INVESTED REVIEW

Investigating Heavy-metal Hyperaccumulation using *Thlaspi caerulescens* as a Model System

MATTHEW J. MILNER and LEON V. KOCHIAN*

Robert W. Holley Center for Agriculture and Health, USDA-ARS, Cornell University, Ithaca, NY 14853, USA

Received: 12 February 2008 Returned for revision: 12 March 2008 Accepted: 26 March 2008 Published electronically: 25 April 2008

• Background Metal-hyperaccumulating plant species are plants that are endemic to metalliferous soils and are able to tolerate and accumulate metals in their above-ground tissues to very high concentrations. One such hyperaccumulator plant species is *Thlaspi caerulescens*, which has been widely studied for its remarkable properties as a heavy metal hyperaccumulator. *Thlaspi caerulescens* has been identified as a model system for the study of metal hyperaccumulation, and its use in phytoremediation has been at the forefront of studies concerning the hyperaccumulation of heavy metals, such as zinc (Zn), cadmium (Cd), and nickel (Ni). In particular, certain ecotypes of *T. caerulescens* can accumulate as much as 30 000 ppm of Zn and approx. 10 000 ppm Cd in the shoot biomass without any signs of toxicity (typical shoot levels are 100–200 ppm Zn and 0.1–10 ppm Cd).

• Scope In this overview, an overview of the findings from recent research aimed at better understanding the physiological mechanisms of *Thlaspi caerulescens* heavy-metal hyperaccumulation as well as the underlying molecular and genetic determinants for this trait will be discussed. Progress has been made in understanding some of the fundamental Zn and Cd transport physiology in *T. caerulescens*. Furthermore, some interesting metal-related genes have been identified and characterized in this plant species, and regulation of the expression of some of these genes may be important for hyperaccumulation.

• Conclusions *Thlaspi caerulescens* is a fascinating and useful model system not only for studying metal hyperaccumulation, but also for better understanding micronutrient homeostasis and nutrition. Considerable future research is still needed to elucidate the molecular, genetic and physiological bases for the extreme metal tolerance and hyperaccumulation exhibited by plant species such as *T. caerulescens*.

Key words: Zn, Cd, Ni, *Thlaspi caerulescens*, hyperaccumulator, phytoremediation, heavy metal.

INTRODUCTION

The ability of terrestrial plants to deal with a broad array of abiotic conditions has allowed certain plant species to adapt to extremely harsh environments. One of the potential abiotic stresses a plant may face is toxic levels of heavy metals in the soil. The typical terrestrial plant has a limited capacity for dealing with excess metals; the main approach that most plants use to deal with heavy metals is storage in the root cell wall and vacuole, thus keeping the heavy metal sequestered from the root cytoplasm and, more importantly, from the shoot which minimizes heavy metal-associated damage to the photosynthetic apparatus. However, there are a number of plant species that have evolved on metalliferous soils and thus are adapted to extreme soil metal environments. Currently there are believed to be around 400 plant species from a number of different families such as the Asteraceae, Brassicaceae, Caryophyllaceae, Poaceae, Violaceae and Fabaceae that possess the ability to tolerate very high levels of heavy metals in the soil and, more importantly, in the plant shoot. The Brassicaceae is the best represented amongst these metal-hyperaccumulator families with 87 Brassica species classified as metal hyperaccumulators. Hyperaccumulation was a term first coined by Brooks et al. (1977) for plants that are endemic to metalliferous soils and are able to tolerate and accumulate metals in their above-ground tissues to very high concentrations (approx. 100 times that of a nonaccumulator plant species). Of these 87 different species in the Brassicaceae, two species in particular, *Thlaspi caerulescens* and *Arabidopsis thaliana*, have been studied extensively for their ability to hyperaccumulate several heavy metals, mainly zinc (Zn), cadmium (Cd) and nickel (Ni). In particular, certain ecotypes of *T. caerulescens* can accumulate as much as 30 000 ppm of Zn and approx. 10 000 ppm Cd in the shoot biomass without any signs of toxicity (typical shoot levels are 100–200 ppm Zn and 0.1–10 ppm Cd).

Contamination of soils with heavy metals is both an environmental problem as well as a risk to human health (Ryan et al., 1982; Mazess and Barden, 1991; Gairola et al., 1992). The remediation of metal-contaminated soils based on relatively destructive engineering-based methods has been estimated to cost approx. 400 billion dollars in the US alone (Salt et al., 1995). Thus there has been interest in recent years in determining whether an understanding of the mechanisms that metal-hyperaccumulator plant species such as *T. caerulescens* employ to absorb, detoxify and store metals can be used to develop plants suited for the remediation of metal-contaminated soils via phytoremediation.

Over the past 10 years as interest in metal-hyperaccumulating plants has increased, *T. caerulescens* has been at the forefront of studies concerning the hyperaccumulation process. Because *T. caerulescens* is a slow-growing plant species that does not generate significant...
shoot biomass, it has been used primarily as a model system for the investigation and identification of the underlying molecular and physiological mechanisms of hyperaccumulation, with the ultimate goal of transferring these mechanisms to higher biomass plant species. In this review, an overview of the physiological mechanisms of heavy-metal hyperaccumulation as well as the underlying molecular and genetic determinants for this trait will be discussed, with a focus on the body of work with *T. caerulescens*.

