First publ. in: New Phytologist 185 (2010), 1, pp. 114-129

Transcriptional regulation of metal transport genes and mineral nutrition during acclimatization to cadmium and zinc in the Cd/Zn hyperaccumulator, *Thlaspi caerulescens* (Ganges population)

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Received: *5 June 2009*
Accepted: *13 August 2009*

New Phytologist (2009)
doi: 10.1111/j.1469-8137.2009.03051.x

Key words: acclimatization, cadmium, gene regulation, heavy metals, inductively-coupled plasma emission spectroscopy, quantitative *in situ* hybridization, zinc.

Summary

• We investigated changes in mineral nutrient uptake and cellular expression levels for metal transporter genes in the cadmium (Cd)/zinc (Zn) hyperaccumulator, *Thlaspi caerulescens* during whole plant and leaf ontogenesis under different long-term treatments with Zn and Cd.

• Quantitative mRNA *in situ* hybridization (QISH) revealed that transporter gene expression changes not only dependent on metal nutrition/toxicity, but even more so during plant and leaf development. The main mRNA abundances found were: *ZNT1*, mature leaves of young plants; *ZNT5*, young leaves of young plants; *MTP1* (= *ZTP1* = *ZAT*), young leaves of both young and mature plants.

• Surprisingly different cellular expression patterns were found for *ZNT1* and *ZNT5*, both belonging to the *ZIP* family of transition metal transporters: *ZNT1*, photosynthetic mesophyll and bundle sheath cells; *ZNT5*, nonphotosynthetic epidermal metal storage cells and bundle sheath cells. Thus, ZNT1 may function in micronutrient nutrition while ZNT5 may be involved in metal storage associated with hyperaccumulation.

• Cadmium inhibited the uptake of Zn, iron (Fe) and manganese (Mn), probably by competing for transporters or by interfering with the regulation of transporter gene expression. Cadmium-induced changes in cellular expression for *ZNT1*, *ZNT5* and *MTP1* could also be part of plant acclimatization to Cd toxicity. Defence against Cd toxicity involved enhanced uptake of magnesium (Mg), calcium (Ca) and sulphur (S).

Abbreviations: CLSM, confocal laser scanning microscope; EXAFS, extended X-ray absorption fine structure; GOI, gene of interest (i.e. the gene for which transcript levels are analysed); ICP-AES, inductively-coupled plasma emission spectroscopy; MT, metallothionein; MTP, metal transport protein (the *Thlaspi* MTPs are highly similar the *Arabidopsis* ZAT and belong to the CDF family); QISH, quantitative whole-mount mRNA *in situ* hybridization; ZAT, Zn transporter of *Arabidopsis thaliana* (a member of the CDF family); ZIP, ZRT/IRT-like protein, sometimes also referred to as 'zinc inducible protein'; ZNT, zinc transporter of *Thlaspi* (while the mammalian ZNTs belong to the CDF family, the *Thlaspi* ZNTs belong to the ZIP family of micronutrient transporters); ZTP, synonym of metal transport protein (MTP).

The authors will provide on request, for nonprofit research, all the biological and chemical materials not commercially available that are used for the experiments reported.

Introduction

Certain heavy metals are well known to be essential micro-elements needed for plants to grow and complete their life cycle. Elevated concentrations of these metals, however, can be toxic and induce inhibition of various plant metabolic processes (as reviewed, for example, by Baumann, 1885; Prasad & Hagemeyer, 1999; Küpper & Kroneck, 2005). Cadmium (Cd) can occur in very high concentrations in the soil that are detrimental or even lethal to most plants, as a result of mining (Van Geen *et al.*, 1997), smelting activities (Buchauer, 1973), dust from car tires (Lagerwerff & Specht, 1970; Fergusson *et al.*, 1980) or application of sewage sludge (McBride *et al.*, 1997). Photosynthetic reactions are some of the most important sites of inhibition by Cd and other heavy metals (Prasad & Strzałka, 1999; Küpper & Kroneck, 2005).

Plants have developed a number of strategies to resist the toxicity of heavy metals (see Prasad & Hagemeyer, 1999; Cobbett & Goldsbrough, 2002 and Küpper & Kroneck, 2005 for recent reviews). Such strategies include the functioning of metal efflux pumps, sequestration in cells and intracellular compartments where metals can do the least harm, and binding of heavy metals inside the cells by strong ligands. Most heavy metal resistant plants, called 'excluders', prevent the accumulation of heavy metals inside their tissues (Baker, 1981). Others actively take up heavy metals, and accumulate them in their shoots up to several per cent of the dry mass. These plants, native to metal-rich soils, are therefore called 'hyperaccumulators' (Brooks *et al.*, 1977). In nature, heavy metal hyperaccumulation can serve as a defence against pathogens and herbivores (Boyd & Martens, 1994; Martens & Boyd, 1994; Boyd *et al.*, 2002; Hanson *et al.*, 2003; Jhee *et al.*, 2005; Palomino *et al.*, 2007). Human interest in them, however, mainly arose from the opportunities to exploit them for the decontamination (phytoremediation) of anthropogenic heavy metal contaminated soils and for the commercial extraction (phytomining) of high-value metals (mainly nickel (Ni)) from metal-rich soils (Baker *et al.*, 1994; McGrath & Zhao, 2003; Chaney *et al.*, 2005). *Thlaspi caerulescens* is probably the best-known Cd/zinc (Zn) hyperaccumulator, accumulating Zn (e.g. citation of the work of Risse in the article of Sachs, 1865; Brown *et al.*, 1995) and Cd in the southern French (Ganges) ecotype (Lombi *et al.*, 2000; Küpper *et al.*, 2004) to several per cent of its shoot dry mass. Hence, it has been proposed that *Thlaspi caerulescens* can be studied as a hyperaccumulator model species to provide basic information about metal metabolism and for designing more effective hyperaccumulators via traditional breeding or genetic approaches (Assunção *et al.*, 2003; Peer *et al.*, 2003, 2006; reviewed by Chaney *et al.*, 2005).

The mechanisms by which plants hyperaccumulate heavy metals in their shoots and prevent phytotoxicity of these metals have been the subject of many studies. Nonetheless, many of these mechanisms are still under debate (Pollard *et al.*, 2002). Enhanced uptake of metals into the root symplasm was found in *T. caerulescens* compared with the related nonaccumulator, *Thlaspi arvense* (Lasat *et al.*, 1996, 1998), and a reduced sequestration into the root vacuoles was associated with the higher root to shoot translocation efficiency of *T. caerulescens* (Shen *et al.*, 1997; Lasat *et al.*, 1998). By contrast, sulphur-containing metal-binding ligands such as phytochelatins were shown not to be relevant for Cd or Zn storage or detoxification in *T. caerulescens*. For example, phytochelatin levels were shown to be lower in this plant than in the related nonaccumulator, *T. arvense* (Ebbs *et al.*, 2002), inhibition of phytochelatin synthesis in hyperaccumulators did not affect their Cd resistance (Schat *et al.*, 2002) and direct measurements of the Cd and Zn ligands by extended X-ray absorption fine structure (EXAFS) showed that most of the Cd in this species is not bound by any strong ligands, but primarily by weak oxygen ligands (Salt *et al.*, 1999; Küpper *et al.*, 2004). Nevertheless, metallothionein genes have been found to be highly expressed in *T. caerulescens* (Papoyan & Kochian, 2004; Roosens *et al.*, 2004), and differences between *T. caerulescens* and *Arabidopsis thaliana* metallothioneins have been examined (Roosens *et al.*, 2004, 2005), but their function remains unclear. Studies of cellular metal compartmentation have shown that in most hyperaccumulators the metal is sequestered preferentially into compartments where they cannot damage metabolic processes. The primary metal storage compartment seems to be the leaf vacuole (Küpper *et al.*, 1999, 2001; Frey *et al.*, 2000; Bidwell *et al.*, 2004; Broadhurst *et al.*, 2004), in particular large highly vacuolated 'metal storage cells' in the epidermis (Küpper *et al.*, 1999, 2001; Frey *et al.*, 2000). In the vacuoles of these metal storage cells, heavy metal concentrations of several hundred mmol l^{-1} can be reached (Küpper *et al.*, 1999, 2001), showing that hyperaccumulation must involve active pumping of the metals into specific storage sites. This is mediated, at least in part, by an up to 200 times higher expression of metal transporter genes in hyperaccumulators compared with related nonaccumulator plants (Lasat *et al.*, 2000; Pence *et al.*, 2000; Assunção *et al.*, 2001; Becher *et al.*, 2004; Papoyan & Kochian, 2004; Weber *et al.*, 2004; Hammond *et al.*, 2006; Van de Mortel *et al.*, 2006; Hanikenne *et al.*, 2008). The main detoxification strategy in hyperaccumulators is clearly not binding to strong ligands but sequestration of the hyperaccumulated heavy metals. Other genes (e.g. those related to stress responses) are also much more highly expressed in hyperaccumulators than in related nonaccumulators, but the relevance for hyperaccumulation is not clear. In contrast to the progress that has been made in finding genes that are expressed at higher

