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Title: # Transition metal transport

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Keywords: heavy metal transport, hyperaccumulation, deficiency, metal homeostasis, evolution

List of abbreviations:

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Abstract

Transition metal transporters are of central importance in the plant metal homeostasis network which maintains internal metal concentrations within physiological limits. An overview is given of the functions of known transition metal transporters in the context of the unique chemical properties of their substrates. The modifications of the metal homeostasis network associated with the adaptation to an extreme metalliferous environment are illustrated in two Brassicaceae metal hyperaccumulator model plants based on cross-species transcriptomics studies. In a comparison between higher plants and unicellular algae, hypotheses are generated for evolutionary changes in metal transporter complements associated with the transition to multicellularity.

1. Introduction

Essential transition metals are required in all plant organs for the activities of numerous metal-dependent enzymes and proteins. According to a search of protein descriptions in The Arabidopsis Information Resource (TAIR) database, the largest number of proteins are functionally associated with zinc (1272), followed by copper (108), iron (106), manganese (12), and nickel (4). Metal-containing proteins of the photosynthetic machinery are particularly abundant in plants, with a predominance of iron, for example in the reaction centres of photosystems I and II, and the occurrence of manganese in the oxygen-evolving complex of photosystem II and copper in plastocyanin [1].

Relative to the amounts accumulated by plants, transition metal ions are substantially less available for uptake by plant roots than other inorganic nutrient ions in most soils [2]. Therefore, plants possess highly effective metal acquisition and uptake systems [3]. The concentrations of metal ions in the soil and their chemical speciation are subject to major fluctuations. This requires the regulation of metal uptake and its coordination with detoxification and storage mechanisms.

Metal uptake, metal partitioning to plant organs and cell types, and metal delivery to metal requiring proteins in different sub-cellular localizations, as well as metal storage in vacuoles and re-mobilization all require the operation of transition metal transporters [3]. Not surprisingly, Arabidopsis mutants with defects in the transport of essential transition metals have severe phenotypes [4-6]. The dynamic range of optimal internal concentrations, within which plants are

unaffected by deficiency or toxicity, is particularly narrow for transition metals [3]. Thus, metal transport and its regulation have to operate with high precision and specificity.

Extensive regulatory cross-talk is to be expected between the transition metal homeostasis network and homeostatic systems of other nutrients [7]. Regulatory interactions between transition metal homeostasis and plant growth, metabolism and development must ensure that metal requirements are met under different environmental conditions in all cell types and at all stages of development [4,8]. Maintaining availability and controlled distribution of metal ions in a plant requires tight control of their binding to organic and inorganic compounds [9].

Most known proteins that mediate the transport of transition metals in plants belong to the following families: the zinc-regulated transporter, iron-regulated transporter protein (ZIP) family, the cation diffusion facilitator (CDF) family, the P_{1B}-type subfamily of P-type ATPases, the natural resistance associated macrophage protein (NRAMP) family, the yellow-stripe 1-like (YSL) subfamily of the oligopeptide transporter (OPT) superfamily, the copper transporter (COPT) family, the Ca^{2+}-sensitive cross complementer 1 (CCC1) family and the iron-regulated protein (IREG) family. Other membrane protein families that have been implicated in transition metal transport are the cation exchangers (CAX) family and three subfamilies of ATP-binding cassette (ABC) transporters, the multidrug resistance-associated proteins (MRP), the ABC transporters of the mitochondria (ATM) and the pleiotropic drug resistance (PDR) transporters [10]. Finally, putative transporters of metal ion ligands, the *At*FRD3 (Ferric Reductase Defective 3) of the large multidrug and toxin efflux (MATE) family and *At*ZIF1 (Zinc Induced Facilitator 1) of the major facilitator superfamily (MFS), have key roles in iron and Zn homeostasis, respectively [11-13]. These plant metal transporter families and their biological roles have been reviewed by other authors recently [2,14-21](review Iron Transport, this issue).

Here we begin by outlining some specific characteristics that distinguish transport processes for essential and toxic transition metals from the transport of other small molecules or ions. We then discuss the issue of transition metal transporter specificity and its implications for metal homeostasis and toxicity. We review the emerging key roles of metal transporters in naturally selected metal hyperaccumulation and discuss the adaptation of the metal homeostasis network to an extreme environment. Finally, we address the adaptive and evolutionary implications of differences in metal transporter complements between organisms. We apologize to all authors whose work is not cited due to space limitations.

2. Membrane transport of transition metal ions and their complexes

For most transporter substrates, such as sodium cations or glucose, a freely diffusible, unbound pool is present on either side of a biological membrane. These substrates can associate with and be released from various binding sites rapidly, based on the affinities of the binding sites and the concentrations of binding sites and substrates.

The distinct chemical properties of transition metal ions require distinct molecular models for their transport across membranes. As soft Lewis acids, transition metal ions exhibit very high binding affinities for electron-pair donors such as carboxylic groups, amino groups and sulfide groups. Complexes of particularly high stability are formed when several electron pair donors are available from one molecule for multi-dentate complexation, i.e. chelation. The solubility of transition metal phosphate salts is very low. A large excess of non-specific binding sites are thus available for metal ions, particularly in the cytoplasm. Moreover, a transition metal ion can displace another metal ion downstream in the Irving-Williams series from its specific binding sites ($Zn^{2+} < Cu^{+} > Cu^{2+} > Ni^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+}$). Consequently, transition metal ions present in free aqueous form in the cytoplasm can disrupt cellular functions.