**PHYSIOLOGY OF METAL HYPERACCUMULATION IN *THLASPI CAERULESCENS***

The metal transport component of hyperaccumulation in *T. caerulescens* appears to involve at least four physiological events. The first is a stimulated metal influx across the root cell plasma membrane, the second reduced metal sequestration in the root vacuole, the third increased loading into the xylem for transport to the shoots and, finally, the fourth involves stimulated metal influx across the leaf cell plasma membrane and sequestration in the leaf vacuole. These transport steps resulting in metal hyperaccumulation are summarized in the model depicted in Fig. 1 for transport in and across the root, and Fig. 2 for transport in the leaf.

Based on a detailed concentration-dependent root Zn-uptake kinetic analysis for *T. caerulescens* compared with a closely related non-accumulator species, *T. arvense*, it was found that the affinity of the root Zn transporter for Zn was similar between the two species (*K_m* values of 6 μM for *T. caerulescens* and 8 μM for *T. arvense*); however, the maximal Zn uptake (*V_max*) in *T. caerulescens* was found to be approx. six times higher (Laset et al., 1996). This suggests that the Zn transporters in roots of *T. caerulescens* are not more efficient at transporting Zn than in non-accumulator plants; instead, it appears that the density of Zn transporters in the root-cell plasma membrane is much higher in *T. caerulescens* versus *T. arvense*. In both *Thlaspi* species, root Zn uptake appears to be regulated by plant Zn status. In *T. arvense*, like other non-accumulators, root Zn uptake increases as the plant transitions from Zn sufficiency to deficiency. *Thlaspi caerulescens*, on the other hand, maintains its much greater root Zn influx in both Zn-deficient and Zn-sufficient plants. Only when *T. caerulescens* is grown on very high Zn concentrations (nutrient solution with 50–1000 μM Zn) does the *V_max* for Zn uptake decrease, but is still maintained at flux values higher than what is seen in *T. arvense*. Another interesting aspect of the relationship between plant metal status and accumulation in *T. caerulescens* comes from the work of Papoyan et al.
In this study, it was found that when *T. caerulescens* was grown on very high Zn levels, the plants were more Cd tolerant and accumulated more Cd. The converse was also true, in that high Cd-grown plants also accumulated more Zn than low Cd-grown plants. This stimulated shoot metal accumulation was associated with enhanced root metal influx, and xylem transport of these metals from the root to shoot. The authors speculated that as xylem loading is a key step in the hyperaccumulation process, the enhanced xylem loading triggered by exposure to high heavy metal levels for extended periods, may translate into improved heavy metal tolerance, as the metals are more efficiently translocated to the shoots where highly effective metal tolerance mechanisms operate. When all of these studies are considered together, it appears that the regulation of expression of micronutrient/heavy metal transporters by plant metal status is altered in an unknown fashion in the hyperaccumulating *Thlaspi* species, resulting in higher expression of several different transporters along the metal transport pathway from the soil to the shoot.

Once the Zn enters the root, the plant deals with the Zn in one of two ways: by storing Zn in the root vacuole or by transporting the Zn radially through the root to be loaded into the xylem for transport to the shoots. Root Zn compartmentation via efflux analysis was studied in both *Thlaspi* species (Lasat *et al.*, 1998). It was found that *T. arvense* stored approx. 2.5 times more Zn in the root vacuole compared with *T. caerulescens* and this vacuolar Zn was released from the vacuole twice as slowly in *T. arvense*. Longer-term studies showed that as much as six times as much Zn was sequestered in the vacuoles of *T. arvense* compared with *T. caerulescens* over a 46-h time period (Lasat *et al.*, 1998). These findings indicate that, along with much higher rates of Zn entry into the root, the hyperaccumulator species also maintains the root Zn in a more mobile pool that is moved to the xylem much more readily.

The next key step regarding the movement of heavy metals from the soil to the shoot is the loading of Zn from xylem parenchyma into xylem vessels for translocation to the shoot. Xylem sap from *T. caerulescens* was found to contain approx. a 5-fold higher Zn concentration compared with the xylem sap from *T. arvense* for plants grown on the same Zn level. While the Zn concentration in the xylem increased with increased external concentrations of Zn, the ratio of xylem sap Zn concentration between the two *Thlaspi* species stayed the same (Lasat *et al.*, 1998). The dramatically greater metal loading into the xylem is a hallmark of metal hyperaccumulators and may be due to the activity of a P-type ATPase, HMA4, which will be discussed in greater detail in the molecular studies section later in this review. These root-associated transport steps are summarized in the model depicted in Fig. 1.

In the shoot, the storage of Zn and Cd to very high levels appears to require some co-ordination between different cell types. The highest concentrations of Zn and Cd are found in...
leaves of T. caerulescens, are summarized in the model depicted in Fig. 2.