levels in hyperaccumulators, the cell-specificity of their expression and regulation in hyperaccumulators has remained largely unknown. Therefore, it remains impossible to judge which of these genes are directly involved in hyperaccumulation by encoding transporters that pump the metal into storage sites, and which may be secondarily upregulated to prevent Zn deficiency in other compartments. Two recent studies have indicated such secondary upregulation for members of the IRT micronutrient transporter family: Küpper *et al.* (2007a) for *ZNT1* in *T. caerulescens* by quantitative whole-mount mRNA *in situ* hybridization (QISH) analysis and Hanikenne *et al.* (2008) for *ZIP4* and *IRT3* by expressing *HMA4* from the Zn hyperaccumulator *Arabidopsis halleri* (*AhHMA4*) under its own promoter in the nonaccumulator *A. thaliana.*

Plant metal toxicity and acclimatization to this stress are important plant traits because they set the boundaries that limit the biotechnological use of hyperaccumulators for the phytoremediation or phytomining of metal-rich soils. *Thlaspi caerulescens* displays a pronounced stress and subsequent long-term acclimatization response to Cd treatment, showing that part of its Cd resistance is inducible (Küpper *et al.*, 2007b). In response to the initiation of Cd-induced stress, metal accumulation was found to be transiently (until acclimatization is achieved) enhanced in specific cells of the leaf mesophyll (Küpper *et al.*, 2000, 2001, 2007b), and these same cells displayed reduced photosynthetic activity (Küpper *et al.*, 2007b). Furthermore, these same cells were found to contain elevated levels of magnesium, which was interpreted as part of a defence against substitution of Mg^{2+} in chlorophyll by heavy metals (Küpper *et al.*, 1996, 1998, 2002).

In the present study, we investigated cellular gene expression (measured as mRNA abundance) of three metal (mainly Zn) transport genes (*ZNT1*, *ZNT5* and *MTP1*) in leaves as the main metal storage organs of hyperaccumulator plants. These genes are known to be much more highly expressed in shoots of *T. caerulescens* compared with nonaccumulators (see the Discussion section for details), making them prime candidates for genes causing the already-known (as mentioned in the previous section) cellular metal sequestration pattern in the shoots of hyperaccumulators. Further, it is well known that the chemical similarity of Cd and Zn results in significant levels of Cd-affinity for binding sites designed for Zn, so that the three selected Zn-transporters should transport or at least bind Cd as well. We investigated the potential role of these genes in acclimatization to Cd stress by looking at changes of their expression depending on the Zn/Cd supply to the plant, the age of the plant and the age of the individual leaf. Further, we analysed whether they may be directly responsible for Cd/Zn hyperaccumulation by comparing cellular distribution of their expression with the known pattern of cellular Zn compartmentation. We analysed this via a newly developed technique for quantitative mRNA *in situ* hybridization (Küpper *et al.*, 2007a) and complemented these expression data with inductively-coupled plasma emission spectroscopy (ICP-AES) measurements of metal content in specific plant tissues, as well as observations of growth.

Materials and Methods

Plant material, culture media and culture conditions

Seeds of *T. caerulescens* J. & C. Presl (Ganges population) were germinated on a mixture of Perlite and vermiculite moistened with deionized water. Three week after germination, seedlings were transferred to the nutrient solution described by Shen *et al.* (1997), but with lower levels of copper (Cu) and manganese (Mn) as described in Küpper *et al.*, 2007a,b). The nutrient solution was aerated continuously. The nutrient solution in the pots (*c.* 0.5 l per plant) was exchanged twice each week. Each treatment was replicated with at least eight plants, four of which were harvested 5 months after germination (i.e. *c.* 4 months of metal treatment), the remaining plant were harvested after 1 yr. The following Cd/Zn treatments were applied: 1, 10, 20 μM Zn (not in all experiments, therefore data not shown except for the supporting information on statistics Tables S1–S4), 450 μM Zn, 10 μM Zn + 10 μM Cd, 10 μM Zn + 50 μM Cd. Plants were grown in a growth room with 14-h daylength and *c.* 24°C : 20°C day : night temperature. Irradiance during the light period was 100 μmol m^{-2} s^{-1} and was supplied by Sylvania GroLux fluorescent tubes.

Harvesting

After fresh weight determination of all parts of the plants, representative samples of all the tissues to be analysed (roots, stems, young petioles, mature petioles, senescent petioles, young leaves, mature leaves, senescent leaves) were obtained, oven-dried (min. 72 h) and dry weights determined for metal analysis. The division into different tissues was done before drying (so that we determined the fresh weight, not the dry weight of the whole plant) because the drying process made the tissues so brittle that reliable tissue fractionation after drying was impossible. 'Young' leaves/petioles were the second to third youngest, not yet fully expanded leaf in the rosette, 'mature' leaves were fully expanded leaves that did not show signs of senescence, and 'senescent' leaves were still living but already starting to become yellow before dying. Roots were briefly rinsed with deionized water (and then blotted dry with paper towels), but not desorbed with calcium nitrate, etc., so that the measured root metal contents also included the metal that was adsorbed to the cell walls.

Quantitative mRNA *in situ* hybridization

For every gene expression study in response to specific metal treatment, leaf age and plant age was investigated by QISH; at least two leaves were collected per plant for each analysis and two tissue samples were excised per leaf. One of the two samples per leaf was hybridized with sense strand oligonucleotide probes, the other with antisense strand oligonucleotide probes. In each of these samples, for each cell type (upper epidermal guard cells, upper epidermal subsidiary cells, upper epidermal storage cells, palisade mesophyll cells, bundle sheath cells, phloem cells, spongy mesophyll cells, lower epidermal storage cells, lower epidermal subsidiary cells and lower epidermal guard cells) usually seven representative cells were selected for numerical analysis (quantification). In this way, during this study about 27 000 cells (around half of them with sense-strand oligos for background subtraction) were individually selected and analysed for their gene expression.

All other details of the sample preparation, the characteristics (design of the sequences and choice of labels) of the oligonucleotide probes, the details regarding microscope optics (including excitation and emission wavelengths), the measuring conditions (data resolution, etc.), the data processing and various tests of the reliability, performance and limitations of the QISH technique are described in details in our recent publication on this technique (Küpper *et al.*, 2007a). It is possible that the hybridization efficiency of our internal standard, 18s rRNA, is lower than for mRNA, leading to an overestimation of mRNA concentrations relative to 18s rRNA. However, this would not affect any of the conclusions of our studies, as the structure of the 18s rRNA and thus the hybridization efficiency would be the same for all cell types and treatments tested, and all genes of interest were normalized to the same internal standard.

Metal analysis

Dried samples were ground and digested with a mixture of HNO_3 and $HClO_4$. Concentrations of Cd, Zn and other elements in the digests were determined using ICP-AES.

Results

Growth and visible symptoms of damage

All of the Zn treatments yielded similar growth for young (5-month-old) plants (Fig. 1, photographs in Fig. S1). In low Zn-grown (1 μM Zn^{2+} in the nutrient solution) plants, the leaves had a slightly yellowish colour suggesting that growth on low Zn may cause borderline Zn deficiency, while observations of the more brittle and somewhat curled leaves of the high Zn-grown plants (450 μM Zn^{2+} in the nutrient solution) suggested that this resulted in a slight Zn toxicity (not shown). After growth for 1 yr (mature plants) under the same conditions, however, the low-Zn and high-Zn plants showed a drastic reduction of growth compared with the control plants grown on 10 μM Zn^{2+}. Another interesting observation was the decrease in the root : shoot ratio at increasing Zn concentrations, both in young and mature plants (Fig. 1).

