport processes. For example, genes encoding a zinc transporter (*ZTP1*; Assunção et al. 2001) and an aquaporin were clearly higher expressed in LC. Furthermore, the expression levels of a gene encoding a γ-tonoplast intrinsic protein were also higher in LC compared to LE (Table 2); this protein could be involved in osmoregulation. Transcripts for a metallothionein-like protein were found that were also less expressed in LC. Two of these genes taking an active part in transport processes also appeared in Table 1 as root cDNA was used for hybridisations.

Corresponding to the responses to biotic stimuli, the expression of a disease resistance gene was downregulated in LC. The gene encoding myrosinase, which is involved in the glucosinolate catabolism and possesses thioglucosidase activity, was less expressed in LC; a gene for a cytochrome P450 enzyme, which is involved in the biosynthesis of glucosinolates (Table 2), was also less expressed in LC.

Generally, the transcript level of a myo-inositol 1-phosphate synthase gene in LC gradually exceeded the level of LE from 0 to 100 μM $ZnSO_4$ (Table 2); this enzyme catalyses the conversion of glucose 6-phosphate to L-*myo*-inositol 1-phosphate (Johnson and Sussex 1995). A similar result could be noticed for a major latex protein (MLP). The expression of a gene encoding a *N*-acetylornithine deacetylase-like protein was higher in LC (Table 2); this protein is a member of the metal-dependent aminoacylase family. The purified enzyme contains a single atom of zinc per monomer, and its activity can be increased greater than 2-fold by the addition of zinc (Javid-Majd and Blanchard 2000).

Zn concentration-dependent genes in LC after hybridisation with shoot cDNA

Compared to the $ZnSO_4$ supply of 2 μM, the expression of the copper/zinc superoxide dismutase gene was repressed when Zn was absent in the medium. The gene was induced when Zn was supplied at 100 and 1,000 μM $ZnSO_4$ (Table 3). In contrast, the iron superoxide dismutase gene was slightly induced when Zn was absent and was clearly downregulated at 100 and 1,000 μM $ZnSO_4$. The arginyl-tRNA synthetase gene was upregulated when Zn was deficient at 100 μM $ZnSO_4$ but was slightly repressed at a Zn concentration of

Table 1 Selected gene expression ratios comparing the two *T. caerulescens* ecotypes LE and LC using root cDNA for hybridisations

Gene annotation	Similarity to *A. thaliana*[c]	**LE0–*LC0***	SD	**LE2–*LC2***	SD	**LE100–*LC100***	SD	LC0[d]	LC2[d]	LC100[d]
Arginyl-tRNA synthetase[a]	67% to At4g26300	**4.2**	0.3	**17.2**	0.7	**14.2**	0.1	226	368	937
cab[a]	100% to At5g01530	**2.0**	0.8	**2.8**	0.3	**5.2**	0.3	82	147	133
Thioredoxin family protein[b]	95% to At5g61440	**2.7**	0.2	**1.7**	0.2	**11.0**	0.6	88	109	173
Zinc transporter[b]	79% to At2g46800	**4.4**	0.8	**3.3**	0.3	**2.8**	0.2	681	838	1,144
Peroxidase putative[a]	68% to At4g21960	**2.0**	0.5	**1.9**	0.2	**2.1**	0.1	5,930	8,991	11,366
Catalase 3[a]	94% to At1g20620	**2.0**	0.2	**2.4**	0.1	**2.7**	0.2	450	495	976
SNF1-related protein kinase[b]	83% to At3g23000	**1.9**	0.1	**2.2**	0.1	**2.7**	0.1	655	1,301	2,884
Copper ion binding (*SKS5*)[a]	94% to At1g76160	**2.2**	0.1	**2.9**	0.2	**2.6**	0.1	378	573	618
Carbonic anhydrase[a]	91% to At1g70410	**0.3**	0.3	**0.3**	0.2	**0.5**	0.2	169	353	410
Basic endochitinase[b]	90% to At3g12500	**0.3**	0.3	**0.3**	0.1	**0.3**	0.2	1,470	2,592	7,538
Nitrate reductase[a]	97% to At1g37130	**0.3**	0.4	**0.3**	0.1	**0.4**	0.2	401	543	800
Class-1 ns-Hb[b]	85% to At2g16060	**0.3**	0.2	**0.2**	0.2	**0.2**	0.2	445	873	632
Iron superoxide dismutase[b]	91% to At4g25100	**0.5**	0.1	**0.4**	0.1	**0.3**	0.2	1,507	2,390	1,495
Metallothionein-like protein[b]	84% to At1g07610	**0.5**	0.1	**0.4**	0.1	**0.3**	0.2	1,995	3,497	6,816
Glutamine synthetase[b]	98% to At1g66200	**0.2**	0.2	**0.5**	0.1	**0.5**	0.2	1,432	3,074	5,217
Alcohol dehydrogenase[b]	69% to At1g77120	**0.1**	0.5	**0.2**	0.2	**0.2**	0.4	1,337	3,417	3,363
Nicotianamine synthase 1[a]	*T. caerulescens*	**0.2**	0.3	**0.4**	0.3	**0.1**	0.3	92	92	129