It is presumed that an important aspect of metal storage involves metal chelation with organic ligands. However, it is still not clear which specific ligands are involved. Salt et al. (1999), using X-ray absorption, obtained results indicating that the form of Zn in T. caerulescens differed depending on the location in the plant. It was suggested that a significant fraction of the Zn within the roots is associated with histidine; in the xylem sap Zn was mostly found as the free hydrated Zn$^{2+}$ ion with a small portion bound to organic acids, and in the shoots Zn was most commonly found associated with organic acids, with smaller fractions found as the free ion and bound to histidine or the cell wall. It would be expected that metals such as Zn and Cd would be associated with sulfur ligands such as those in cysteine, glutathione or phytochelatins. Küpper et al. (2004), based on X-ray absorption spectroscopy analysis, suggested that a large portion of the Cd in the leaves of T. caerulescens was bound to sulfur ligands. On the other hand, using $^{113}$Cd-NMR, Ueno et al. (2005) presented findings suggesting a significant portion of the leaf Cd was bound to organic acids, mainly malate. Malate is not a particularly strong ligand for Cd, thus Ueno et al. (2005) speculated that efficient transport of Cd into the vacuole, which already contains fairly high malate concentrations, would facilitate the malate–Cd interaction. While there were already large amounts of malate in the vacuole, Cd treatment did not facilitate any increases in leaf malate levels.

It would be expected that phytochelatins, that are believed to play a role in plant Cd tolerance, would be involved in Cd hyperaccumulation in T. caerulescens. However, in a study comparing phytochelatin levels in leaves of T. caerulescens and T. arvense when plants were exposed to high Cd levels, no differences were found in leaf or root phytochelatin levels in these two plant species (Ebbets et al., 2002). A recent study looking at the effect of inhibition of phytochelatin biosynthesis on Cd tolerance showed that the T. caerulescens ecotype, Prayon, exhibited an increased sensitivity to Cd stress when a key enzyme in the phytochelatin synthetic pathway, $\gamma$-glutamylcysteine synthetase, was inhibited, suggesting that phytochelatins may play at least an initial role in Cd tolerance (Herández-Allica et al., 2006). It is interesting to note that, in the same study, the Gange ecotype, which accumulates considerably more shoot Cd than other T. caerulescens ecotypes (see discussion below), showed no change in Cd tolerance when $\gamma$-glutamylcysteine synthetase was inhibited, suggesting Gange may employ a different Cd tolerance mechanism than other T. caerulescens ecotypes. The authors speculated that a low-molecular-weight thiol other than phytochelatins that has not yet been identified might play a role as a Cd ligand in the Gange ecotype.

As mentioned above, T. caerulescens ecotypes from the south of France, such as Gange, accumulate much more leaf Cd than other ecotypes (with Prayon being the most widely studied ‘other’ ecotype), while all of the ecotypes are approximately equal in their ability to hyperaccumulate Zn. A detailed kinetic analysis of root Cd and Zn influx showed there was an approximate 5-fold larger $V_{\text{max}}$ for root Cd uptake in Gange versus Prayon in short-term radiotracer studies, while the $K_m$ value for Cd uptake was not different (Lombi et al., 2001). Comparison of Zn influx between the two ecotypes showed no differences in either $V_{\text{max}}$ or $K_m$ (Lombi et al., 2001). These physiological studies suggested there is a high-affinity Cd uptake transporter in the roots of the Gange ecotype that is not present in Prayon, which contributes to the enhanced Cd hyperaccumulation in Gange. It is not clear what the identity of this Cd transporter is. However, in a subsequent study, Lombi et al. (2002) showed that the imposition of iron (Fe) deficiency on the two ecotypes specifically induced high-affinity root Cd uptake in Gange. They also found that the root Fe uptake transporter IRT1 that has also been shown to transport Cd (Connolly et al., 2002; Vert et al., 2002; Cohen et al., 2004), was induced by Fe deficiency in roots of Gange but not Prayon. These findings are described in more detail in the section on the molecular biology of metal hyperaccumulation.

FIELD AND SOIL STUDIES

Some interesting field studies have been performed over the past several years that may indicate that, under certain conditions, despite its low biomass production, it might be possible to use T. caerulescens for the phytoremediation of certain metal-contaminated soils. It appears that certain variables such as season length, method of sowing seed, and soil pH have effects on the Zn and Cd extraction capabilities of T. caerulescens from the soil (McGrath et al., 2006; Yanai et al., 2006). It was found that a soil pH of between 5 and 6 seems to be optimum to facilitate Cd extraction as well as plant biomass production (Yanai et al., 2006). Also leaving the plants over winter increased the biomass production almost 5-fold and also increased the amount of Zn taken up by 57% versus that of a single 4-month growing period (McGrath et al., 2006). There was no difference seen in Cd accumulation between the 4- and 14-month growth conditions. In terms of the sowing of T. caerulescens plants, there was a $>2$-fold increase in the Cd and Zn taken up from the soil when the plants were sown as seedlings from a subplot versus that of planting seeds directly into the soil (McGrath et al., 2006). These few studies indicate that more agronomically based research on the practical aspects of metal hyperaccumulation and phytoremediation is needed.
With regards to metal hyperaccumulation from the soil, it has been suggested in earlier studies that *T. caerulescens* may be able to mine the soil for metals more efficiently than non-accumulator plants (Knight et al., 1997; McGrath et al., 1997). That is, it may be that *T. caerulescens* may have access to a different, more tightly bound pool of soil metals than non-accumulating plants, and it has been speculated that this may involve root organic acid exudation as a means of increasing metal availability in the rhizosphere. However, it should be mentioned that in hydroponic studies, where all the Zn and Cd are available, *T. caerulescens* still accumulates much more Zn and Cd than *T. arvense*, even at low Zn/Cd concentrations (Lasat et al., 1996; Pence et al., 2000).