The addition of Cd to the nutrient solution (containing 10 μM Zn^{2+}) always caused a reduction of growth, but the extent of inhibition strongly varied with the age of the plants (Fig. 1). While young plants only reached 28% and 3% of the shoot fresh weight of the control plants at 10 and 50 μM Cd^{2+} in the nutrient solution, respectively, in mature plants an acclimatization to the Cd toxicity was observed. After 1 yr of growth, the plants on 10 μM Cd^{2+} reached 52% of the control shoot fresh weight, and the plants grown on 50 μM Cd^{2+} reached > 5%. This was associated with a reversal of the Cd-dependent change in the root : shoot ratio. In young plants, exposure to higher Cd levels reduced the root : shoot ratio, similar to what was observed for growth on high Zn. In contrast to the effect of toxic levels of Zn, in mature Cd-stressed plants, the root : shoot ratio was much higher compared with the control (Fig. 1). The acclimatization of plants to Cd was further visible as much less leaf chlorosis was seen in the mature plants, compared with the significant leaf chlorosis seen in young, Cd-stressed plants (shown in Küpper *et al.*, 2007b).

Metal uptake and compartmentation in different plant organs

The present study revealed new features regarding the pattern of Cd and Zn hyperaccumulation in *T. caerulescens* (Ganges population). Unless stated otherwise, all effects stated below were statistically significant with $P < 0.05$ according to three-way ANOVAS with subsequent multiple pairwise (meaning pairs of groups like 'all mature vs all young plants' or '10 μM Zn treatment vs. 1 μM Zn treatment' rather than comparisons of all groups vs a 'reference') comparisons via the Holm–Sidak method with adjustment of the significance level for multiple comparisons (Table S1). First, at low Zn concentrations in the medium, the leaves contained significantly more Zn than the petioles (paired *t*-test, $n = 16$ leaf/petiole pairs, $P < 0.01$), while at high Zn concentrations this difference disappeared (not shown). For both young and mature plants a very strong increase in leaf Cd and Zn concentrations can be seen in the comparison of young, mature and senescent leaves. This was most pronounced for Zn, with *c.* fourfold more Zn seen in senescent compared with young leaves of young plants grown on 10 and 450 μM Zn, but an increased accumulation during maturation/ageing was also observed for Cd (Fig. 2).Growth on high Zn also resulted in a greater increase in root Zn accumulation compared with the shoots

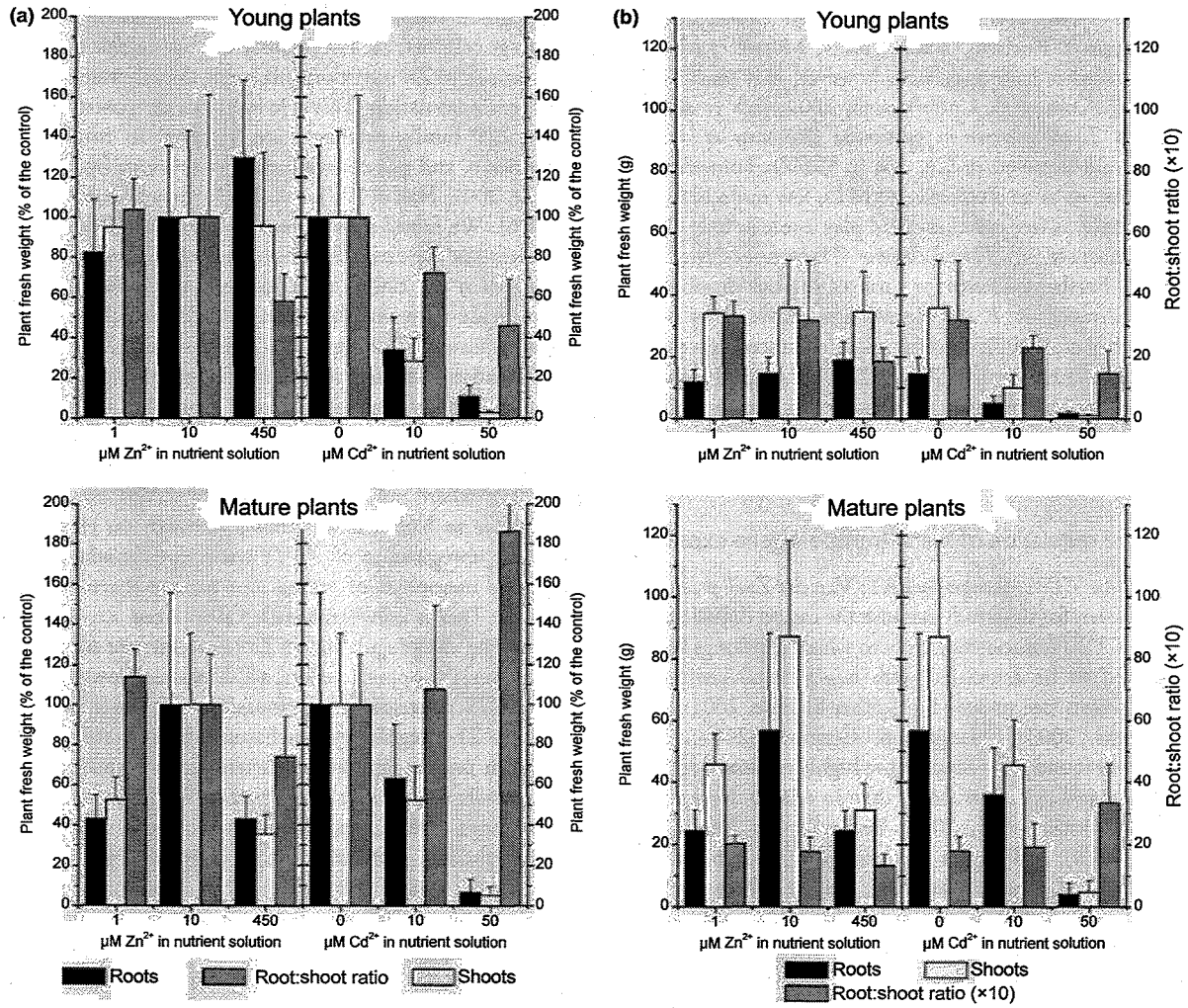

Fig. 1 Effects of cadmium (Cd)/zinc (Zn) treatment on fresh weights of young and mature *Thlaspi caerulescens* plants. Each value represents average + SE of four plants (unless a plant died). The Cd treatments included the control level (10 μM) of Zn. Top, young (5 month old) plants; bottom, mature (1 yr old) plants. (a) Values relative to the control. (b) Absolute values.

(Fig. 2, Zn; Table S1, Zn), probably owing to Zn adsorption/precipitation on the root surface. For plants grown on 10 μM Zn^{2+} in the nutrient solution, the Zn concentration in senescent and dead leaves was two to three times higher than in the roots, while for plants grown on 450 μM Zn^{2+} the roots contained almost seven times more Zn than the dead and senescent leaves. A similar tendency was observed for Cd accumulation (Fig. 2, Cd; Table S1, Cd). Further, shoot Cd concentrations were generally higher in young plants compared with mature plants (Fig. 2, Cd; Table S1, Cd). This trend was much more evident in plants grown on 50 μM Cd compared with 10 μM Cd, which excludes the possibility that this response was caused by Cd depletion in the nutrient solution. After plant growth on 50 μM Cd, dead and senescent leaves contained *c.* 25 300 ppm Cd in young plants, compared with 12 600 ppm in mature plants (Fig. 2). Finally, addition of Cd to the medium caused a significant decrease in shoot Zn uptake (> 50%) compared with plants grown without Cd (Fig. 2, Zn; Table S1, Zn).

The different Cd/Zn treatments also resulted in significant changes in the uptake of other mineral nutrients. Despite the somewhat similar chemistry of Ca, Cd and Zn, no changes in the uptake of calcium (Table S1, Ca), magnesium (Fig. 2, Mg; Table S1, Mg), potassium (Table S1, K) or sulphur (Fig. 2, S; Table S1, S) were observed in response to Zn deficiency or excess. Only Mn concentrations in the shoot decreased with increasing Zn in the medium, particularly in mature plants (Fig. 2, Mn, Table S1, Mn). The addition of Cd to the medium, by contrast, caused enhanced uptake of Mg, Ca and S into the shoots (Fig. 2, Cd, Mg and S; Table S1, Ca, Cd, Mn, S). For Mg, this enhancement of uptake was similar for both young and