Zero, 2 and 100: used $ZnSO_4$ concentrations. Italic-typed ecotype: less (≤0.5) or more (≥2.0) expressed. Bold-typed gene: also expressed in Table 2. Values are average ratios of three biological replicates combined with a dye swap each

SD standard deviation

[a] Shoot cDNA library

[b] Root cDNA library

[c] AGI gene code

[d] Spot intensity

1,000 μM when compared to 10 μM $ZnSO_4$ (Table 3). The major latex protein-related gene was repressed at all comparisons of the different Zn concentrations.

Comparison of microarray results and qRT-PCR

To validate the results of the microarray experiments, the transcript levels of nine differentially expressed genes were quantified by RT-PCR relative to the 60S ribosomal protein L13 gene (Tables 4 and 5). A significant correlation was found between the microarray and the qRT-PCR data, both with root and shoot cDNA (Tables 4 and 5). The magnitude of the relative changes in transcript abundance did not differ greatly between the two techniques with exception of the following genes: The ratios obtained for the arginyl-tRNA synthetase gene by qRT-PCR were generally lower than that obtained by array analysis for both root and shoot cDNA. Array data of class-1 ns-Hb did not agree well with the results of the qRT-PCR for experiments with 0 and 2 μM $ZnSO_4$. However, occasional differences in the gene expression data found by microarray technologies versus qRT-PCR are known in the literature (Holland 2002; Czechowski et al. 2004).

Discussion

Performing hierarchical clustering of the microarray results revealed different patterns of gene expression among the two different *T. caerulescens* ecotypes LC and LE (Tables 1 and 2). Genes with no hit to the *A. thaliana* proteome and/or unknown functions (Table 2; Supplementary Table) were found that show higher or lower levels of expression between the two ecotypes under particular Zn treatments (root cDNA, 13.7% out of 168 regulated genes; shoot cDNA, 11.5% out of 113 regulated genes). This is of special interest because these genes are metal responsive and may be a unique feature of the hyperaccumulator plant *T. caerulescens*. According to Rigola et al. (2006), a fraction of around 8% of their *Thlaspi* unigenes, which are the basis of the used microarray, has no detectable similarity with *Arabidopsis* or other databases. Obviously, comparing the two ecotypes through microarray analysis is an appropriate tool to bring unknown genes into focus, which might be '*Thlaspi* specific'. Rigola et al. (2006) started to study the genes with no detectable similarity to other plants. All in all, mostly genes with a putative function were found to be differentially expressed.