In the soil solution, free metal ion concentrations are in the sub-micromolar to nanomolar range [2]. High affinities of plasma membrane metal uptake systems allow metal entry into cells, mostly from the pool of free transition metal cations in the soil solution [2,3,22]. After entering the cell through a plasma membrane metal uptake system, the cytoplasmic movement of transition metals by a random sequence of binding and dissociation events would render metal homeostasis slow and inefficient. When a transition metal ion has bound to a binding site, a process that can be reasonably fast (rate constants $\geq 10^{8}$ s^{-1}, compared to ~ 10^{9} s^{-1} for Ca^{2+}), the release is slow (rate constants < 10^{-2} s^{-1}, compared to ~10^{3} s^{-1} for Ca^{2+}; determined by lower ionic radii of transition metal cations compared to Ca^{2+}, coordination symmetries, and several other factors)[23]. This further emphasizes the need for intracellular metal trafficking and tight control over the binding partners of transition metal ions.

Based on metal binding affinities of apo-metalloproteins and metal sensors, cytoplasmic concentrations have been estimated at between 10^{-21} and 10^{-18} M for free aqueous copper ions [24], and at around 10^{-15} M for free aqueous zinc ions [9,25]. This corresponds to less than one cytoplasmic free metal ion per cell. It has been concluded from this that there is no persistent pool of free transition metal ions, from which intracellular metal transporters can bind metal ions. Instead,

intracellular metal transporters are thought to acquire their metal ion substrates through interaction with metallochaperone shuttle proteins [26].

The role of copper metallochaperone proteins in Cu trafficking and the interactions of copper metallochaperones with copper transport proteins and target apo-metalloproteins are well conserved across cyanobacteria, plants, yeast and humans [2,26]. For example, the cytoplasmic yeast Cu metallochaperone *Sc*ATX1 delivers Cu(I) to the *Sc*CCC2 P_{1B}-type Cu-ATPase that transports Cu into late Golgi or post-Golgi vesicles. By analogy, a yeast-two-hybrid assay suggested the interaction of the cytoplasmic *At*ATX1 with *At*RAN1, the post-Golgi localized P_{1B}-type Cu-ATPase transporter required for the delivery of Cu to the ethylene receptor [27,28]. Metallochaperones for zinc or iron, however, have not been identified so far. In plants, low-molecular-weight chelators, such as nicotianamine, glutathione, phytochelatins, histidine or citrate, are important metal chelators and often required for long-distance and inter-cellular metal transport, or for the sub-cellular compartmentalization of metals [2,3]. However, a metallochaperone-like metal trafficking role has not yet been experimentally demonstrated for any low-molecular-weight chelator. Instead, a subset of known metal transporters transports metal-chelate complexes and not metal cations (see below).

Transport properties of plant metal transporters mediating metal influx into the cytoplasm, for example the Zn transporter *At*ZIP1, have been analyzed upon heterologous expression in yeast by measuring metal uptake into yeast cells [29]. Heterologous expression in oocytes of *Xenopus laevis* has been used in electrophysiological approaches to determine transport properties and mechanisms for the maize yellow-stripe 1 protein, the uptake system for phytosiderophore-iron chelates and a related protein from *A. thaliana* [30,31]. The establishment of functional assays for the direct analysis of transition metal transporter functions is still a major bottleneck. Especially for internal membrane transition metal transporters, transport functions have so far largely been inferred from phenotypic yeast complementation and mutant phenotypes, such as metal sensitivity or changes in bulk tissue metal accumulation.

In one of the few direct studies of a transition metal effluxer from the cytoplasm, metal-dependent ATP hydrolysis and extra-vesicular Zn depletion have been measured using microsomal vesicles prepared from the yeast *Saccharomyces cerevisiae* expressing *At*HMA2. Metal-dependent ATPase activity was maximal in the presence of Zn or Cd. Half-maximal ATPase activities were reached at 0.11 µM Zn and at 0.031 µM Cd in the presence of an excess of 20 mM cysteine, which likely formed metal complexes in the assay mixture [32]. This highlights the fact that to date, there is

insufficient knowledge of the *in vivo* substrates of metal transporters, or the metallochaperones or metal complexes delivering metal ions as substrates of metal transporters.

3. Metal specificity of transition metal transport in the plant metal homeostasis network

Among the functionally characterized transition metal transporters, metal selectivity is generally lower in the transporters mediating metal influx into the cytoplasm from an extra-cytoplasmic compartment. For example, *At*ZIP2 and *At*ZIP4 enhance cellular accumulation of Zn^{2+} as well as Cu^{2+} [29,33]. The best characterized member of this same protein family, *At*IRT1 known as the primary root iron uptake system of *A. thaliana*, transports a wide range of divalent transition metal cations, including toxic Cd^{2+} ions [34]. Consequently, plants appear to lack full control over the composition of the cocktail of metals entering their cytoplasm, making them somewhat vulnerable to metal imbalances and toxic metal cations present in the soil as contaminants [35]. Inside the plant, there is a need to differentiate between the transition metal cations of different elements, either by selective cytoplasmic metal chelation and trafficking or by internal transporter selectivity.