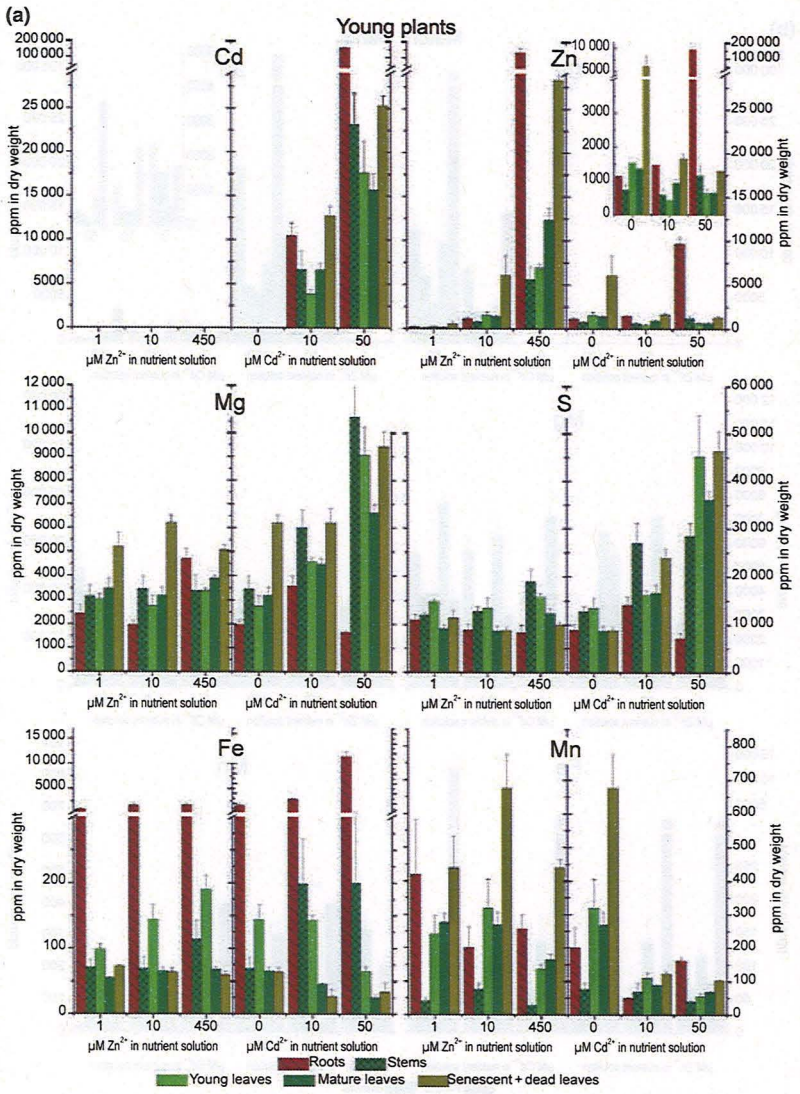

Fig. 2 Concentrations of selected elements in young and mature *Thlaspi caerulescens* plants. Each value represents the average + SE of four plants (unless a plant died). The cadmium (Cd) treatments contained the control level (10 µM) of zinc (Zn). (a) Young (5 months old) plants; (b) mature (1 yr old) plants. Fe, iron; Mg, magnesium; Mn, manganese; S, sulphur.

mature plants, while for S it was much stronger in young plants (Fig. 2a vs. b). The root concentrations of all these elements remained constant in all Cd/Zn treatments (Fig. 2, Mg and S). The iron (Fe) content of mature and senescent leaves significantly decreased under Cd toxicity (Fig. 2, Fe; Table S1, Fe). This was probably related to enhanced retention of Fe in the roots, which generally contained much (> 10 times) more Fe than the shoots (Fig. 2, Fe), likely owing to passive adsorption in cell walls. However, problems with Fe translocation as a result of Cd toxicity could also explain the strongly increased petiole/leaf ratio of Fe concentrations at all leaf developmental stages in the 50 µM Cd and in dead and senescent petioles/leaves in the high-Zn treatment (not shown). Manganese concentrations in both roots and shoots strongly decreased at increasing concentrations of Cd or Zn; at 10 µM Cd^{2+} in the nutrient solution the leaf concentrations of Mn were reduced to less than half of the control treatment (Fig. 2, Mn, Table S1, Mn).

Regulation of metal transport gene expression as analysed by QISH

As for the ICP-MS data, all trends described in the following were statistically significant according to ANOVA analyses, with subsequent all-pairwise comparisons using the Holm–Sidak method with adjustment of the significance level for multiple comparisons. The results of all two-way ANOVAS are listed in Tables S2–S4. Further statistical tests are described in the text.

MTP1 In young leaves (that are a few days old and not fully expanded) of both young and mature plants, *MTP1* transcript levels were highest in epidermal metal storage cells,

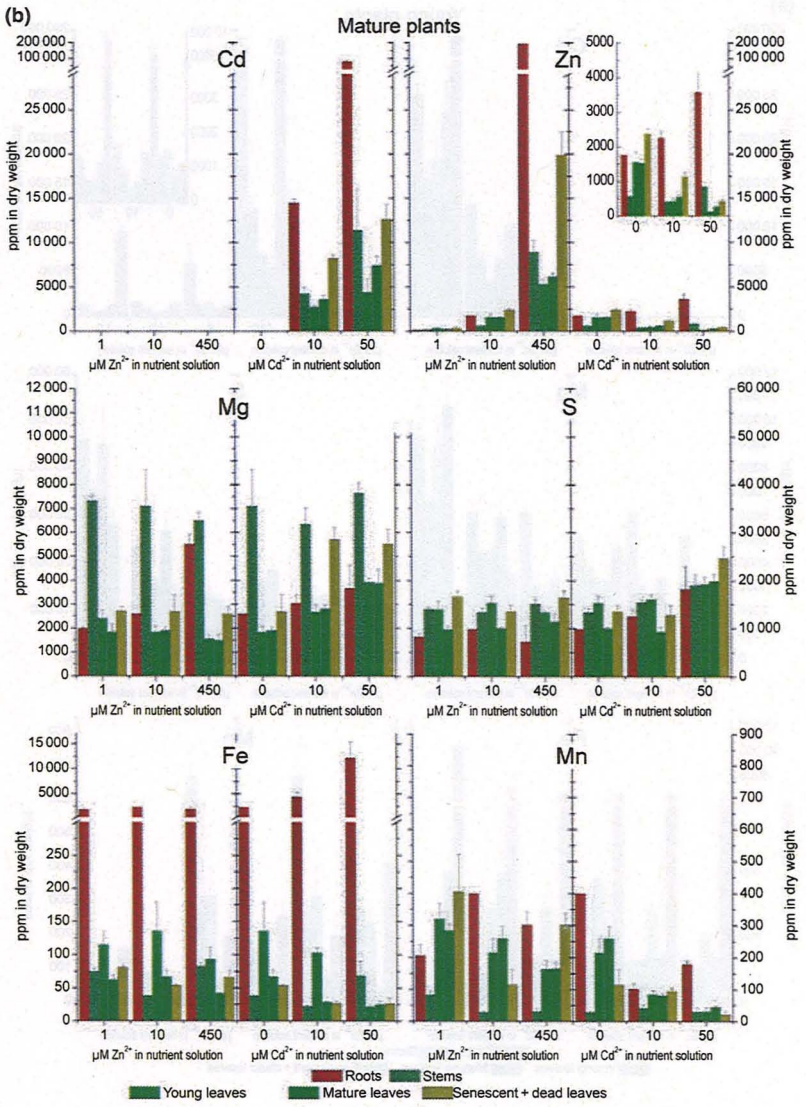

Fig. 2 Continued.

bundle sheath cells and spongy mesophyll cells (Figs 3, 4). Epidermal storage cells can easily be distinguished from all other epidermal cell types by their much larger size and their characteristic shape, as shown in Fig. 4. In mature (fully expanded, but not senescent) leaves in both young and mature plants, *MTP1* expression in the epidermal storage cells disappeared almost completely, while the transcript levels in bundle sheath and mesophyll cells remained quite similar to those measured in young leaves (Fig. 3).

In young leaves, no obvious effects of the metal treatments on *MTP1* transcript levels were observed; most differences were not statistically significant (Table S2). Only in the palisade mesophyll and bundle sheath cells did some comparisons between individual treatments yield statistically significant differences (Table S2), but even those did not show a clear trend (Fig. 3). Noise in the MTP1 data was high not only because of true differences in mRNA levels between individual leaves, but also because the tissue contained antisense strand mRNA (verified by Northern blots, Fig. S2) that made the quantification less accurate, since it relies on the equation

$$\frac{c(MTP1\ \text{mRNA})}{c(18s\ \text{rRNA})} = \left(\frac{fl(MTP1_a)}{fl(18s\ \text{rRNA}_a)} - \frac{fl(MTP1_s)}{fl(18s\ \text{rRNA}_a)}\right) \cdot flr \qquad \text{Eqn 1}$$

(a, antisense strand probe; c, concentration; fl, fluorescence measured in the CLSM; flr, fluorescence ratio measured in the CLSM of the *MTP1* probe to the internal standard in an equimolar solution of the two probes; s, sense strand probe).