Table 2 Selected gene expression ratios comparing the two *T. caerulescens* ecotypes LE and LC using shoot cDNA for hybridisations

Gene annotation	Similarity to *A. thaliana*[c]	**LE0–*LC0***	SD	**LE2–*LC2***	SD	**LE100–*LC100***	SD	LC0[d]	LC2[d]	LC100[d]
Arginyl-tRNA synthetase[a]	67% to At4g26300	**8.7**	0.2	**4.7**	0.6	**13.9**	0.3	292	149	251
Major latex protein-related[b]	80% to At1g14960	**17.9**	0.5	**12.9**	0.2	**26.3**	0.3	240	289	71
N-Acetylornithine deacetylase-like protein[a]	88% to At4g17830	**4.1**	0.2	**3.3**	0.1	**4.8**	0.1	1,560	940	557
Zinc transporter[b]	79% to At2g46800	**2.3**	0.2	**2.1**	0.1	**2.4**	0.1	5,093	3,788	2,052
γ-Tonoplast intrinsic protein[a]	91% to At3g26520	**1.8**	0.1	**1.7**	0.1	**1.7**	0.1	14,523	14,021	8,554
Myo-inositol 1-phosphate synthase[a]	96% to At4g39800	**2.3**	0.1	**2.9**	0.2	**3.3**	0.0	769	1,484	333
Fructose bisphosphate aldolase[a]	96% to At2g21330	**1.5**	0.1	**1.8**	0.1	**2.0**	0.1	11,056	18,643	9,133
Catalase 2[a]	89% to At4g35090	**1.9**	0.1	**2.4**	0.1	**3.1**	0.1	3,140	3,018	1,150
Aquaporin[b]	91% to At2g39010	**1.5**	0.1	**2.1**	0.1	**3.9**	0.1	201	138	111
Metallothionein-like protein[b]	84% to At1g07610	**0.5**	0.1	**0.6**	0.2	**0.5**	0.2	197	214	160
Glutathione transferase[b]	91% to At2g30860	**0.4**	0.1	**0.5**	0.1	**0.4**	0.0	5,093	5,772	2,552
AP2 domain-containing protein[b]	67% to At3g16770	**0.4**	0.1	**0.6**	0.1	**0.6**	0.1	224	336	192
Myrosinase[a]	85% to At5g25980	**0.6**	0.1	**0.4**	0.1	**0.5**	0.2	3,493	2,159	1,169
Disease resistance protein-related[b]	83% to At1g33590	**0.6**	0.1	**0.5**	0.1	**0.6**	0.0	1,100	562	561
MCM3 homologue[b]	83% to At5g46280	**0.6**	0.1	**0.3**	0.1	**0.3**	0.1	639	258	133
Unknown[a]	not found	**0.3**	0.1	**0.2**	0.1	**0.2**	0.1	584	384	261
Cytochrome P450 family (CYP83B1)[a]	86% to At4g31500	**0.3**	0.1	**0.2**	0.1	**0.1**	0.1	137	81	45

Zero, 2 and 100: used $ZnSO_4$ concentrations. Italic-typed ecotype: less (≤0.5) or more (≥2.0) expressed. Bold-typed gene: also expressed in Table 1. Values are average ratios of three biological replicates combined with a dye swap each

SD standard deviation

[a] Shoot cDNA library

[b] Root cDNA library

[c] AGI gene code

[d] Spot intensity

Referring to the Venn diagram (Fig. 1), hybridisation with root cDNA resulted in more high and low expressed genes with ratios of >2.0 or <0.5, even though the microarray consists of 1,700 root and 2,700 shoot cDNAs. Mere around one fifth of the high and low regulated genes were identically extracted by cDNA of both tissues. Thus, performing microarray experiments hybridised separately with root and shoot cDNA leads to varying regulated genes directing attention to different metabolic processes. In the following, strongly regulated genes that were selected from

Table 3 Selected gene expression ratios comparing different $ZnSO_4$ treatments of LC using shoot cDNA for hybridisations