Based on the limited knowledge available to date, metal selectivity appears to be more pronounced in transporters that export transition metals from the cytoplasm. For example, according to the available data, the transporters *At*MTP1 and *At*MTP3 are largely specific for the export of Zn from the cytoplasm into the vacuole [19,36-38]. A distinct phylogenetic group of the same CDF protein family, including the vacuolar-membrane localized *Stylosanthes hamata* MTP8 (previously *Sh*MTP1), appear to be specific for Mn export from the cytoplasm [39]. Furthermore, the Arabidopsis P_{1B}-type ATPases HMA2, HMA3 and HMA4 have been reported to export Zn and Cd, but not Fe or Mn, from the cytoplasm across the plasma membrane [4,32,40].

The specificity of transition metal export from the cytoplasm may serve to establish specificity through the differential storage of transition metals in specific tissues or cell types [35,41,42]. In combination with this, the broad substrate specificity of cytoplasmic transition metal importers, for example of the ZIP and NRAMP families [5,42], may ensure their universal potential to fulfill different transport functions in different plant organs or at different developmental stages, without perpetuating the consequences of limited metal transporter specificity throughout the plant.

4. The role transition metal transport processes in naturally selected metal hyperaccumulation

Metal hyperaccumulator plants accumulate and detoxify extraordinarily high levels of metal ions in their above-ground tissues. Metal hyperaccumulation and metal exclusion are two opposed physiological strategies used by plants to grow and reproduce on soils containing high, potentially toxic levels of transition metal ions of geogenic or anthropogenic origin [43]. The ecology and physiology of metal hyperaccumulator plants have been investigated since the 1970s, but the molecular mechanisms underlying metal hyperaccumulation have only been addressed by researchers recently. Especially for Zn hyperaccumulation, a picture is beginning to emerge which attributes a central role to metal transport proteins, improving our mechanistic understanding not only specifically of metal hyperaccumulation but also of the plant metal homeostasis network in general.

Two model hyperaccumulator species of the Brassicaceae have been used in particular for molecular studies – the Zn/Cd hyperaccumulator *Arabidopsis halleri* and *Thlaspi caerulescens*, a species with considerable natural variation in hyperaccumulation and tolerance of Zn, Cd and Ni between populations. Together with the non-accumulator *Arabidopsis lyrata*, *A. halleri* forms the sister clade of the Zn-sensitive, non-accumulating model species *A. thaliana*. *A. halleri* and *A. thaliana* are estimated to have diverged approximately 3.5 to 5.8 million years ago, and *T. caerulescens* and *A. thaliana* approximately 20 million years ago [44]. Compared to *A. thaliana*, *A. halleri* and *T. caerulescens* show on average about 94% and 88.5% conservation of nucleotide sequences within coding regions, respectively [45,46]. This has made it possible to use genomics tools developed for *A. thaliana* for the molecular study of the two hyperaccumulator species.

In the past three years, microarray-based cross-species comparisons have been performed of the transcriptomes of *A. halleri* or *T. caerulescens* and related non-accumulator species, i.e. *A. thaliana*, *A. lyrata* or *Thlaspi arvense* [44-50]. These studies identified candidate genes that may be involved in the physiological processes governing metal accumulation and associated tolerance. Interestingly, there is a common core set of metal transporter and metal homeostasis genes which are more highly expressed in both hyperaccumulator species compared to their non-accumulator relatives. We will

focus here on this convergent core set of candidate genes encoding members of the ZIP, CDF, HMA and NRAMP transporter families as well as FRD3 and NAS2, a ligand transporter and an isoform of nicotianamine synthase, respectively (Figure 1).

4.1 ZIP family of membrane transport proteins

In roots and/or shoots of *A. thaliana*, transcript levels of several genes encoding ZIP family transporters, namely *AtZIP1* to *AtZIP5*, *AtZIP9* to *AtZIP12* and *AtIRT3*, are increased under Zn-limiting conditions [29,33,44,46]. In both hyperaccumulators, *A. halleri* and *T. caerulescens*, the genes *ZIP4*, *ZIP10* and *IRT3* are highly expressed in roots under conditions of sufficient Zn supply [44-46,51-53]. Nevertheless, the expression of these genes remains responsive to changes in Zn supply and decreases with increasing external Zn in both species.

ZIP4 and *IRT3* have also been found to be highly expressed in shoots of *T. caerulescens* [49]. In shoots of *A. halleri*, *ZIP4* and *IRT3* expression is increased under Zn deficiency, but not above levels observed in leaves of Zn-deficient *A. thaliana* [44]. *AhZIP4*, *AhZIP10* and *AhIRT3* are most likely single copy genes [44]. The *T. caerulescens* homologues of *AtZIP4* and *AtIRT3* have previously been isolated in molecular approaches involving screens of cDNA libraries and were named *ZNT1* and *ZNT2*, respectively [51-53]. *ZNT1/TcZIP4* can mediate Zn and Cd influx when expressed in yeast [51,52].

In *A. halleri*, the high but Zn-responsive expression of *ZIP4* and *IRT3* is closely linked with Zn status, particularly in roots [44]. Enhanced root metal uptake mediated by ZIP family proteins is likely to be a factor necessary but not sufficient for hyperaccumulation.

