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Review

# Metal tolerance and hyperaccumulation: Costs and trade-offs between traits and environment

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ABSTRACT

Metal hyperaccumulation is a trait present in over 450 higher plant species. Hyperaccumulators are also tolerant to metals, but hyperaccumulation and tolerance are genetically independent traits. The ecological and biological significance of hyperaccumulation is not clear yet. To provide new insights, this review examines recent literature, in particular focusing on the Cd and Zn hyperaccumulator species *Arabidopsis halleri* (L.) O'Kane and Al Shehbaz and *Thlaspi caerulescens* J. et C. Presl. in comparison with the model plant species *Arabidopsis thaliana* (L.) Heynh. The main aspects considered in the discussion on hyperaccumulation and tolerance involve: (i) uptake of metals, (ii) vacuolar sequestration, (iii) xylem loading, and (iv) chelation with ligands. The review discusses the advancement of knowledge obtained through genetic analysis and molecular biology, together with the use of transgenic approaches and transcriptomics. The most important genes which have been correlated to hyperaccumulation and tolerance in plant species are described and discussed. From the in depth analysis of published results, the main topics for future research are highlighted. Ecological relevance of the hyperaccumulation and tolerance traits in the environment is discussed, with the advantages they can confer to individuals, the possible disadvantages, and the trade-offs between these genetic traits and the environmental conditions.



**Contents**



## 1. Introduction

Plants use inorganic substances in the soil mostly for nutrition. Several metals and metalloids, which are essential for biochemical and cellular processes, such as Zn, Cu, Fe, Mg and Mn, are taken up to different extents. They may be essential for plant growth, but

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**Table 1**
Occurrence of metals relevant as environmental contaminants in uncontaminated soils and plant tissues, compared with the values chosen as threshold for definition of hyperaccumulator plant species.

| Element | Average range in soils[a] (mg/kg dry weight) | Average range in plant tissues[b] (mg/kg dry weight) | Threshold for hyperaccumulators[c] (mg/kg dry weight) |
|---|---|---|---|
| Mercury | <0.1[d] | 0.005–0.2 | 1000 = 0.1% |
| Selenium | 1–2 | 0.01–0.2 | 1000 = 0.1% |
| Cadmium | 1–2 | 0.03–0.5 mg/kg | 100 = 0.01% |
| Copper | 2–60 | 2–20 | 1000 = 0.1% |
| Nickel | 2–200 | 0.4–4 | 1000 = 0.1% |
| Chromium | 5–1000[d] | 0.2–1 | 1000 = 0.1% |
| Lead | 10–150 | 0.1–5 | 1000 = 0.1% |
| Zinc | 25–200 | 15–150 | 10000 = 1% |
| Manganese | 100–4000[d] | 1–700 | 10000 = 1% |

[a] Lepp (1981).
[b] Markert (1996).
[c] McIntyre (2003).
[d] Adriano (2001).

excessive doses can become toxic (Marschner, 1995; Marmiroli and Maestri, 2008). Other trace elements with no known biological role can enter into plant tissues and damage normal processes, either passively or due to their similarity with essential ions (Garland and Wilkins, 1981; Antosiewicz, 2005; Krämer et al., 2007). There is a broad range of metals concentrations in soils, depending on their origin and usage (Table 1); their chemical forms, mobility and availability to the plant altogether condition the ecology of the plant–soil interactions, and element concentrations in the plants are also highly variable (Table 1). Some plant species at times show extreme tolerance to one or more trace elements; for example, on calamine and serpentine soils, some species thrive in the presence of exceedingly high concentrations that are lethal to most species. This tolerance derives either from mechanisms leading to exclusion of excess metal (avoidance), or is linked with the plant structures which can detoxify and/or sequester the excess of toxic ions inside the cells (true tolerance, *sensu* Baker, 1981).

Many plant species have now been found that are capable of accumulating metals in above-ground tissues at concentrations which are significantly higher than those present in the soil. They have been termed hyperaccumulators when the metal concentrations are 50–100 times higher, depending upon the metal, than in non-accumulating plants (Table 1) (Baker and Brooks, 1989; McGrath and Zhao, 2003). The standard definition of hyperaccumulation implies uptake of metals from the soil at high rates, translocation and accumulation of the same in shoot organs, stem and leaves. This definition differentiates hyperaccumulators from other plants which accumulate much of the metal in their roots, thus excluding or limiting translocation to shoots. Examples of the latter include ecotypes of *Silene maritime* With. (Baker, 1978), *Agrostis stolonifera* L. (Wu and Antonovics, 1975), and woody plants such as *Pinus radiata* D. Don. (West, 1979), *Salix* and *Populus* species (Dickinson and Pulford, 2005; Giachetti and Sebastiani, 2006). Hyperaccumulator species are thus quite distinct, although many phenotypes of the ultramafic flora have not been taxonomically identified at species level (Reeves et al., 2007). Hyperaccumulation is widespread in over 450 species across families, orders and genera of vascular plants, from ferns to trees. Furthermore, a single specific element or multiple elements may be hyperaccumulated, adding an extra fund of complexity to the topic. For the last 30 years, the potential to exploit hyperaccumulation traits for purposes of phytoremediation has spurred intensive research into its biochemical, physiological and genetic basis. However, considering the wide distribution of hyperaccumulation throughout the plant kingdom, it is unlikely that the same mechanism underlies the phenomenon in all species.