There have been a few studies suggesting that rhizosphere metal availability plays a role in metal hyperaccumulation. For example, when *T. caerulescens* was grown in axenic versus non-sterile soil, the axenically grown plants accumulated 25–42% less Zn in the shoots, suggesting that rhizosphere microbes may play a role in metal availability for the hyperaccumulator (Whiting et al., 2001). Also, Ingwersen et al. (2006) found that *T. caerulescens* can increase the availability of extractable Cd in the rhizosphere primarily through the kinetically limited desorption of Cd from soil particles. However, a number of other studies did not find that *T. caerulescens* has any special properties with regards to mining the soil for heavy metals. Hammer et al. (2006) found the *T. caerulescens* did not accumulate more metal due to an increased pool of metals in the soil solution as has been suggested above. They found that *T. caerulescens* accessed the same soil metal pools as that of the non-accumulator, *Brassica napus*, under the same conditions. With regards to root exudation of organic acids, Zhao et al. (2001) found no significant differences in organic acid exudation from the roots of *T. caerulescens* compared with *T. arvense*. Based on the evidence that *T. caerulescens* has the same abilities as non-accumulators with regards to increasing metal availability in the rhizosphere, it was then suggested that it might be possible to amend the soil with materials such as EDTA or citrate to increase metal solubility, and facilitate better metal extraction by *T. caerulescens*. However, McGrath et al. (2006) found that the addition of certain amendments such as EDTA may actually decrease biomass production of the hyperaccumulators, thus reducing their effectiveness, possibly by increasing the solubility of metals such as Cu to levels that are toxic to the hyperaccumulator. A number of other possible amendments such as NTA and citric acid were also looked at under field conditions and seemed to have no effect on increasing metal content or growth of *T. caerulescens* (McGrath et al., 2006).

**GENETIC ANALYSIS OF METAL HYPERACCUMULATION**

A recent review of the *Brassica* family as a whole investigated various factors such as lifecycle, self-incompatibility, genetic resources and plant size to help find the best species within this family in order to begin to investigate the genetic basis for both Ni and Zn hyperaccumulation (Peer et al., 2006). To date, only a few genetic resources such as mapping or diversity populations have been developed for *T. caerulescens*, and only very recently have the first studies, which focused on quantitative trait locus (QTL) analysis of metal accumulation, been published.

The first QTL analysis study involved the phenotyping of an F₂ population generated from a cross between a higher Zn-accumulating *T. caerulescens* ecotype from a non-metalliciferous location (Lellingen), and a relatively lower Zn-accumulating ecotype from a calamine soil (La Calamine). Two major QTL were found for increased root Zn accumulation, with each parent in the population contributing one of the QTL. The two QTL explained 22% and 17% of the variation in root Zn accumulation, respectively (Assunção et al., 2006). The second publication looked at QTLs for both root and shoot Cd and Zn accumulation for a mapping population from a parent from a Pb/Cd/Zn-contaminated site near La Calamine, Belgium, while the second parent was selected from a site with similar soil characteristics near Ganges, France (Deniau et al., 2006). The authors identified eight total QTL between the two populations, with the Gange ecotype contributing six of the QTL. Two QTL for root Cd accumulation and two for root Zn accumulation were identified, as well as three shoot Zn-accumulation QTL and one for shoot Cd accumulation. These QTL explained 24% of the variation in Zn content and 60% of the variation in Cd content within the population. It is interesting to note that there was overlap of QTL for root Zn and Cd accumulation as well as for shoot Zn and Cd accumulation. Obviously the genetic analysis of the metal hyperaccumulation trait is in its infancy, but these studies suggest that metal hyperaccumulation in *T. caerulescens* is a relatively complex, quantitative trait.

**MOLECULAR BIOLOGY OF METAL HYPERACCUMULATION IN T. CAERULESCENS**

**Molecular analysis of metal transporters**

Major advances in the identification of a number of plant micronutrient/heavy metal transporter families has occurred, and new transporters such as the ZIP (ZRT/IRT like Protein), CDF (Cation Diffusion Facilitator), Nramp (Natural Resistance and Macrophage Protein) and HMA (Heavy Metal ATPase) families have been identified recently, primarily in the model plant species *A. thaliana*. However, to date only a handful of these transporters have been identified and characterized in *T. caerulescens*.