Two other genes encoding ZIP transporters have been identified as candidates in the transcriptomic studies of hyperaccumulators: *ZIP9* and *ZIP6*. In *A. thaliana*, *ZIP9* is strongly responsive to Zn deficiency, its expression being upregulated in roots and induced in shoots. In roots of *A. halleri*, *ZIP9* is highly expressed under Zn sufficient conditions, thus, its Zn-responsiveness appears to be partially lost in roots [44,46,47]. In shoots of *A. halleri*, *ZIP9* expression is only detectable under Zn deficiency, albeit at a lower level than in *A. thaliana* [44]. *AhZIP9* is most likely to be encoded by two gene copies [44]. This opens the possibility that one of the copies lost Zn-responsive transcriptional regulation accounting for the high expression levels in roots at sufficient Zn supply, whereas the other copy retained its Zn-responsiveness. Contrary to the findings in *A. halleri*, *ZIP9*

expression in *T. caerulescens* roots is Zn-responsive and appears not to be higher at sufficient Zn than in *A. thaliana* roots [46].

Different from several other members of the ZIP family, *ZIP6* transcript levels are not upregulated under Zn deficiency in *A. thaliana*. Compared to non-accumulators, *ZIP6* is more highly expressed in the shoots of both *A. halleri* and *T. caerulescens* and in the roots of *A. halleri* [44,45,48,49]. In *A. halleri*, *ZIP6* appears to be a two-copy gene with constitutively high transcript levels showing no metal-dependent regulation [44,45]. Interestingly, in a microarray study comparing *A. halleri*, *A. lyrata* and Zn-accumulating and non-accumulating F3 progeny of a cross between these two species, the data filtering procedure combined with the lack of Zn-dependent transcriptional regulation of *ZIP6* resulted in the identification of *ZIP6* as the only *ZIP* family candidate gene for a role in Zn hyperaccumulation [48].

4.2 CDF family

In plants, transporters of the CDF family appear to mediate the cytoplasmic efflux of transition metal cations such as Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+} or Mn^{2+} and have been named MTP for “metal tolerance protein”. Phylogenetically, the majority of known plant CDFs belong to two of four distinct groups, group I and III [36].

Compared to non-accumulator species three genes encoding CDFs are highly expressed in *A. halleri* and *T. caerulescens*: *MTP1* (group III), *MTP8* (group I) and *MTP11* (group I) [44-46]. Group III CDF proteins are most likely Zn transporters [21]. *MTP1* has been identified in transcriptomic approaches as a candidate gene in shoots and roots of *A. halleri* and in roots of *T. caerulescens*. *Ah*MTP1 was also isolated in a screen for proteins from *A. halleri* that are able to complement a Zn-sensitive yeast mutant [54]. The expression of *AhMTP1* is constitutively high over a range of external Zn supplies, especially in leaves [54]. In *A. thaliana*, the much lower expressed *AtMTP1* contributes to basal Zn tolerance and basal Zn accumulation in leaves [19]. The genome of *A. halleri* contains at least three loci harbouring *MTP1* gene copies. Two of these loci account for the high *AhMTP1*transcript levels and have been shown to co-segregate with Zn tolerance in a segregating backcross 1 population of an inter-specific cross between *A. halleri* and *A. lyrata* [54]. *Thlaspi caerulescens MTP1* has been cloned previously from a cDNA library and was named *ZTP1* [53]. Compared to *ZTP1* from *T. arvense*, *ZTP1/TcMTP1* was found to be more highly expressed in leaves and roots of three different accessions of *T. caerulescens*, with the expression in leaves being slightly higher than in roots.

MTP11 and especially MTP8 are close homologues of *Sh*MTP8 (formerly *Sh*MTP1), which conferred Mn tolerance when expressed in yeast and when ectopically overexpressed in *A. thaliana* [39]. Little is known about the proteins encoded by *MTP8* and *MTP11* of *A. halleri* or *T. caerulescens*. *MTP8* does not appear to be Zn-responsive in either species, but is upregulated in response to Cd and Cu in roots and to Cu in shoots of *A. halleri* [44,46]. It is possible that MTP8 and MTP11 contribute to the homeostasis of other metals than Zn. The high transcript levels of *MTP8, MTP11* and other candidate genes with putative functions in Fe, Cu and Mn homeostasis suggest the need to adjust the homeostasis of these metals in Zn hyperaccumulators. In this context, it is interesting to note that in an experiment employing a range of different metal-supplemented nutrient solutions, *T. caerulescens* had the ability to not only accumulate Zn, Cd and Ni, but also other metal ions, for example Mn and Co in shoots, and Cu, Fe and Pb in roots [55].

4.3 P_{1B}-type ATPases transporting transition metals

The heavy-metal-transporting P_{1B}-ATPases (HMAs) translocate cations out of the cytoplasm across biological membranes using energy from the hydrolysis of ATP. Genes encoding two HMAs of the group of Zn/Cd/Pb/Co divalent cation transporting P_{1B}-ATPases, *HMA3* and *HMA4*, display high expression levels in both hyperaccumulators *A. halleri* and *T. caerulescens* [44-46,49]. *Ah*HMA3 and *Ah*HMA4 are both able to confer partial Zn tolerance to a Zn-sensitive yeast strain. *Ah*HMA4 can also confer Cd tolerance when expressed in yeast [44,45]. In *A. halleri*, HMA4 proteins are encoded by more than one gene copy [44]. *Tc*HMA4 was previously identified through screening of cDNA expression libraries for clones conferring Cd tolerance to yeast [56,57] and was shown to mediate the efflux of Cd from yeast cells [56]. In both hyperaccumulator species, *HMA4* expression is approximately 2 to 3-fold higher in roots than in shoots. In *A. halleri*, expression levels appear to be relatively constant over different external Zn concentrations. In *T. caerulescens* roots, *HMA4* transcript levels are increased in response to high Zn and high Cd, and also under Zn deficiency [44,56,57].