Gene annotation	Similarity to *A. thaliana*[c]	**LC2–*LC0***	SD	**LC2–*LC100***	SD	**LC10–*LC1000***	SD	*LC0*[d]	*LC100*[d]	*LC1000*[d]
Cu/Zn superoxide dismutase[a]	86% to At1g08830	**0.5**	0.2	**2.0**	0.2	**1.5**	0.1	700	1,598	1,720
Arginyl-tRNA synthetase[b]	67% to At4g26300	**3.6**	0.6	**10.4**	0.7	**0.7**	0.1	602	662	478
Major latex protein-related[a]	80% to At1g14960	**0.6**	0.3	**0.6**	0.3	**0.5**	0.5	237	201	151
Iron superoxide dismutase[b]	92% to At4g25100	**1.6**	0.2	**0.6**	0.1	**0.6**	0.1	4,361	2,048	1,351
Iron superoxide dismutase[a]	92% to At4g25100	**1.6**	0.2	**0.5**	0.2	**0.4**	0.1	1,969	488	484

Zero, 2, 10, 100 and 1,000: used $ZnSO_4$ concentrations. Italic-typed concentration: induced or repressed. Values are average ratios of three biological replicates combined with a dye swap each. Repression, ≤0.5; induction, ≥2.0

SD standard deviation

[a] Root cDNA library

[b] Shoot cDNA library

[c] AGI gene code

[d] Spot intensity

Table 4 Comparison of gene expression levels found in microarray experiments with that obtained by quantitative RT-PCR analysis

Gene annotation	LE0–*LC0*		LE2–*LC2*		LE100–*LC100*	
	Microarray	qRT-PCR	Microarray	qRT-PCR	Microarray	qRT-PCR
Arginyl-tRNA synthetase[a]	4.2±0.3	3.1±1.3	17.2±0.7	2.5±0.0	14.2±0.1	3.1±0.7
Zinc transporter[b]	4.4±0.1	2.5±1.8	3.3±0.3	1.7±0.6	2.8±0.2	2.3±0.3
Copper/zinc superoxide dismutase[b]	0.8±0.1	0.6±0.2	2.0±0.1	1.4±0.4	1.0±0.2	0.8±0.1
Aquaporin[b]	0.8±0.7	0.8±0.4	1.6±0.2	1.1±0.4	1.5±0.1	1.2±0.6
Major latex protein-related[b]	0.6±0.4	0.6±0.2	0.7±0.2	0.7±0.2	1.5±0.2	2.2±1.2
Iron superoxide dismutase[b]	0.5±0.1	0.4±0.1	0.4±0.2	0.1±0.1	0.3±0.2	0.2±0.0
Metallothionein-like protein[b]	0.5±0.1	0.4±0.1	0.4±0.1	0.2±0.0	0.3±0.2	0.2±0.2
Cytochrome P450 family (CYP83B1)[a]	0.6±0.4	0.4±0.0	0.6±0.3	0.5±0.4	0.7±0.2	0.6±0.2
Class-1 ns-Hb[b]	0.3±0.2	0.1±0.0	0.2±0.2	0.1±0.0	0.2±0.2	0.2±0.0
Correlation of microarray and qRT-PCR ratios	0.980**		0.827**		0.776*	

Ratios refer to root cDNA. Italic-typed ecotype: less or more expressed. qRT-PCR values are average values from two biological replicates calculated out of three technical replicates. Target gene expression was normalised to the control gene expression. For detailed description of qRT-PCR data evaluation, see "Materials and Methods". Statistical analysis of normal distribution and Pearson correlation was performed with SPSS 15.0 for Windows

*$p<0.05$; **$p<0.01$

[a] Shoot cDNA library

[b] Root cDNA library

4,400 root and shoot genes are discussed in more detail according to their biological processes.

Responses to oxidative stress

Although Zn is not a redox active metal, it has been shown in previous studies that an excess of Zn can lead to oxidative damage and can induce antioxidative defence mechanisms (Weckx and Clijsters 1997; Cuypers et al. 2001). A constitutively higher level of defence mechanisms against oxidative stress in LC could be an important feature for the adaptation to metalliferous soils.