The first transporter gene cloned from *T. caerulescens* was ZNT1 by Pence et al. (2000). *TcZNT1* is a member of the ZIP family of transport proteins. The first members of this family were identified based on homology to the high-affinity Zn-uptake transporter in yeast, ZRT1, and the Arabidopsis Fe transporter, IRT1. *TcZNT1* is a putative plasma membrane-localized transporter that can mediate Zn and Cd uptake when expressed in yeast (Pence et al., 2000). *TcZNT1* shares high homology with ZRT1, the yeast high-affinity Zn-uptake transporter, as well as *AtZIP4*.
from Arabidopsis thaliana. It was found that changes in root TcZNT1 expression induced by changes in plant Zn status closely paralleled changes in the kinetics of root Zn uptake, leading the authors to speculate that TcZNT1 may be a major transporter mediating root Zn and Cd uptake from the soil (Pence et al., 2000). One of the hallmarks regarding expression of this gene in T. caerulescens versus expression of its homologue in non-accumulators such as T. arvense and A. thaliana is that it is expressed to much higher levels in roots and shoots of T. caerulescens (Fig. 3).

Furthermore, the response of TcZNT1 expression to changes in plant Zn status is different to that in non-accumulator plants. As seen in Fig. 3, TaZNT1 is expressed to very low levels in roots and shoots of a non-accumulator plant (T. arvense) grown on sufficient (10 μM) Zn. However, when these plants were made Zn deficient, TaZNT1 expression increased, presumably to synthesize more Zn transporters to facilitate Zn uptake. The same pattern is true for the Arabidopsis homologue, AtZIP4 (Grotz et al., 1998). In T. caerulescens, expression of TcZNT1 is very high in both Zn-deficient and -sufficient (1 μM Zn-grown) plants. Because T. caerulescens is relatively Zn inefficient, and requires higher Zn levels to achieve Zn sufficiency (Levent et al., 2003), it may be that growth on 1 μM Zn may not yield totally Zn-sufficient plants (although there are no observable symptoms of Zn deficiency in these plants). Or, when grown on this particular Zn concentration, the plants may behave as though they are physiologically Zn deficient as the metal is being effectively tied up as part of the hyperaccumulation phenotype. When T. caerulescens plants are grown at very high Zn levels (50–1000 μM), one sees a significant down-regulation of TcZNT1 expression (Fig. 3). However, even at these very high Zn levels, TcZNT1 expression is still significantly higher than that of its homologues in T. arvense or Arabidopsis. Thus it has been suggested that hyperexpression of TcZNT1 and other metal-related genes involves an alteration in the regulation of these genes by plant metal status, and this hyperexpression may play a role in hyperaccumulation (Pence, 2002; Letham et al., 2005).

It is interesting to note that, although in Arabidopsis ZIP4 is the closest homologue to TcZNT1, sharing 90% DNA sequence identity as well as 81% homology at the amino acid level, it may function as a different metal transporter. TcZNT1 was cloned via complementation of yeast mutants defective in endogenous Zn transporters (the zrt1/zrt2 mutant) and thus could not grow on low Zn (Pence et al., 2000). TcZNT1, at least in yeast, was shown to be a high-affinity Zn uptake transporter and low-affinity Cd uptake transporter. Furthermore, Grotz et al. (1998) showed that AtZIP4 could not complement the same zrt1/zrt2 Zn uptake-deficient yeast mutant. AtZIP4 was later shown to complement the cfr1 yeast mutant, which is defective in Cu uptake, thus allowing the mutant to grow under Cu-limiting conditions (Wintz et al., 2003). These findings suggest that AtZIP4 is involved in Cu uptake and not Zn/Cd uptake. Furthermore, its expression was shown to be induced not only by Zn deficiency, but also by Cu deficiency (Wintz et al., 2003). Furthermore, the cell-specific expression of these transporters also may suggest they have different roles than initially suggested. In the root, at least for AtZIP4 expression, transgenic Arabidopsis expressing the AtZIP4 promoter::GUS construct indicates that AtZIP4 expression is localized to the stele, thus suggesting a role in trans-root metal transport and not metal uptake from the soil as previously suggested (Fig. 4; M. J. Milner and L. V. Kochian, unpubl. res.). The same experiment for the TcZNT1 promoter::GUS reporter has not yet been completed. As mentioned above, metal accumulation in the T. caerulescens leaf is much higher in epidermal cells (except guard cells) compared with other leaf types. However, Küpper et al. (2007), using a quantitative in situ hybridization technique, found that TcZNT1 is not expressed in the leaf epidermal cells. Instead, it is preferentially expressed in leaf mesophyll, bundle sheath and guard cells, leading them to postulate it plays a role in normal leaf Zn nutrition and not metal hyperaccumulation in T. caerulescens.