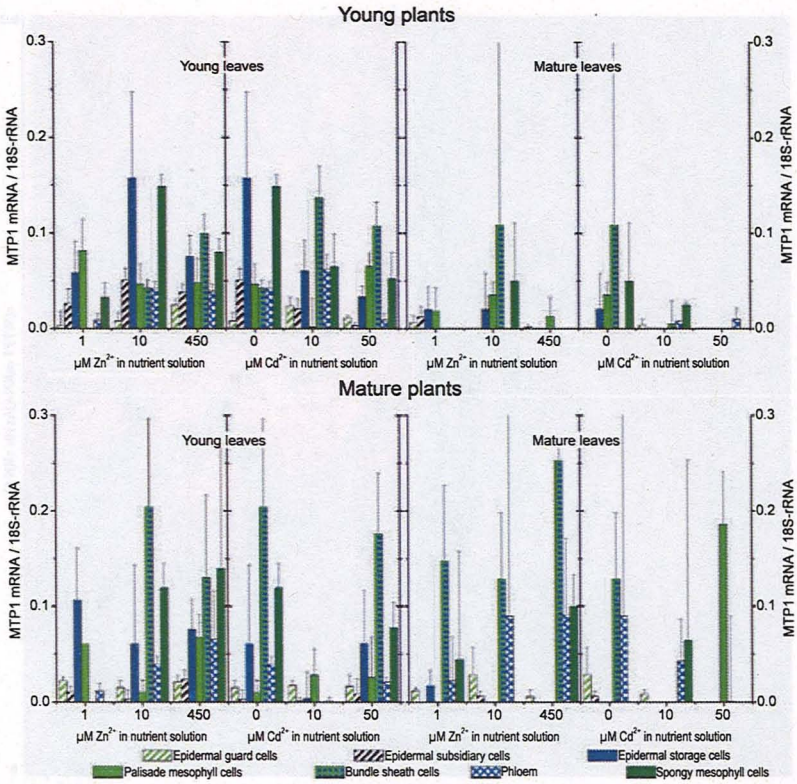

Fig. 3 Comparison of *MTP1* mRNA concentrations in young (not fully expanded, third youngest leaf of rosette) and mature (fully expanded, but not senescent) leaves of young (3–5 months old) and mature (1 yr old) *Thlaspi caerulescens* plants grown on different concentrations of zinc (Zn^{2+}) and cadmium (Cd^{2+}) in the nutrient solution. The colours of the bars indicate the function of the tissue they represent: blue, a function in metal transport and storage; green, photosynthetic activity. Molar mRNA concentration ratios ± SE are presented (see Methods section for details of statistics). The ratio of the concentrations of the mRNA for *MTP1* to *18S* rRNA was determined from the following equation:

$$\frac{c(MTP1\ \text{mRNA})}{c(18s\ \text{rRNA})} = \left(\frac{fl(MTP1_a)}{fl(18s\ \text{rRNA}_a)} - \frac{fl(MTP1_s)}{fl(18s\ \text{rRNA}_a)}\right) \cdot flr \qquad \text{Eqn 2}$$

(a, antisense strand probe; c, concentration; fl, fluorescence measured in the CLSM; flr, fluorescence ratio measured in the CLSM of the *MTP1* probe to the internal standard in an equimolar solution of the two probes; s, sense strand probe).

In mature leaves of both young and mature plants, the addition of Cd to the nutrient solution caused a cessation of *MTP1* expression in the bundle sheath cells (Fig. 3, Table S2). In mature leaves of young plants, Cd also decreased the expression of *MTP1* in the other cell types investigated, while in mature leaves of mature plants *MTP1* expression in the palisade mesophyll increased, while it decreased in all other cell types (Fig. 3, Table S2). In mature leaves of young and mature plants, higher *MTP1* expression in epidermal storage cells was observed in Zn-deficient plants compared with the other treatments (statistically significant only in young plants, Table S2).

ZNT1 Generally, *ZNT1* mRNA abundance was greater in mature than in young leaves (Fig. 5), which was highly significant both for young plants (three-way ANOVA, $n = 3629$, $P < 0.001$) and mature plants (three-way ANOVA, $n = 2211$, $P < 0.001$). Further, *ZNT1* expression was also higher in photosynthetic cells (palisade and spongy mesophyll, guard cells and bundle sheath cells) compared with transport (phloem) or nonphotosynthetic epidermal cells (subsidiary and storage cells). Of all three genes analysed, *ZNT1* transcript levels in mature leaves of young plants were by far the highest (Fig. 6).

In young leaves of young plants, *ZNT1* was primarily expressed in bundle sheath cells (Küpper *et al.*, 2007a; Table 1 and Fig. 6 (left column), statistics in Table S3). With increasing leaf age, the expression of *ZNT1* extended first to the spongy mesophyll cells around the bundle sheath. Then, in fully mature leaves of young plants, maximal transcript levels were found in the leaf mesophyll, but *ZNT1* expression also strongly increased in other cell types compared with young leaves (Figs 5,6). In mature leaves of mature plants, *ZNT1* expression levels were lower than in mature

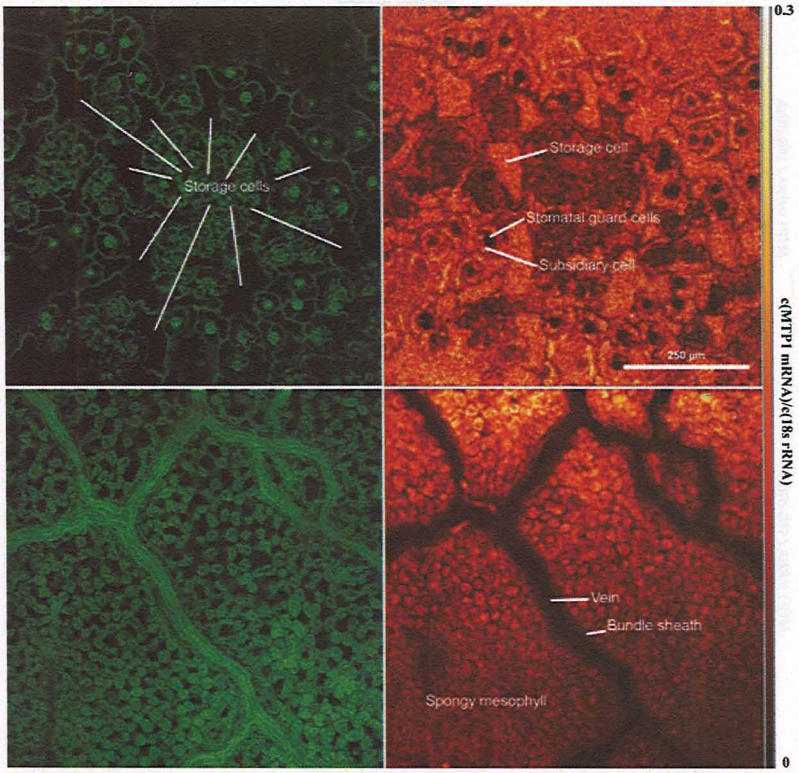

Fig. 4 Spatial distribution of the mRNA abundance of *MTP1* in *Thlaspi caerulescens* Ganges leaves (*c.* 4-month-old plants) zinc (Zn)-replete nutrition (10 µM Zn), as analysed by quantitative whole-mount mRNA *in situ* hybridization (QISH). These images show the ratio

$$\frac{c(MTP1\ mRNA)}{c(18s\ rRNA)} \approx \left(\frac{fl(MTP1_a)}{fl(18s\ rRNA_a)}\right) \cdot flr \qquad \text{Eqn 3}$$

(a, antisense strand probe; c, concentration; fl, fluorescence measured with the CLSM; flr, fluorescence ratio of probe for the gene of interest (here *MTP1*) to the internal standard (here *18s rRNA*) probe in an equimolar solution of the two probes). Please note that this type of ratio imaging does not allow for a subtraction of nonspecific binding as well as binding to genomic DNA (Küpper *et al.*, 2007a), making it less accurate than the fully corrected data presented in Figs 3, 5 and 7. The scale bar applies to all panels. Top, upper epidermis; bottom, spongy mesophyll. Left, green autofluorescence images to show the tissue structure; right, quantitative whole-mount mRNA *in situ* hybridization (QISH) ratio images.

leaves of young plants (three-way ANOVA, $n = 2741$, $P < 0.001$); the decrease was greater in the mesophyll compared with the bundle sheath cells (Fig. 5). Interestingly, the opposite trend was observed for young leaves, which had higher *ZNT1* mRNA levels in mature plants than in young plants (three-way ANOVA, $n = 1827$, $P < 0.001$).