Compared to LE, the transcript levels of an arginyl-tRNA synthetase were much higher in both roots and

Table 5 Comparison of gene expression levels found in microarray experiments with that obtained by quantitative RT-PCR analysis

Gene annotation	LE0–*LC0*		LE2–*LC2*		LE100–*LC100*	
	Microarray	qRT-PCR	Microarray	qRT-PCR	Microarray	qRT-PCR
Major latex protein-related[a]	17.9±0.5	25.3±16.4	12.9±0.2	10.4±0.4	26.3±0.3	48.4±50.7
Arginyl-tRNA synthetase[b]	8.7±0.2	3.0±2.6	4.7±0.6	2.7±2.7	13.9±0.3	3.7±1.2
Zinc transporter[a]	2.3±0.2	2.3±0.3	2.1±0.1	2.4±1.2	2.4±0.1	3.4±0.4
Aquaporin[a]	1.5±0.1	0.9±0.3	2.1±0.1	2.7±0.2	3.9±0.1	4.6±0.9
Copper/zinc superoxide dismutase[a]	0.8±0.1	0.6±0.2	1.5±0.3	2.8±2.3	1.7±0.2	1.8±0.0
Iron superoxide dismutase[a]	0.7±0.1	0.5±0.1	0.4±0.2	0.3±0.2	0.8±0.1	0.7±0.4
Iron superoxide dismutase[b]	0.7±0.1	0.6±0.0	0.4±0.2	0.3±0.2	0.7±0.1	0.7±0.4
Class-1 ns-Hb[a]	1.1±0.1	0.2±0.1	1.1±0.1	0.5±0.1	0.9±0.1	0.8±0.7
Metallothionein-like protein[a]	0.5±0.1	0.3±0.1	0.6±0.2	0.6±0.2	0.5±0.2	1.2±0.6
Correlation of microarray and qRT-PCR ratios	0.938*		0.969*		0.902*	

Ratios refer to shoot cDNA. Italic-typed accession: less or more expressed. qRT-PCR values are average values from two biological replicates calculated out of three technical replicates. Target gene expression was normalised to the control gene expression. For detailed description of qRT-PCR data evaluation, see "Materials and Methods". Statistical analysis of normal distribution and Pearson correlation was performed with SPSS 15.0 for Windows

*$p<0.01$

[a] Root cDNA library

[b] Shoot cDNA library

shoots of LC in all array experiments (Tables 1 and 2). This induction was in a fewer pronounced manner and was also confirmed by qRT-PCR (Tables 4 and 5). Arginyl-tRNA synthetase could be a sensor of arising oxidative stress (Lee et al. 1983) because according to Wiebauer et al. (1979), sulfhydryl groups of aminoacyl-tRNA synthetases can be easily oxidised. Unver et al. (2008) also found higher expression levels of an arginyl-tRNA synthetase when the boron tolerant plant, *Gypsophila perfoliata* L., was treated with an excess boron concentration. A higher expression of this gene seems to be an important feature of the metal tolerant ecotype LC indicating a better adjusted adaptation to oxidative stress caused by heavy metals. By examination of the expression of arginyl-tRNA synthetase within LC, this gene was induced in LC shoots when Zn was omitted and at moderately elevated Zn concentrations (Table 3).

Another gene with low spot intensities belonging to the thioredoxin family was highly expressed in LC, especially at 100 μM $ZnSO_4$. This thioredoxin family protein is 95% similar to the *A. thaliana* gene product At5g61440 that is described as a chloroplastic thioredoxin. Thioredoxins are small proteins that catalyse protein folding and are involved in the regulation of the redox environment of the cell (Gelhaye et al. 2005). Furthermore, the thioredoxin system is involved in reactive oxygen species (ROS) detoxification through several peroxiredoxin isoforms, glutathione peroxidases and peptide methionine sulfoxide reductases (Rouhier et al. 2004). Indeed, a higher expression of a putative peroxidase gene was also found in the LC roots and even with a remarkably high spot intensity (Table 1). Additionally, this expression profile also applies to a catalase 3 gene in the roots and a catalase 2 gene in the shoots (Tables 1 and 2). Catalases are the major H_2O_2 scavengers that remove the bulk of cellular H_2O_2 (Inzé and Van Montagu 2002). The expression of a gene encoding an iron superoxide dismutase (FSD1), however, which is involved in removing superoxide radicals (O^{2-}) by the conversion to O_2 and H_2O_2, was plainly downregulated compared to LE (Table 1). However, it is principally possible that the biological function of FSD1 has been transferred to Cu/Zn superoxide dismutases (SODs; Alscher et al. 2002). For example, when Zn was deficient, less Cu/Zn-SOD transcripts were found in LC, while FSD1 transcripts were slightly upregulated. This situation was reversed when Zn concentrations were high enough to allow the enzymatic activity of Cu/Zn SODs (Table 3).