In *A. thaliana*, *At*HMA2 and *At*HMA4 have been implicated in the root-to-shoot translocation of Zn, possibly by mediating xylem loading [4,58,59]. Both transporters have been proposed to localize to the plasma membrane [4,58]. It is possible that the *A. halleri* and *T. caerulescens* homologues of *At*HMA4 perform an analogous function. Thus, the high expression of *AhHMA4* and *TcHMA4* in roots could account, at least in part, for the high root-to-shoot Zn flux in these accumulator species.

HMA4 may also contribute to Cd hyperaccumulation in some accessions of *T. caerulescens* and *A. halleri*. Moreover, AhHMA4 and TcHMA4 may contribute to Zn and Cd tolerance by mediating cytoplasmic metal efflux in root and leaf cells.

4.4 NRAMP transporters

Characterized plant members of the NRAMP family transport divalent metal cations into the cytoplasm. In *A. halleri* and *T. caerulescens*, *NRAMP3* is highly expressed in roots. *AhNRAMP3* is also highly expressed in shoots, whereas *NRAMP1* and *NRAMP5* are highly expressed in shoots of *T. caerulescens* [44,46-49]. The primary biological function of *A. thaliana* NRAMP transporters appears to be in Fe homeostasis. When expressed in yeast, AtNRAMP1, AtNRAMP3 and AtNRAMP4 can mediate the uptake of Fe, Mn and Cd [60]. *In planta*, AtNRAMP3 and AtNRAMP4 mediate the remobilization of Fe from vacuolar stores, and their expression is upregulated under Fe starvation [5,42]. To date, *A. thaliana* NRAMP proteins have not been directly implicated in Zn transport. However, the *A. thaliana nramp3-1* mutant accumulates Mn and Zn in roots when grown at low Fe [42]. It remains to be investigated whether the NRAMP transporters highly expressed in metal hyperaccumulators have a direct role in Zn or Cd hyperaccumulation or ensure the maintenance of Fe or Mn homeostasis.

4.5 The transport of chelators and metal chelates

A gene encoding another transport protein primarily involved in Fe homeostasis of *A. thaliana*, FRD3, is highly expressed in roots of *A. halleri* and *T. caerulescens* at the transcript level. *FRD3* is also highly expressed in leaves of *A. halleri* when compared to *A. thaliana* [44,46]. *A. thaliana frd3* mutants display constitutive Fe-deficiency responses despite enhanced root Fe uptake, leading to the accumulation of metals (see also review on Iron Transport in this issue)[11]. *At*FRD3 is expressed in the root pericycle and operates as an effluxer of citrate, a low molecular weight chelator molecule [61]. Thus, *At*FRD3 is needed to maintain root-to-shoot Fe mobility [12,61]. It is possible that in *A. halleri* and *T. caerulescens*, FRD3 contributes to Fe homeostasis and, in particular, to Fe mobility in the presence of xylem Zn concentrations that are likely to be substantially higher than in non-accumulator species like *A. thaliana* [62]. The need for a higher metal chelation capacity in the xylem could also explain the high transcript levels of *FRD3* in the

hyperaccumulators. Thus, it cannot be ruled out that FRD3 contributes to the homeostasis of other metals, including Zn.

The severe phenotypes of the *A. thaliana frd3* and *zif1* mutants, which are defective in a ligand and a putative ligand or Zn-chelate transporter, respectively, illustrate the functional importance of low molecular weight chelator molecules in the metal homeostasis network [13,61]. A chelator that appears to have an important role in metal hyperaccumulation is nicotianamine (NA), which is synthesized from three molecules of S-adenosyl-L-methionine by nicotianamine synthase (NAS) and can form complexes with several divalent metal cations [63]. Physiologically, NA has been linked with Fe and Cu, but also Ni and Zn homeostasis, and is involved in the mobility of metal ions in vascular tissues and between cells [2,16,63].

Both *A. halleri* and *T. caerulescens* highly express *NAS2*, encoding one isoform of NAS, in their root tissues [46,47]. High expression of *NAS* genes was also found in the shoots, of *NAS3* in *A. halleri* and of *NAS1* and *NAS4* in *T. caerulescens* [45,49]. *AhNAS2* is only expressed under Zn deficiency in shoots whereas in roots, *AhNAS2* transcript levels are constitutively high over a broad range of external Zn concentrations [44]. This suggests a divergence of *AhNAS2* regulation between shoots and roots, similar to that found for *AhZIP9*. *AhNAS2* and *AhNAS3* can complement Zn-sensitive phenotypes when expressed in yeast [45,47]. Roots of *A. halleri* were found to contain around 3-fold higher levels of NA than roots of *A. thaliana* [47]. A similar result has been obtained for *T. caerulescens* in comparison to *T. arvense*, albeit after exposure to non-toxic concentrations of Ni [64]. Together, the available data indicate that NA plays an important role in the metal ion homeostasis of hyperaccumulators. Indeed, enhanced Ni tolerance and higher accumulation of Ni were observed in leaves of *A. thaliana* ectopically overexpressing *TcNAS1* compared to wild-type plants [65].