Changes in Zn nutrition generally had surprisingly little effect on *ZNT1* expression. The downregulation at high Zn levels observed earlier (Pence *et al.*, 2000; Küpper *et al.*, 2007a) was now only found in the mature leaves of young plants in the comparison of Zn-deficient vs control plants (Fig. 5, statistics in Table S3). Increasing Zn from 10 µM to 450 µM caused upregulation of *ZNT1* in all cell types of mature leaves of young plants, and in the bundle sheath and phloem cells of mature leaves in mature plants (Fig. 5). When plants were grown on elevated Cd, upregulation of *ZNT1* transcript abundance was observed in the spongy mesophyll of young and mature leaves of young plants at both 10 µM and 50 µM Cd (Fig. 5, Table S3). However, the opposite effect (i.e. a Cd-induced downregulation of *ZNT1*) was found in young and mature leaves of mature plants (Fig. 5).

ZNT5 The present study showed that *ZNT5* is mainly expressed in young leaves of young plants (Figs 7, 8, three-way ANOVA, $n = 2391$, $P < 0.001$). The *ZNT5* expression pattern in the epidermis of these leaves (Fig. 8) closely resembled the pattern of heavy metal (Zn, Ni) accumulation that was earlier reported for *T. caerulescens* (Küpper *et al.*, 1999; Frey *et al.*, 2000) and *Thlaspi goesingense* (Küpper *et al.*, 2001), where particularly large epidermal cells were identified as the main storage sites of the accumulated metal. These metal storage cells were found to contain much higher levels of *ZNT5* mRNA than all other epidermal cells (Figs 7,8, statistics in Table S4). This pattern remained almost constant at different levels of Zn nutrition

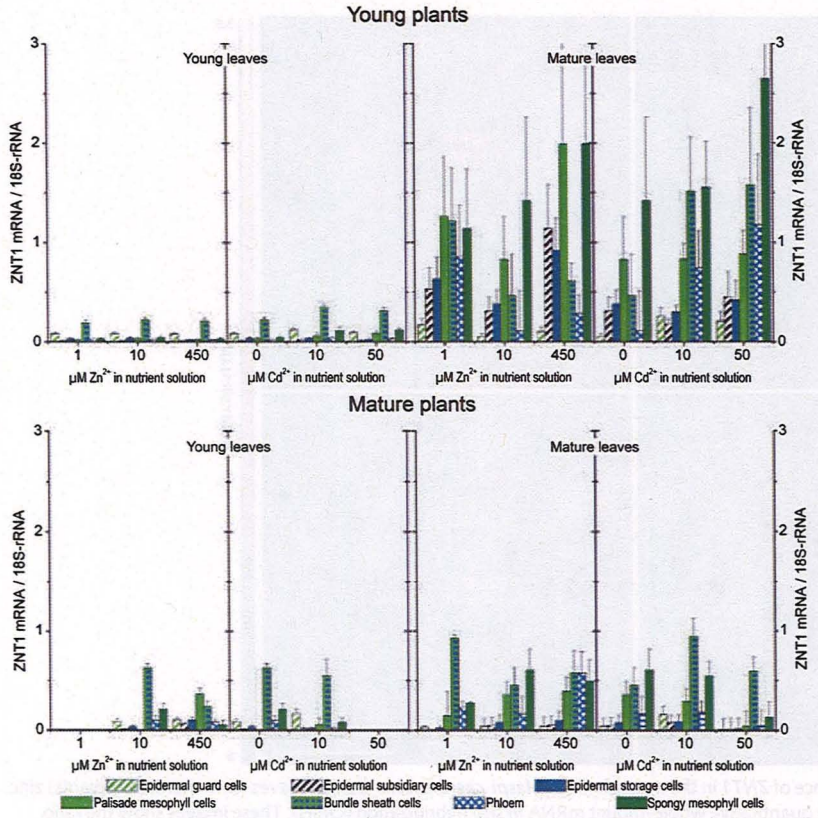

Fig. 5 Comparison of transcript levels of *ZNT1* mRNA in young and mature leaves of young and mature *Thlaspi caerulescens* plants grown on different concentrations of zinc (Zn^{2+}) and cadmium (Cd^{2+}) in the nutrient solution. Molar mRNA concentration ratios ± SE are presented. See the legend to Fig. 3 for equation for the concentration ratio of *ZNT1* mRNA to *18S rRNA*.

of the plants. Only at 450 µM Zn was a moderate decrease of *ZNT5* mRNA abundance observed, which affected *ZNT5* expression levels almost equally in all tissues. Elevated Cd concentrations, however, caused a strong decrease of *ZNT5* mRNA concentrations in both main expression sites: epidermal storage cells and bundle sheath cells (Fig. 7, Table S4). This decrease was less pronounced in other cell types (Fig. 7).

In mature leaves of young plants, *ZNT5* expression was generally very weak; only in the mesophyll and in bundle sheath cells were measurable *ZNT5* transcript levels found (Fig. 7). *ZNT5* expression was decreased further in plants grown on either low or high Zn or high Cd concentrations (Fig. 7, Table S4). In mature plants, *ZNT5* expression decreased even further, so that no reliable quantification was possible (data not shown).

Discussion

Long-term acclimatization of heavy metal hyperaccumulator plants to toxic levels of heavy metals in the soil is crucial for phytoremediation, since part of the metal resistance in hyperaccumulator plants is inducible (Küpper *et al.*, 2007b), and only after acclimatization do the plants grow at a rate sufficient to produce enough biomass for successful phytoremediation. The long-term effects of this acclimatization are of further importance because phytoremediation has to be conducted with mature plants grown for significant periods on contaminated soils (Chaney *et al.*, 2005; McGrath *et al.*, 2006). Here we investigated two key aspects of this acclimatization: changes in plant metal uptake/accumulation and changes in cellular expression levels of metal transporter genes. We analysed those changes during whole-plant and leaf ontogenesis under different supplies of Zn and Cd for up to 1 yr. This study provided insights into the regulatory network of metal uptake and trafficking, involving a delicate age- and cell-specific differential regulation of metal transporter genes, which proved to be very different even for closely related genes. This complicated regulation will make transformation of high-biomass nonaccumulators via gene transfer from hyperaccumulators even more difficult than previously thought (e.g. review of Chaney *et al.*, 2005).

Changes in metal uptake owing to variation of Cd/Zn supply

The present study has reinforced the view that Cd enters plant cells via Zn transporters, because adding Cd to the nutrient solution strongly decreased Zn uptake into the shoots. A similar competition between Cd and Zn uptake in *T. caerulescens* was found recently by Assunção *et al.*

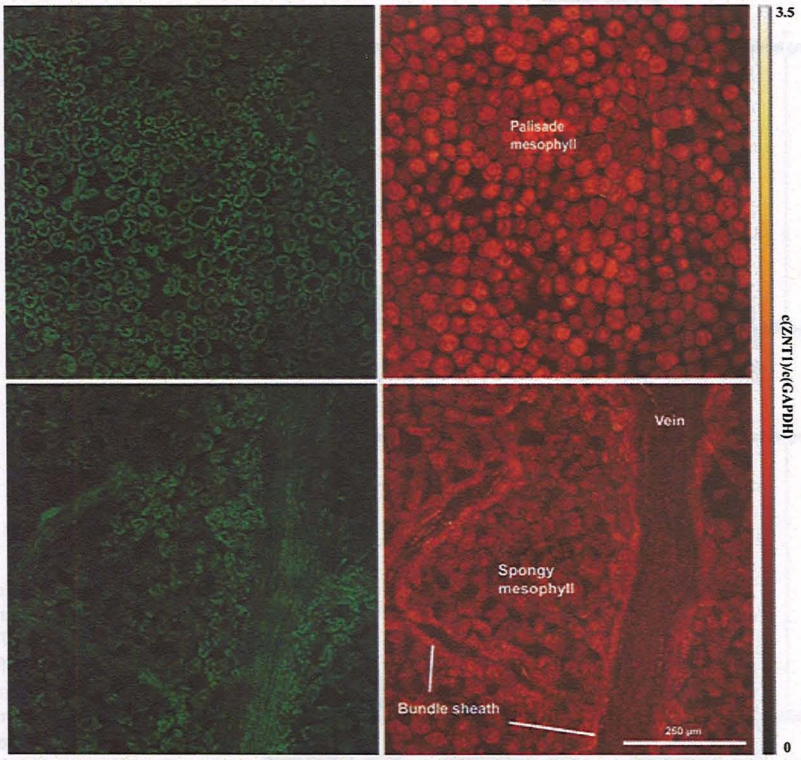

Fig. 6 Spatial distribution of the mRNA abundance of *ZNT1* in the mesophyll of *Thlaspi caerulescens* Ganges leaves (*c.* 4-month-old plants) zinc (Zn)-replete nutrition (10 µM Zn), as analysed by quantitative whole-mount mRNA *in situ* hybridization (QISH). These images show the ratio

$$\frac{c(ZNT1\ \text{mRNA})}{c(cGAPDHmRNA)} \approx \left(\frac{fl(ZNT1_a)}{fl(cGAPDHmRNA_a)}\right) \cdot flr \qquad \text{Eqn 4}$$

(Symbols used in this equation and limitations of such ratio images are explained in the legend to Fig. 4). The scale bar applies to all panels. Top, palisade mesophyll, bottom: spongy mesophyll. Left, green autofluorescence images to show the tissue structure; right, quantitative whole-mount mRNA *in situ* hybridization (QISH) ratio images.