In response to general stress, another protective component might be the SNF-1-related protein kinase, which could be involved in responses to nutritional and/or environmental stress (Hardie et al. 1998). This enzyme, with a higher expression in LC, plays a role in intracellular signalling. This kinase is able to phosphorylate and, consequently, inactivate NR post-translationally (Sugden et al. 1999). Additionally, the transcription levels of the NR gene were downregulated in LC compared to LE (Table 1). At the transcriptional level, NR is regulated by the availability of the substrate nitrate and by the end product glutamine (Kaiser et al. 1999). NR activity seems to also be sensitive to H_2O_2 (Sharma and Dubey 2005). As the ecotype LC obviously possesses more transcripts for ROS catalysing enzymes, LC might need less transcripts for NR compared to the more ROS stressed ecotype LE at identical Zn concentrations. As a useful side effect, LC could be better protected since the presence of nitrite can lead to the formation of NO, which can further react with active oxygen species to produce the strong oxidant peroxynitrite (Morot-Gaudry-Talarmain et al. 2002). According to Ohwaki et al. (2005), the expression of the class-1 ns-Hb gene is closely associated with that of the NR gene. Both array and qRT-PCR data demonstrate a very similar regulation of NR and class-1 ns-Hbs (Tables 1 and 4) when the both *Thlaspi* ecotypes were compared. The role of class-1 ns-Hbs during the process of nitrate reduction has been discussed in relation to the energy status of the cells (Nie and Hill 1997). Wang et al. (2000) hypothesised that ns-Hbs may act to protect the plants from certain toxic metabolites that are produced during nitrate reduction. Assuming a common nitrate-signalling pathway in plants (Sakakibara et al. 1997), the glutamine synthetase gene is actually less expressed in LC roots compared to LE, just like the NR gene (Table 1).

It is worth mentioning that the expression of a glutathione transferase gene was diminished in LC when shoot cDNA of the two ecotypes were compared (Table 2). Glutathione transferases are reported to be induced upon exposure to heavy metals in soybean seedlings (Ulmasov et al. 1995) and in rice roots (Moons 2003). Plant glutathione transferases fulfil functions involving glutathione to conjugate natural products, counteract oxidative stress, which could very well be reduced in LC (see above), mediate isomerase reactions and act as binding proteins of endogenous toxins and hormones (Edwards et al. 2000).

Transport mechanisms

Assunção et al. (2003b) demonstrated that the total plant Zn accumulation rates of LE were 3- to 5-fold higher than the rates of LC at 1 and 10 μM $ZnSO_4$. The increased transcript levels for metallothioneins, which perform intracellular complexation of metals, in LE compared to LC (Tables 1 and 2) might be caused by the elevated accumulation rate of Zn in LE (Assunção et al. 2003b). Array data for metallothionein was confirmed by qRT-PCR (Tables 4 and 5). According to Hassinen et al. (2009), metallothioneins of *T. caerulescens* are not primarily involved in Zn accumulation but may contribute to the metal-adapted phenotype. Nicotianamine, which forms highly stable chelates with Cu, Fe and

Zn, is essential for metal mobility in the phloem and intercellular space (Ling et al. 1999; Takahashi et al. 2003). For instance, *Arabidopsis* NAS1 catalyses the synthesis of a nicotianamine molecule from three molecules of *S*-adenosylmethionine (Suzuki et al. 1999). Increased expression levels of a nicotianamine synthase gene (*Tc-NAS1*) were found in LE compared to LC even though the spot intensities were very low (Table 1). Hybridisation with labelled root cDNA uncovered the expression of this *Tc-NAS1* gene by microarray analysis even though Mari et al. (2006) found a restriction of the *Tc-NAS* gene expression to the aerial part of *T. caerulescens* in their experiments.