Transporters of the YSL transporter family, which mediate the cellular uptake of metal-NA complexes, have been characterized in *A. thaliana* [14-16](see review Iron Transport in this issue). In transcriptomic studies, *YSL6* was identified as a candidate gene in *A. halleri* shoots, and *YSL7* in *T. caerulescens* roots [44,46]. In a recent study on YSL transporters from *T. caerulescens*, *TcYSL3*, *TcYSL5* and *TcYSL7* were found to be highly expressed in both roots and shoots, with *TcYSL3* and *TcYSL7* expression being localized around the vascular tissue in roots [66]. So far, YSL transporters have been mostly implicated in Fe homeostasis [14,15], and it is unclear whether they contribute to the homeostasis and accumulation of Zn or Cd or to the physiological balance of other divalent metal cations in *T. caerulescens* and *A. halleri*.

The transcriptomic and single gene studies reviewed here have significantly advanced our understanding of the molecular mechanisms underlying hyperaccumulation, mainly of Zn, in *A. halleri* and *T. caerulescens*. Although metal hyperaccumulation is likely to have evolved independently in the two hyperaccumulator species, both of them share a convergent core set of highly expressed homologous candidate genes for metal hyperaccumulation and associated hypertolerance. This suggests that redundancy in the plant metal homeostasis network is low and interdependence is high so that the network has to be modified in a defined direction at specific nodes to result in a specific phenotype.

5. Evolutionary trends in plant metal transporter complements

The recent completion of several algal and higher plant genome sequences allowed the identification of complete complements of transition metal transport protein families of the metal homeostasis network in these organisms. Their comparison reveals that unicellular algae possess the basic tools to maintain cellular metal homeostasis. As such, they represent powerful models to study the functions and the interactions of different metal transport and intracellular trafficking systems [21]. However, higher plants evolved adaptations of their metal homeostasis network reflecting the need for long-distance transport and for the proper distribution of metals between different organs and cell types. During evolution, the adaptation to different environmental factors has influenced the composition of the transition metal transporter complements of photosynthetic organisms. What are the specific components that distinguish the metal homeostasis networks of higher plants from those of unicellular algae?

5.1 Long- distance transport

Several protein families contribute to long-distance transport and distribution of metals in plants. In *A. thaliana*, the Zn(II)/Cd(II) or divalent cation transporting P_{IB}-type ATPAses *At*HMA2 and *At*HMA4 play a central role in root-to-shoot zinc translocation, possibly by xylem loading or by unloading metal ions from xylem parenchyma cells [4,58,59]. Both genes are mainly expressed in the root and shoot vascular tissues, and the corresponding proteins localize to the plasma membrane

[4,58]. An *hma4* single mutant accumulates less zinc in the shoot than wild-type plants, and an *hma2hma4* double mutant displays a strong zinc deficiency phenotype in the shoot [4,58].

Copper(I) or monovalent cation-transporting P_{IB}-type ATPAses are ubiquitous, whereas the occurrence of divalent cation-transporting HMAs is apparently limited to prokaryotes and higher plants. A discontiguous MegaBLAST search did not retrieve sequences homologous to the divalent cation-transporting P_{1B}-type ATPases in the moss *Physcomitrella patens* in the NCBI trace archives (January 2007). Similarly, unicellular algae only possess the Cu(I)-transporting P_{IB}-type ATPAses that are involved in copper delivery to the chloroplast or the ER, but lack the divalent cation-transporting HMAs [17,21].

The proteins of the IREG family are also potentially involved in the long-distance transport of transition metals in plants. Three IREG proteins (*At*IREG1 to 3) are encoded in the *A. thaliana* genome. These are homologous to the mammalian IREG1 protein (or Ferroportin1), which mediates the transport of iron from the basolateral surface of the enterocytes to the blood stream [67]. Whether the *A. thaliana IREG* genes encode iron transporters involved in loading vessels with iron remains to be elucidated. Recently, *At*IREG2 was shown to have a role in nickel transport into the vacuole under iron-deficiency [68]. An *IREG1* homolog is also present in the genome of the unicellular red alga *Cyanidioschyzon merolae*, but is lacking in *Chlamydomonas reinhardtii* and *S. cerevisiae* [21]. Further data is needed to verify whether an IREG1-like protein was present in the common ancestor of photosynthetic and non-photosynthetic eukaryotes, but was maintained primarily in multicellular organisms. It is hypothesized that an IREG1-like protein has been maintained in *C. merolae* as an adaptation to its environment, where an iron efflux function might be important to cope with high iron availability of the acidic sulphur-rich hot springs [21]. Similar to the situation for IREG proteins, a homologue of the *S. cerevisiae* CCC1 protein, which transports iron into the vacuole, is encoded in the genomes of *A. thaliana* (*AtVIT1*) [41], rice and *Cyanidioschyzon merolae*, but not in the *C. reinhardtii* genome.

Other key transport proteins in the plant metal homeostasis network are YSL transporters, which transport complexes of metals with NA and related low-molecular-weight organic ligands. The functional characterization of *At*YSL1 to *At*YSL3 in *A. thaliana* and OsYSL2 in rice implicated these proteins in the distribution of transition metals in the plants: the genes are expressed in vascular tissues and are regulated depending on the iron status, and the corresponding proteins are essential for proper metal accumulation in the seeds [14,15,31,69,70]. Algae do not synthesize NA or NA-derived phytosiderophores, and are lacking NAS- and YSL-encoding genes [21]. Recently,

NA synthesized by a functional nicotianamine synthase was detected in the filamentous fungus *Neurospora crassa*, in which it was suggested to be involved in the cell-to-cell distribution of Zn and other micronutrients *via* the incomplete septas of the hyphae [71]. A BLAST search identifies several YSL-related genes in the *N. crassa* genome (Table 1). It can be hypothesized that at least some of them might be involved in the transport of NA or NA-derived-siderophore-bound metal.