(2008), while Lombi *et al.* (2000) found no influence of Zn supply on Cd uptake in the Ganges ecotype of *T. caerulescens*. In the present study, addition of Cd to the nutrient solution further decreased the uptake of Cu, Fe and Mn; the last element was also affected by increased Zn concentrations. Such an inhibition of the uptake of essential micronutrients could contribute to Cd toxicity, as reported previously (Yoshihara *et al.*, 2006; Mendoza-Cozatl *et al.*, 2008). It could be caused not only by inhibited uptake, but also by Cd-stimulated heavy metal efflux as a Cd-resistance strategy that could also lead to the efflux of other ions (Migocka & Klobus, 2007). One reason why this was not

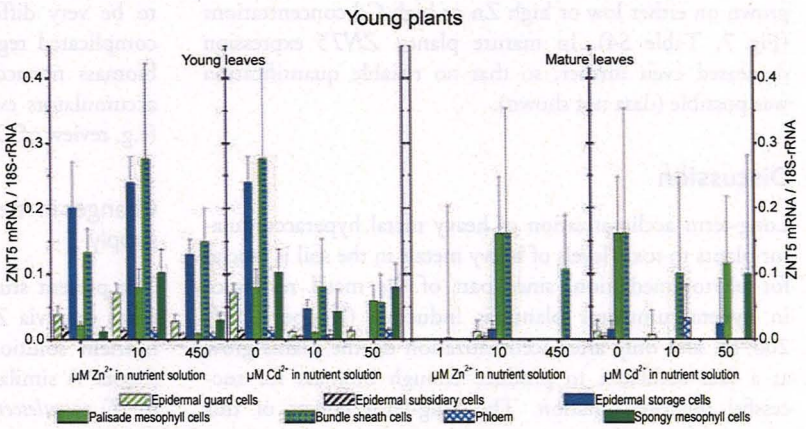

Fig. 7 Comparison of transcript levels of *ZNT5* mRNA in young and mature leaves of young and mature *Thlaspi caerulescens* plants grown on different concentrations of zinc (Zn^{2+}) and cadmium (Cd^{2+}) in the nutrient solution. Molar mRNA concentration ratios ± SE are presented (see Methods section for details of statistics). See the legend to Fig. 3 for equation for the concentration ratio of *ZNT5* mRNA to *18S rRNA*.

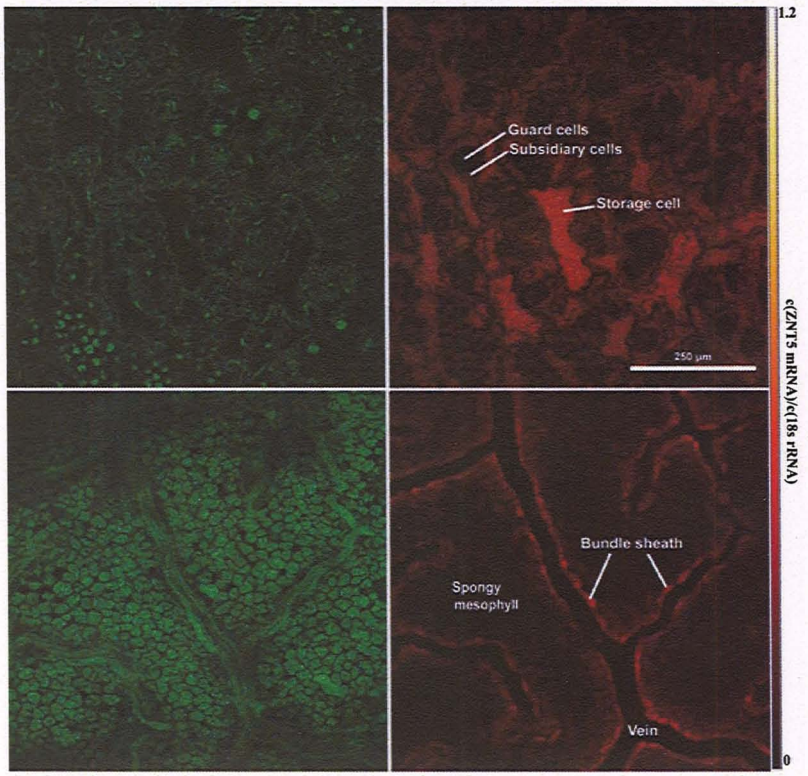

Fig. 8 Spatial distribution of the mRNA abundance of *ZNT5* in the mesophyll and epidermis of *Thlaspi caerulescens* Ganges leaves (*c.* 4-month-old plants) at high zinc (Zn) nutrition (450 µM Zn), as analysed by quantitative whole-mount mRNA *in situ* hybridization (QISH). These images show the ratio

$$\frac{c(ZNT5\ mRNA)}{c(18S\ rRNA)} \approx \left(\frac{fl(ZNT5_a)}{fl(18S\ rRNA_a)}\right) \cdot flr \qquad \text{Eqn 5}$$

(Symbols used in this equation and limitations of such ratio images are explained in the legend to Fig. 4). The scale bar applies to all panels. Top, epidermis; bottom: spongy mesophyll. Left, green autofluorescence image to show the tissue structure; right, quantitative whole-mount mRNA *in situ* hybridization (QISH) ratio image.

observed by Papoyan *et al.* (2007) could be the much shorter metal treatments (2 months instead of the 4 months and 12 months used here).

When plants were grown on elevated Cd concentrations that led to visible Cd toxicity symptoms and growth reduction, increased uptake of Mg, Ca and S was found. Enhanced Mg uptake may diminish Cd toxicity because more Mg in chloroplasts could protect them against Cd-binding to chlorophyll (Küpper *et al.*, 1996, 2002) and to catalytic sites of enzymes. This was proposed previously for Mg vs Cd in *A. halleri* (Küpper *et al.*, 2000) and Ni in *T. goesingense* (Küpper *et al.*, 2001). These two plant species reacted to Cd- and Ni-toxicity by transporting additional Mg into mesophyll cells, but not into other cell types. Similarly, enhanced uptake of Ca could stop replacement of Ca by Cd in proteins, which might contribute to Cd-induced inhibition of photosynthesis (Faller *et al.*, 2005).

The enhanced uptake of sulphur during exposure to toxic Cd concentrations could be related to the synthesis of Cd-binding sulphur ligands such as glutathione, metallothioneins (MTs) and phytochelatins (Cobbett & Goldsbrough, 2002). At first, this seems to be at variance with previous results of other studies. It was found, for example, that Cd in *T. caerulescens*, in contrast to nonaccumulators, is mainly associated with weak oxygen/nitrogen ligands, not with sulphur ligands (Küpper *et al.*, 2004). Further, it was shown that Cd induction of phytochelatin synthesis in *T. caerulescens* is weaker than in the related nonaccumulator, *T. arvense* (Ebbs *et al.*, 2002), and even a complete inhibition of phytochelatin synthesis did not affect Cd resistance in *T. caerulescens* and other hypertolerant species (Schat *et al.*, 2002). Nevertheless, it could be that Cd detoxification in *T. caerulescens* does involve MTs as transient transport ligands, which would mean that only a small proportion of the total accumulated Cd is bound to sulphur ligands at any point in time. This would be consistent with the EXAFS data (Küpper *et al.*, 2004) and might furthermore explain why such genes are so highly expressed in

T. caerulescens compared with related nonaccumulators (Papoyan & Kochian, 2004; Van de Mortel *et al.*, 2006). Other studies have suggested, however, that plant MTs in general (Cobbett & Goldsbrough, 2002; Jack *et al.*, 2007) and in *T. caerulescens* in particular (Roosens *et al.*, 2004, 2005) are involved in Cu, not in Cd/Zn homeostasis. In addition, or as an alternative to MTs, low molecular weight thiols such as free cysteine could play a role in long-distance Cd transport in *T. caerulescens* (Hernandez-Allica *et al.*, 2006). Further evidence for a role of sulphur metabolism in Cd resistance of *T. caerulescens* was provided by Van de Mortel *et al.* (2008), who found an enhanced expression of the sulphate transporter SULTR2, 1, although these authors studied an ecotype of *T. caerulescens* that does not accumulate Cd (Cd levels remained in the low ppm range as for the nonaccumulator *A. thaliana*).