The second gene cloned from T. caerulescens was ZTP1/MTP1, which was identified based on homology to the Arabidopsis transporter, ZAT1, which is thought to be involved in loading Zn into the vacuole (van der Zaal et al., 1999; Assunção et al., 2001; Mäser et al., 2001; Persans et al., 2001). ZTP1 shares high homology to MTP1, a metal transporter from the Ni hyperaccumulator, T. goesingense, which is a member of the CDF family of cation transporters. However, TgMTP1 localizes to the plasma membrane, while TcZTP1 is thought to localize to the tonoplast, based on homology to AtZAT1.
Another metal transporter that has received significant attention in *T. caerulescens* is TcHMA4, which was first identified via yeast complementation and screening of transgenic yeast for increased Cd tolerance (Bernard et al., 2004; Papoyan and Kochian, 2004). TcHMA4 is a member of the P-type ATPase superfamily, and more specifically, the P1B subfamily of ATPases that are purported to transport heavy metals. TcHMA4 was found to be expressed primarily in roots and its expression is induced by both Zn-deficiency and high-Zn treatments, as well as in response to high Cd (Papoyan and Kochian, 2004). The Arabidopsis homologue of TcHMA4 has been characterized in detail and has been shown to be expressed primarily in roots and its expression is induced by both Zn-deficiency and high-Zn treatments, as well as in response to high Cd (Papoyan and Kochian, 2004). The Arabidopsis homologue of TcHMA4 has been characterized in detail and has been shown to be expressed primarily in the root stele and is believed to be involved in loading of Zn into the xylem for transport to the shoots (Hussain et al., 2004; Verret et al., 2004; Sinclair et al., 2007). Overexpression of AtHMA4 also leads to increased accumulation of Zn and Cd in the shoots of the transgenic arabidopsis plants, further suggesting HMA4 plays a role in loading metals into the xylem (Verret et al., 2004). Also when both AtHMA4 and its close relative, AtHMA2 are both knocked out in Arabidopsis, reduced Zn accumulation in the shoot is seen (Hussain et al., 2004). As efficient translocation of metals from the root to the shoot is a hallmark of metal hyperaccumulators, it has been suggested that TcHMA4 may play a critical role in heavy metal transport to the shoot during hyperaccumulation (Papoyan and Kochian, 2004). Support for this hypothesis comes from work from another metal hyperaccumulator, *Arabidopsis halleri*. In a QTL mapping study in a cross between *A. halleri* and *A. lyrata* (a related non-accumulator), a major Cd-tolerance QTL co-located with AhHMA4 (Courbot et al., 2007).

Molecular studies of potential metal-binding ligands

As discussed above, physiological and biochemical studies have failed to clearly identify metal binding ligands which would be expected to be associated with the large concentrations of Zn and Cd accumulated in the leaves of hyperaccumulators such as *T. caerulescens*. These physiological/biochemical studies have focused on organic acids, amino acids and phytochelatins. However, a few molecular studies have provided some circumstantial evidence that other organic compounds may play a role as metal-binding ligands. In the yeast complementation study described above that identified TcHMA4 as a protein that conferred Cd tolerance in yeast, members of another family of genes, the metallothionein (MT) family, were also found to confer Cd tolerance to yeast (Papoyan and Kochian, 2004). Metallothionines are cysteine-rich, low-molecular-weight, metal-binding proteins that can form mercaptide bonds with various metals and have been implicated in metal homeostasis primarily in

![Fig. 4. AtZIP4::GUS expression in roots of Zn-deficient transgenic Arabidopsis plants. The root GUS expression pattern from transgenic Arabidopsis transformed with the AtZIP4 promoter-GUS. Plants were grown for 2 weeks on a modified Johnson’s solution with no Zn. (A) Root tip – the first centimetre of the primary root. (B) The primary root from 1 cm to 2 cm back from the root tip. (C) The primary root from 2 cm to 3 cm back from the root tip. (D) Lower magnification of the first 2 cm of the primary root.](https://academic.oup.com/aob/article/102/1/3/173040)
mammals (Cobbett and Goldsborough, 2002). Metallothioneins have been found in a wide range of organisms crossing a number of kingdoms, with all of the plant MTs falling in one of two main subclasses. For these two main groups, classification is based on where the cysteine residues thought to be involved in the binding of the various metal ions are located (Cobbett and Goldsborough, 2002).

The first member of the MT family that was studied in some detail in *T. caerulescens* was TcMT3 (Roosens et al., 2004). TcMT3 is more highly expressed in the shoot but seems to have a basal level of expression throughout the plant under a wide variety of conditions. Furthermore, TcMT3 expression is induced by Cd exposure. However, with regard to function, questions have arisen regarding the possible role of TcMT3 in metal hyperaccumulation. Based on functional studies in yeast, it appears that TcMT3 confers much greater levels of tolerance to Cu than Cd, and did not confer a measurable increase in Zn tolerance. The increase in yeast Cu tolerance (in a Cu-sensitive mutant background) was more strongly conferred by TcMT3 compared with AtMT3 (Roosens et al., 2004). The authors speculated that possibly TcMT3 plays a role in metal tolerance by allowing *T. caerulescens* to maintain normal Cu homeostasis under conditions where high levels of Cd and Zn occur in the cytoplasm. Roosens et al. (2005) also studied and characterized two other MTs in *T. caerulescens*, TcMT1 and TcMT2, in comparison with their Arabidopsis *thaliana* homologues. Constitutive expression of TcMT1 and TcMT2 was considerably higher than expression of their homologues in Arabidopsis. However, interestingly, with regards to functional metal-tolerance assays in yeast, although both TcMT1 and TcMT2 conferred increased tolerance to Cd, Zn and Cu, the Arabidopsis homologues were either able to confer an equivalent level of tolerance to these metals for AtMT2 (compared with TcMT2), or a greater degree of Cd and Zn tolerance (for AtMT1). Based on these findings, the authors again speculated that in *T. caerulescens*, MTs might be playing a role in maintaining proper Cu nutrition/homeostasis in the face of Cd and Zn hyperaccumulation.