5.2 Expansion of multi-gene families

In general, the sizes of the transition metal transporter families increase with the complexity of the photosynthetic organism. This is likely to contribute to cell-, tissue- and developmentally specific expression of transporter genes and allows the fine-tuning of the metal homeostasis network in a multicellular organism (Table 1). For example, *C. reinhardtii* and *C. merolae* possess a single putative Zn-transporting MTP (group III) [21], whereas four (*At*MTP1 to *At*MTP4) are present in the *A. thaliana* genome. Although the *At*MTP1 and *At*MTP3 proteins share the same subcellular localization (i.e. the vacuolar membrane) and function (i.e. the storage of zinc in the vacuole), their genes display distinct tissue expression patterns in leaves and especially in roots, and MTP1 and MTP3 have differing physiological functions in Zn partitioning in the plant [35,38].

A similar trend is also observed in fungi. The unicellular yeast *S. cerevisae* possesses a smaller transition metal transporter complement than the filamentous *N. crassa* [72] (Table 1). However, contradicting this general trend, some large metal transporter families are present in unicellular organisms. A striking example is the presence of 14 putative ZIP transporters encoded in the genome of *C. reinhardtii* [21]. A similar number of ZIP proteins are encoded in the human (14) and *A. thaliana* (17) genomes, whereas other unicellular organisms possess only a limited number of *ZIP* genes (e.g. four in the red alga *Cyanidioschyzon merolae* or five in yeast). The abundance of *ZIP* genes in *C. reinhardtii*, a flagellate living in water and soils, might reflect a need to adapt quickly to a fluctuating environment, changing metal transport requirements during the complex life cycle, transporter substrate diversification (e.g. Mn), or a reduction from a multicellular life form during the evolutionary history of this organism.

5.3 Iron uptake strategies

Plants and algae use divergent iron uptake strategies that might reflect past or present differences in metal ion availability in their environment. In algae, such as *C. reinhardtii* or *Dunaliella salina*, Fe(III) is first reduced by ferric chelate reductases, then high affinity iron uptake is mediated by a multi-copper ferroxidase that reoxidizes Fe(II) to Fe(III) and an iron permease that transports Fe(III) into the cell, as it occurs in yeast and mammals [73,74]. In these species, iron uptake is dependent on copper availability, and the re-oxidation step confers a high metal specificity to this high-affinity iron uptake system.

Higher plants have abandoned this seemingly ancestral system and have developed two alternative copper-independent iron acquisition strategies, which allow different plants to cope with the range of iron availabilities and oxidation states characteristic of their habitats (see review Iron Transport in this issue). It is noteworthy that also algae, such as *C. reinhardtii*, possess copper-independent iron uptake systems [73]. In analogy with the role of the ZIP protein *At*IRT1 as the primary Fe(II) uptake system of *A. thaliana*, some of the ZIP proteins encoded in the *C. reinhardtii* genome possibly participate in these alternative iron uptake systems [21]. In summary, copper-independent iron uptake systems are widespread in photosynthetic organisms, and come at the cost of a reduced metal specificity [30,34].

5.4 Putative Regulatory domains in transition metal transporters

To maintain and supply physiological concentrations of transition metals within cells and organs, it is essential that plants tightly regulate their metal homeostasis network both at the transcriptional and posttranslational levels. These regulatory mechanisms remain largely unknown.

Compared to their prokaryotic homologues, many plant metal transporters have acquired additional cytoplasmic domains. These extensions might be involved in metal sensing, interactions with metallochaperones or the regulation of the protein activity and/or subcellular localization. For example, in comparison to its prokaryotic homologues, *At*MTP1 possesses an extended histidine-rich cytosolic loop between transmembrane helices IV and V, which has been proposed to participate in zinc-binding and exhibits characteristic amino acid sequence differences between hyperaccumulator and non-accumulator MTP1 proteins [19,54,75]. A recent *in vitro* thermodynamic analysis of metal binding to a similar, shorter histidine-rich sequence motif present

in a cytoplasmic loop of IRT1 demonstrated a low affinity for most divalent transition metal cations and a high affinity for Fe^{3+} [76].

The three *A. thaliana* divalent cation-transporting HMAs (*At*HMA2, -3 and -4) possess large C-terminal cytosolic domains that are missing in their prokaryotic homologues. These contain several cysteine dipeptides, and the C-termini of *At*HMA2 and *At*HMA4 additionally contain long stretches of histidine residues. These domains constitute putative metal-binding sites and might serve as heavy metal sensors or domains for interactions with regulatory proteins [17,77]. Interestingly, although the core domains of the HMA4 proteins are highly similar, the HMA4 cytosolic C-terminal domains of both hyperaccumulators *A. halleri* and *T. caerulescens* exhibit strong sequence differences compared to their *A. thaliana* homolog [44,56,57].