Cellular regulation of metal transport gene expression

MTP1 (= ZTP1, similar to ZAT; Van der Zaal *et al.*, 1999) was chosen for this study because the cation diffusion facilitator (CDF) transporter family to which it belongs has been reported to be associated with heavy metal resistance in plant shoots (see reviews by Gaither & Eide, 2001; Hall & Williams, 2003; Colangelo & Guerinot, 2006). MTP1 itself was found to be much more highly expressed in *T. caerulescens* and *A. halleri* shoots than in related nonaccumulators (Assunção *et al.*, 2001; Becher *et al.*, 2004; Dräger *et al.*, 2004). MTP1 was also shown to be involved in Zn tolerance and accumulation in *A. thaliana* (Desbrosses-Fonrouge *et al.*, 2005). Our current data only partly support the earlier conclusion that this gene is important for metal resistance and hyperaccumulation. The expression in epidermal metal storage cells certainly points in this direction, but an almost complete cessation of its expression in those cells in mature leaves shows that it can have such a role only in young leaves, although the mature and senescent leaves are clearly those accumulating most of the hyperaccumulated metal. It is possible that expression of this gene is switched off when the vacuoles should be full. The higher *MTP1* expression in epidermal storage cells in mature leaves of Zn-deficient plants compared with the other treatments is a clear hint in this direction. Also, Desbrosses-Fonrouge *et al.* (2005) found MTP1 expression only in tissues containing dividing, differentiating and expanding cells.

ZNT1, a member of the ZIP (Zrt, Irt-like proteins; Grotz *et al.*, 1998) family of micronutrient transporters, was the first metal transport gene that was shown to be highly overexpressed in roots and shoots of *T. caerulescens* compared with the nonaccumulator, *T. arvense* (Pence *et al.*, 2000; Assunção *et al.*, 2001). This high expression was confirmed here and in our first QISH study (Küpper *et al.*, 2007a) in young plants. As discussed in detail in this previous publication, high *ZNT1* expression in photosynthetic mesophyll and guard cells but not in the Zn-accumulating epidermal storage cells that are not photosynthetic suggests that ZNT1 functions in Zn nutrition and is not directly responsible for hyperaccumulation. This is in line with the general view that the ZIP family members are involved in micronutrient nutrition, and not detoxification (see reviews by Gaither & Eide, 2001; Hall & Williams, 2003; Colangelo & Guerinot, 2006). We found in the present study that *ZNT1* downregulation at elevated Zn concentrations (Pence *et al.*, 2000; Küpper *et al.*, 2007a) occurred only when the comparison was made between Zn-deficient vs Zn sufficient plants in young leaves of young plants. By contrast, *ZNT1* mRNA abundance increased in mature leaves of high-Zn grown plants, and no downregulation of expression in response to increasing Zn was found in mature plants regardless of leaf age. This is consistent with the study of Assunção *et al.* (2001), where no downregulation of *ZNT1* expression in response to high Zn was found. Growth on elevated Cd caused upregulation of *ZNT1* transcript abundance in the spongy mesophyll of young and mature leaves of young plants. This is consistent with Cd-induced Zn deficiency, signalling an upregulation of Zn micronutrient transporters. However, the opposite effect, a Cd-induced downregulation of *ZNT1* expression, was found in all leaves of mature plants. The regulatory mechanism behind this response remains unclear, and at the current state of knowledge it is impossible to speculate as to whether the Cd sensing is direct or indirect. No Cd-specific direct sensing is known so far (phytochelatin synthase is activated by blocked thiols, but the blocking need not be caused by binding of Cd; Vatamaniuk *et al.*, 2000). But this does not mean that it does not exist. Since ZNT1 appears to be responsible for Zn nutrition of the leaf mesophyll, in addition to other mechanisms of Cd toxicity (see reviews by Prasad & Hagemeyer, 1999 and Küpper & Kroneck, 2005), Cd-induced downregulation of ZNT1 in the spongy mesophyll could lead to Zn deficiency and in this way contribute to the oxidative stress and chlorosis that is observed in plants stressed by Cd toxicity or Zn deficiency (Hacisalihoglu & Kochian, 2003).

Further, surprisingly different cellular expression patterns were found for the fairly closely related transporters, *ZNT1* and *ZNT5*, both belonging to the ZIP family of micronutrient transporters and thus predicted to be involved in uptake of Zn as an essential micronutrient. While *ZNT1* was expressed mainly in photosynthetic cells in line with this function, *ZNT5* was highly expressed in nonphotosynthetic epidermal metal storage cells as well as in the bundle sheath cells, with very low expression in mesophyll cells. This expression pattern for *ZNT5* suggests that it may be involved in the active Cd/Zn sequestration into these heavy metal storage sites (i.e. *ZNT5* may play an important role in hyperaccumulation). When plants were grown on nutrient solution containing Cd, a downregulation of *ZNT5* transcript abundance was observed, again (like for ZNT1,

as mentioned earlier) suggesting that Cd interacts with the same regulatory system that normally senses the cellular Zn status in influencing *ZNT5* gene expression. *ZNT5* was included in this study because a preliminary screen showed a relatively high expression of this ZIP family member in shoots of *T. caerulescens* (M. J. Milner & L. V. Kochian, unpublished), while a previous published study (Plaza *et al.*, 2007) suggested that it is expressed only in roots. The latter interpretation may have been caused by the choice of the plant material used for sampling, because in the current study high *ZNT5* expression in leaves occurred only in one of the four leaf/plant age combinations, namely the young leaves of young plants. In a study published after our experiments for the current study were performed (see the Materials and Methods section), ZNT5 expression was again found in the shoots of *T. caerulescens*, and was four times higher than in *T. arvense* according to quantitative PCR (Hammond *et al.*, 2006).

These data lead to a final important finding of this study. Our experiments revealed that heavy metal transporter gene expression drastically changes not only as a function of metal nutrition/toxicity, but often is even more dependent on the age of the whole plant and the individual leaf. Thus, *ZNT1* mRNA was abundant mainly in mature leaves of young plants, *ZNT5* mRNA was abundant mainly in young leaves of young plants and *MTP1* was about equally expressed in young leaves of both young and mature plants. This strong dependency of metal transporter expression on the developmental stage of the whole plant and the individual leaf was previously unknown, and is important for future studies on mechanisms of hyperaccumulation. It clearly indicates that meaningful data for gene regulation under conditions relevant for phytomining and/or phytoremediation cannot be obtained using quick experiments with young seedlings, but require long-term studies involving mature plants. These should also reveal whether the strong age-dependency of gene expression is an essential prerequisite for hyperaccumulation, or whether it is only useful for the success of the plants in its natural environment (e.g. preventing too- high metal concentrations in flowers and seeds). If the hyperaccumulation phenotype involves such regulatory mechanisms, it will be even harder than previously believed to transfer this phenotype to nonaccumulator high-biomass plant species.

Acknowledgements

The authors thank Laura Ort Seib for help with preparing ICP-AES samples and with QISH data analysis, and Holly Manslank and Jon Shaff for help with digesting samples for ICP analysis. The work of H.K. was supported by a Feodor-Lynen-Fellowship of the Alexander von Humboldt Association.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Images of mature *Thlaspi caerulescens* plants shortly before harvesting.

Fig. S2 Northern blot of mRNA with radioactively labelled oligonucleotides.

Table S1 Three-way ANOVAS of ICP-MS data with subsequent multiple pairwise comparisons

Table S2 Two-way ANOVAS and subsequent all-pairwise comparison procedures for MTP1 QISH data

Table S3 Two-way ANOVAS and subsequent all-pairwise comparison procedures for ZNT1 QISH data

Table S4 Two-way ANOVAS and subsequent all-pairwise comparison procedures for ZNT5 QISH data

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