Intensive spots were observed for the transcript encoding the γ-tonoplast intrinsic protein, also called tonoplast aquaporin, which facilitates the osmotically driven transport of water (Engel et al. 1994). This gene and an aquaporin gene, which is probably located in plasma membranes, were expressed in LC at higher levels when the shoot cDNAs were compared (Table 2). A higher expression of the aquaporin gene was also confirmed by qRT-PCR (Table 5). Aquaporins are usually sensitive to heavy metals (Eckert et al. 1999). For example, $ZnCl_2$ is known to block the pore of water channels (Tazawa et al. 1996). Thus, LC grown on metalliferous soil could avoid troubles with water transport because LC shoots obviously express more transcripts that are related to water channels in tonoplasts and in plasma membranes than LE. According to Aharon et al. (2003), symplastic water transport via plasma membrane aquaporins even represents a limiting factor for plant growth and vigour under favourable conditions. Enhanced symplastic water transport, however, has a deleterious effect during drought stress.

Distinctly more transcripts for a Zn transporter (*ZTP1*; Assunção et al. 2001) similar to the *Arabidopsis* Zn transporter, which is a member of the CDF family, were detected in the root and shoot cDNA of LC compared to LE (Tables 1 and 2). These results were verified by qRT-PCR (Tables 4 and 5). CDF family members could confer heavy metal tolerance by affecting heavy metal efflux from the cytoplasm either out of the cell or into an internal compartment (Kim et al. 2004). Expression in the root cDNA of LC was the highest when Zn was deficient and decreased with increasing Zn concentrations (Table 1). In the shoots, however, the induction ratio of LC always remained around 2.3 at all $ZnSO_4$ concentrations tested (Table 2). Recently, we reported that the movement of Zn in the roots of LE seemed to be supported by an induction of the metal transporter *TcZNT5* (88% similarity to *AtZIP5*; At1g05300) when Zn is absent (Plessl et al. 2005). However, at 100 μM $ZnSO_4$, this Zn transporter gene was repressed within LE, indicating a reduction of Zn movement, while in the roots of LC, repression was only evident at 1,000 μM $ZnSO_4$. This is in agreement with the moderate tolerance of LE against high Zn concentrations as found by Assunção et al. (2003b).

Responses to abiotic or biotic stimuli

As seen above, LC can clearly cope better with reactive oxygen species due to Zn stress than LE. Reactive oxygen species also contribute to signalling for the activation of defence responses (Dat et al. 2000). The expression levels of genes that correspond to defence mechanisms against abiotic or biotic stressors were indeed lower in the whole LC plant compared to LE. Thus, transcripts of a basic endochitinase, which responds to fungi, were reduced in the roots of LC compared to LE. Furthermore, the transcripts for myrosinase, also known as β-thioglucoside glucohydrolase, were diminished in the shoots of LC (Table 2). The hydrolysis products of glucosinolates, catalysed by myrosinase, may have important roles in the defence of the plant against microorganisms and insects (Bones and Rossiter 1996; Rask et al. 2000). Additionally, glucosinolate levels in the shoots of LC could be decreased during transcription because the gene expression of a cytochrome P450 (CYP83B1), which possesses a functional role in the biosynthesis of glucosinolates (Naur et al. 2003), was also lower in the shoots of LC as demonstrated by the array data (Table 2) and qRT-PCR (Table 5). This fits very well with previous observations of Noret et al. (2007) that the non-metallicolous populations of *T. caerulescens* generally have higher glucosinolate levels than metallicolous populations from southern France or from Belgium.