A truncated version of *At*HMA4 lacking the C-terminus was shown to be more active in yeast than the wild-type protein [59], suggesting that this domain might have an autoinhibitory activity. This is reminiscent of the P_{3A}-type H^+ ATPase *At*AHA2, in which the autoinhibitory activity of the C-terminus is relieved by phosphorylation that allows a subsequent interaction with a 14-3-3 protein [78]. A putative 14-3-3 binding motif can be identified in the C-terminal cytosolic domain of *At*HMA3 *in silico* (T. Hoffmann and U. Krämer, unpublished data).

6. Conclusions and outlook

In the future it will be of central importance to identify the proteins or low-molecular-weight chelators involved metal trafficking and to characterize their molecular interactions with transition metal transporters. The identification of *in vivo* substrates and the detailed characterization of biochemical properties of transition metal transporters is another important challenge. A better understanding of the determinants and mechanisms of metal specificity is required both at the transporter protein level and at the whole-plant level. For example, what is the extent to which the gene products governing naturally selected hyperaccumulation and hypertolerance of chemically similar transition metals, such as Zn, Cd and Ni, differ or overlap?

After the identification of a first core set of candidate genes for naturally selected metal hypertolerance and hyperaccumulation, the functions of the encoded proteins and their homologues in closely related non-accumulator plants have to be analyzed at the biochemical level and within

the plant metal homeostasis network. Dissecting the molecular alterations underlying the high expression levels of candidate genes in metal hyperaccumulators will be an eminent future research direction.

Finally, the availability of additional full genome sequences of alga and lower and higher plant species will reveal differences between their metal homeostasis networks and provide an increasingly differentiated basis for the generation of hypotheses concerning the evolution of metal homeostasis networks.

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Figure legends

Figure 1

The common core set of transition metal transporter genes highly expressed in both hyperaccumulator species *A. halleri* and *T. caerulescens*. In cross-species microarray-based transcript profiling studies, these genes displayed significantly higher transcript levels in both metal hyperaccumulator species than in a related non-accumulator species. Putative physiological functions for the encoded transporter proteins are indicated, based on published data from homologous membrane transporters regarding transport direction (cytoplasmic influx or efflux), transported metal and tissue-specific and subcellular localisation. The minimal pathway of a metal

in its oxidized form into leaf cells involves the processes highlighted in bold. Note that *ZIP9* was only found to be highly expressed in *A. halleri* roots, and *NRAMP1/NRAMP5* only in shoots of *T. caerulescens*. However, *ZIP9* constitutes a main candidate gene for constitutively enhanced Zn uptake in *A. halleri* roots [44,47], and another member of the *NRAMP* family, *NRAMP3*, is highly expressed in *A. halleri* shoots. One other metal homeostasis gene, *NAS2* (At5g56080) encoding a nicotianamine synthase, was found to be highly expressed in roots of both hyperaccumulator species (see section 4.5). In the figure, the common names of the following *A. thaliana* genes are given: *ZIP4*, At1g10970; *ZIP6*, At2g30080; *ZIP9*, At4g33020; *ZIP10*, At1g31260; *IRT3*, At1g60960; *MTP1*, At2g46800; *MTP8*, At3g58060; *MTP11*, At2g39450; *HMA3*, At4g30120; *HMA4*, At2g19110; *NRAMP3*, At2g23150; *NRAMP1*, At1g80830; *NRAMP5*, At4g18790; *FRD3*, At3g08040. Blue colour denotes Zn-responsive genes with increased transcript levels upon Zn deficiency; orange colour denotes Zn homeostasis genes with mainly constitutive expression; green colour denotes genes that are more likely to be involved in the homeostasis of metal ions other than Zn. Superscripts denote the following microarray-based studies: 1, *A. halleri* shoots [45]; 2, *A. halleri* roots [47]; 3, *A. halleri* shoots and roots [48]; 4, *A. halleri* shoots and roots [44]; 5, *T. caerulescens* shoots [49]; 6, *T. caerulescens* roots [46]; 7, whole seedlings of *A. halleri* [50].

Tables

Table 1. Metal transporter family sizes in unicellular and multicellular eukaryotes.

	Protein families								
Organisms	CDF/MTP	ZIP	COPT/CTR	P_{IB}-type ATPases	YSL	FTR	NRAMP	IREG1	CCC1
S. cerevisae	5	5	2	2	-	1	3	-	1
N. crassa	9	7	2	3	+ ?	1	2	-	1
H. sapiens	9	14	2	2	-	-	2	1	-
C. merolae	3	4	1	2	-	4	3	1	1
C. reinhardtii	5	14	1	4	-	1	3	-	-
A. thaliana	12	17	5	8	8	-	7	2-3	1
O. sativa	10	12	4	9	19	-	14	1-3	1

The data used to assemble this table were collected from various sources (January 2007). *S. cerevisiae* : PlantsT database (http://plantst.genomics.purdue.edu/), *N. crassa* : BLAST search on the release 3 of the genome (http://www.broad.mit.edu/annotation/genome/neurospora/Home.html) and in [72], *H. sapiens* : Human transporter database (http://lab.digibench.net/transporter/Family.html), *C. merolae* and *C. reinhardtii* in [21], *A. thaliana* : TAIR6 release (http://www.arabidopsis.org) and PlantsT database, *O. sativa*: BLAST search on TIGR release 5 (http://www.tigr.org/tdb/e2k1/osa1/index.shtml) and in [70,77]. + : identified by BLAST search, - : not found. For ABC transporters and CAX antiporters refer to [21].

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