Several studies have focused attention on the role of the non-protein amino acid, nicotianamine (NA), in metal tolerance in *T. caerulescens*. NA has been shown to be a chelator of several micronutrient metals and has been suggested to be involved in the movement of micronutrients and heavy metals throughout the plant (Stephan and Scholz, 1993). In ecotypes of *T. caerulescens* that also hyperaccumulate Ni, Mari et al. (2006) found via yeast complementation for Ni tolerance that the gene encoding nicotianamine synthase, TcNAS1, conferred high levels of Ni tolerance when expressed in yeast. TcNAS1 was found to be expressed only in the shoots and induced in as little as 6 h after treatment of Ni. However, after this same 6-h exposure to Ni, high levels of NA were found in the roots, and NA-Ni complexes were also found in the xylem sap. This led the authors to speculate that, in response to Ni, NA is translocated to the roots where it chelates the absorbed Ni and facilitates its transport to the shoot. Further evidence for a role for TcNAS1 in Ni hyperaccumulation came from studies where TcNAS1 was over-expressed in transgenic *A. thaliana* plants (Piannelli et al., 2005). This resulted in a significant increase in both plant Ni tolerance and Ni accumulation in the shoot. These findings suggest that a number of transporters need to be involved in the movement of both free NA and the NA-metal complexes in the plant. One possible family of transporters for this role is the YSL (Yellow Stripe Like) family, where the first member of this family was identified as the putative Fe-phytosiderophore uptake transporter in maize roots, while other members were hypothesized to be involved in transport of NA–metal complexes (Curie et al., 2001). Three members of the YSL family have been characterized in *T. caerulescens*, named TcYSL3, TcYSL5 and TcYSL7 based on sequence homology to Arabidopsis YSLs (Gendre et al., 2007). All three genes were shown to be more highly expressed in *T. caerulescens* than their arabidopsis counterparts. Both TcYSL3 and TcZYL7 were found to be expressed in the root stele, associated with the xylem. Functional analysis in yeast demonstrated that TcYSL3 had the ability to transport both Ni–NA and Fe–NA complexes into yeast. Based on these findings, the authors speculate that TcYSL3 may be involved in long-distance Ni translocation in *T. caerulescens*.

**Molecular studies on ecotypic variation in Cd hyperaccumulation**

As described above in the section on the physiology of hyperaccumulation, *T. caerulescens* ecotypes from the south of France such as the Gange ecotype are more effective Cd hyperaccumulators than other ecotypes, with the Prayon ecotype often used as the comparison. Lombi et al. (2002) noted that when both Gange and Prayon were made Fe deficient, an increase in Cd accumulation was seen. This property had been studied previously for non-accumulator species, and it was shown in those studies that the root Fe transporter, IRT1, whose expression is induced by Fe deficiency, can also function as a Cd transporter (Cohen et al., 1998; Connolly et al., 2002; Vert et al., 2002). In *T. caerulescens*, this Fe deficiency-induced increase in Cd accumulation was shown to be much greater in Gange versus Prayon, and a role for TcIRT1 was suggested (Lombi et al., 2002). More recently, comparison of TcIRT1 in *T. caerulescens* with *A. thaliana* in arabidopsis has shown that there are actually two versions of the IRT1 gene in *T. caerulescens*, and both the Gange and Prayon ecotypes harbour both a full-length and a truncated version of the gene in their genomes. The Gange ecotype only expresses the full-length version of TcIRT1 while the Prayon ecotype only expresses the truncated version. Interestingly, both the full-length and truncated versions of TcIRT1 from the two ecotypes of *T. caerulescens* do not effectively transport Cd, compared with AtIRT1. Thus the authors concluded that TcIRT1 is not the high-affinity root Cd transporter identified from the previous physiological comparisons of root Cd influx in Gange versus Prayon (Plaza et al., 2007). Hence the molecular and physiological...
basis for the increased Cd accumulation in ecotypes such as Prayon is still a mystery.