Another argument for an improved Zn stress tolerance of LC is the downregulation of the transcript level for a disease resistance protein in the shoots. This protein contains a series of leucine-rich repeats and is one of the specificity determinants of the plant immune response (Belkhadir et al. 2004). Moreover, the expression of an AP2 domain-containing protein encoding gene was reduced in the shoot cDNA of LC compared to LE (Table 2). This protein could act as a transcription factor since it contains an AP2 DNA-binding motif. The regulation of this putative transcription factor by disease related stimuli, by wounding and by abiotic stresses has been shown (Gutterson and Reuber 2004).

Energy pathways and other physiological processes

Surprisingly, higher transcript levels of a cab gene were determined in LC compared to LE after hybridisation with the root cDNA (Table 1). According to Chang and Walling (1992), a very low level of chlorophyll a/b-binding protein mRNA was found in soybean roots. Spot intensities of this gene were indeed low in LC and almost undetectable in LE. Usually, this nuclear encoded protein is involved to the

energy transfer processes and is involved in light harvesting and photoprotection (Bassi and Caffarri 2000).

A key enzyme of Zn-binding mechanisms could be the *myo*-inositol 1-phosphate synthase (INPS), which is involved in the biosynthesis of a mixed salt called phytate. Phytate is a common storage form of phosphates and cations in higher plants (Maga 1982). Benaroya et al. (2004) describe that mRNA of INPS increases in *Azolla filiculoides* plants when Zn^{2+} is added to the growth medium. In fact, an increasing expression ratio of this enzyme was shown in the LC shoots that correlated with rising Zn concentrations (Table 2). According to Zhao et al. (1998), however, binding of Zn with phytate is unlikely to play a significant role in the Zn tolerance in the shoots of *T. caerulescens*. Performing hybridisations with the shoot cDNA also showed a higher expression of a MLP-related gene in LC compared to LE. This induction was confirmed by qRT-PCR (Table 5). It seems that the transcript level of the MLP-related gene in LC was the highest at a Zn concentration of 2 μM (Table 3). Little is known about the biological function of MLP-related proteins. MLPs constitute a family of highly conserved, low molecular weight polypeptides (Nessler et al. 1985). Sagner et al. (1998) reported that in *Sebertia acuminata*, Ni is mainly localised in the phloem, with the laticifers and latex showing the highest concentrations of Ni in the plant. It remains to be seen if this observation is also relevant for the Cd/Zn hyperaccumulator plant *T. caerulescens*.

The transcription level of a copper ion-binding gene (*SKS5*) was clearly increased in the roots of LC over that in the roots of LE (Table 1). Multicopper binding proteins belong to a large family (SKS), and it is speculated that *SKS6* gene is expressed in response to environmental stimuli (Jacobs and Roe 2005). The *SKU5* gene was mainly expressed in the expanding root tissue of *Arabidopsis*, and the protein was shown to be localised to the plasma membrane and cell wall (Sedbrook et al. 2002). The reduced expression of *SKS5* in the ecotype LE might indicate a diminished root development. For example, root growth of the poplar clone 'Villafranca' was reduced up to 70% due to a high Zn supply (Lingua et al. 2008).

Conclusion

By comparative transcript profiling of the two *T. caerulescens* ecotypes LC (metalliferous soil) and LE (non-metalliferous soil), unknown and putative genes have been identified that are related to metal tolerance. The expression profiles of the two ecotypes suggest that LC has a higher capacity to cope with reactive oxygen species and to avoid the formation of peroxynitrite than LE. Furthermore, the formation of phytate, increased transcription of genes encoding for water channel components and for a Zn transporter, is conducive to explain the higher Zn tolerance of LC compared to LE. Thus, the increased Zn tolerance of LC was reflected by a lower expression of the genes involved in disease and defence mechanisms. Besides, metal chelator genes were less expressed in LC. Nevertheless, the genes described in this paper only mirror a selection of the putative genes involved in Zn adaptation.

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Conflict of interest The authors declare that they have no conflict of interest.

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