Global analysis of the T. caerulescens transcriptome

As described above for specific genes in T. caerulescens such as TcZNT1, TcHMA4 and TcMTP1, their expression is much higher than the expression of their homologues in related non-accumulator plant species. This has led to the speculation that this ‘hyperexpression’ of specific metal-related genes may play a role in metal hyperaccumulation (see, for example, Pence et al., 2000; Pence 2002). Subsequently, using microarray technology, global analysis of the T. caerulescens transcriptome has shown this to be a broad response involving many genes. Several studies have compared the transcriptome of T. caerulescens and a related non-accumulator using commercially available Arabidopsis gene chips. Hammond et al. (2006) conducted a shoot transcriptome comparison between T. caerulescens and T. arvensis using the Affymetrix Arabidopsis thaliana GeneChip array. The authors painstakingly conducted the necessary proof of concept analysis to validate that cross species hybridization to a genome wide array of a model species was able to yield reproducible results. In this study, a number of genes previously mentioned such as ZNT1, MTP1, and HMA4 all showed much higher expression in the hyperaccumulator. Additionally, a number of other genes encoding transporters from the ZIP, CDF and HMA families were also hyperexpressed in T. caerulescens. Previously, a similar comparative transcriptome analysis had been conducted between a different Zn/Cd hyperaccumulator, A. halleri, and A. thaliana using the gene chip arrays (Becher et al., 2004; Weber et al., 2004). That study showed that hyperexpression may be a general property of metal hyperaccumulators, as a large number of genes was also found to be more highly expressed in A. halleri compared with A. thaliana. Interestingly only 16 of the genes shown to be more highly expressed in A. halleri also exhibited elevated expression in T. caerulescens, suggesting this set of genes might be important for hyperaccumulation in both plant species.

A comparison of the root transcriptomes of T. caerulescens versus A. thaliana using the Agilent Arabidopsis 3 60-mer oligonucleotide microarray identified a number of previously identified and also novel genes showing elevated expression in T. caerulescens (van de Mortel et al., 2006). Many of these genes have been previously been identified as playing a role either in metal transport or sequestration. However a number of other novel genes were also looked at. For example, 131 transcriptional regulators were identified exhibiting at least a 5-fold increase in expression in T. caerulescens compared with Arabidopsis. Interestingly, the authors also identified a suite of hyperexpressed genes involved in lignin biosynthesis. This finding correlated with increased lignification/suberization of the endodermal cell layer and even an occasional observation of two endodermal cell layers in roots of T. caerulescens. Based on these observations, the authors suggested the more strongly developed endodermis in the hyperaccumulator may function to minimize the back movement of metals accumulated in the stele during the trans-root processes resulting in metal loading into the xylem.

FUTURE RESEARCH AND CONCLUDING REMARKS

While significant progress has been made in understanding the physiology of metal hyperaccumulation since Brooks et al. (1977) first coined the term, hyperaccumulator, there is still much more to be done to understand this fascinating process. Progress has been made in understanding some of the fundamental transport physiology, and some of the notable genes involved in metal uptake and transport, as well as in possible mechanisms of metal tolerance have been identified. With the strong evidence that HMA4 and/or HMA2 may play a key role in metal loading into the xylem, which appears to be important in hyperaccumulators as they very efficiently translocate most of their absorbed metal to the shoot, more work on this specific transport step is needed. Furthermore, it is still not clear whether common or separate mechanisms are employed for the hyperaccumulation of Zn, Cd and sometimes Ni in this plant species. The significant difference in Cd hyperaccumulation between ecotypes from different regions of the world needs to be exploited more fully as an experimental tool to better understand Cd hyperaccumulation.

Although a number of different metal transporters have been implicated in metal hyperaccumulation, the transport function and regulation at the transcriptional and post-transcriptional levels for most of these transporters is still quite poorly understood. As pointed out earlier, sequence similarity may not be the best predictor of transport function. The comparison between TcZNT1 and AtZIP4, while closely related at the DNA and protein sequence level, appear to transport different micronutrient/heavy metals and their expression is also influenced by the plant status for different metals. It is clear that a better understanding is needed of the relationship between transporter structure and function, as well as the regulation of these transporters.

One of the most striking features of metal hyperaccumulators is the hyperexpression of whole suites of both metal-related, and nonmetal-related genes. It appears that this hyperexpression is important to the hyperaccumulation phenotype. However, almost nothing is known about the molecular basis for hyperexpression. Is this trait controlled by a small group of unique trans-acting factors, or by trans-factors acting in concert with promoter elements unique to hyperaccumulators?

A better understanding of metal hyperaccumulation in T. caerulescens will certainly benefit from the development of more and better genetic resources. To date, only a handful of QTL analysis studies has been conducted with relatively small mapping populations. This is certainly an area of research that requires much more development.

While a number of soil-based trials have been conducted under controlled conditions, the real application in the field still needs to be investigated further. A few studies recently have looked at the feasibility of using this plant species for the remediation of moderately Cd contaminated soils and
have shown some promise (McGrath et al., 2006; Yanai et al., 2006). Also with the wide amount of natural variation in different populations of *T. caerulescens*, increased selection for traits of interest may help improve its phytoremediation capacity. However, to date, only remediation of Ni-contaminated sites using hyperaccumulator species other than *T. caerulescens* has been shown to be economically viable (Chaney et al., 2005).

*Thlaspi caerulescens* is a fascinating and useful model system not only for studying extreme metal hyperaccumulation, but also for better understanding micronutrient homeostasis and nutrition. We have only started to understand the mechanisms underlying this unique plant trait and there certainly are interesting times ahead. More broadly, with the wide diversity of plants as well as ecological niches that allow for life to flourish under harsh conditions, understanding the molecular and physiological basis that allow these extremeophiles to function in these unique niches could provide considerable basic information that will be useful both for improving crop production on marginal and degraded soils, and also for developing plants well suited for environmental remediation.

**LITERATURE CITED**