Some plants widely distributed in Europe have long been recognised as bearing typical features of metal hyperaccumulators, notably *Thlaspi caerulescens* J. et C. Presl. (Pb, Zn, Cd, Ni), *Alyssum bertolonii* Desv. (Ni, Co) and *Arabidopsis halleri* (L.) O'Kane and Al Shehbaz. *A. halleri* is found on soils with high levels of Zn, Cd and Pb; the plants constitutively accumulate Zn and Cd, but do not accumulate Pb (Bert et al., 2002). These species all belong to the Brassicaceae, which provides good ground for direct comparisons with *Arabidopsis thaliana* (L.) Heynh, taking advantage of its described genomic resources (www.arabidopsis.org). Already this is a widespread practice for delving into the *T. caerulescens* genome, although more limited in *A. bertolonii*. In the way that *A. thaliana* is the favoured model in plant biology, genetics and physiology, *T. careulescens* and *A. halleri* have become model plants for the issues related to hyperaccumulation. Literature searches reveal hundreds of papers analysing hyperaccumulation in these or sister species. Recent reviews and research papers are devoted to the description of hyperaccumulation genetic and physiological mechanisms in *T. careulescens* and in *A. halleri* (Macnair, 2002; Ramos-Onsins et al., 2004; Yang et al., 2005; Basic et al., 2006; Clemens, 2006; Krämer et al., 2007; Willems et al., 2007; Milner and Kochian, 2008; Memon and Schroeder, 2009; Verbruggen et al., 2009), but none have focused on the ecological and environmental relevance of these mechanisms for a broader range of hyperaccumulators.

The present review focuses on Cd and Zn hyperaccumulation in *A. halleri* and *T. caerulescens* in comparison with the model plant species *A. thaliana* in the context of advancement of knowledge through genetic analysis and molecular biology, together with the use of transgenic approaches and transcriptomics. Most of the works cited in this review have been produced by groups working in Europe, and by scientists who have been cooperating in the framework of the activities financed by the European Commission: Framework research programmes, and COST Actions 837 and 859. They serve as a representation of the results which may be achieved when interactions and exchanges among research groups are fostered and encouraged.

## 2. Basic mechanisms in hyperaccumulation

Transport of inorganic ions occurs in plants through both apoplastic and symplastic pathways, although the relative importance of each is not yet fully elucidated (White et al., 2002). Even though the apoplastic route is of relevance, physiological studies recognise three main steps in hyperaccumulation involving symplastic transport: (i) metal active transport across plasma membranes in roots, (ii) metal entry in the symplast during translocation from root to shoot, and (iii) metal chelation and sequestration in specific cell compartments within leaves.

The elucidation of the genetic and molecular aspects building up these processes constitutes a considerable task. Different

approaches have been used to identify genes and functions involved in the shaping of hyperaccumulation phenotype: (i) analysis of natural genetic variation; (ii) analysis of genetic segregation in crosses among plants with contrasting phenotypes; (iii) mapping of major genes or quantitative trait loci (QTLs); (iv) isolation of mutants; (v) isolation and characterization of proteins and compounds correlated with the hyperaccumulating phenotype; (vi) comparative analysis of regulation for specific genes in *A. halleri*/*T. caerulescens* and *A. thaliana*; (vii) functional analysis by complementation assays of yeast mutants sensitive to metals; (viii) construction of transgenic plants; (ix) global analysis of the *T. caerulescens* and *A. halleri* transcriptome using microarray technologies.

### 2.1. Analysis of natural genetic variation and segregation

The ecotypes of *A. halleri* are not strikingly different in hyperaccumulation and tolerance, but rather there is a continuous variation in natural populations (Pauwels et al., 2007). Therefore, segregation is better observed in interspecific crosses, usually performed with *Arabidopsis lyrata* ssp. *petraea* (L.) O'Kane and Al Shebhaz [also called in some papers *Arabidopsis petraea* (L.) V.I.Dorof.]. On the contrary, in *T. caerulescens* genetic variation for hyperaccumulation has been documented within the species. Literature reports intraspecific crosses between *T. caerulescens* ecotypes, or interspecific crosses of *T. caerulescens* with *T. arvense* L.

The conclusions of these studies were that, in *A. halleri*, tolerance to Cd and accumulation of Cd segregate as independent traits (Bert et al., 2002, 2003), whereas tolerance to Cd and Zn, and accumulation of Cd and Zn, cosegregate. Therefore, in this species, there are two or more genes for Cd and Zn accumulation but only one for Cd and Zn tolerance. A lack of genetic correlation between accumulation and tolerance has been found in *T. caerulescens* towards Zn, Cd and Ni (Assunção et al., 2003; Zha et al., 2004; Yang et al., 2005; Richau and Schat, 2009). Therefore, in this species there is a good chance for the genetic and physiological mechanism involved in hyperaccumulation to be distinct from tolerance; though, a correlation between these two aspects does apply to other species (Yang et al., 2005). Genetics has been applied to the analysis of tolerance, more often than that of hyperaccumulation. After studying natural *T. caerulescens* genotypes, it has been proposed that a single gene exerts a major control on Zn accumulation, with a dominant allele which rules hyperaccumulation (Frérot et al., 2003). On the other hand, other authors asserted that a continuous distribution of this hyperaccumulation phenotype might be indicative of the segregation of a quantitative trait (Assunção et al., 2003).

### 2.2. QTL mapping

Recently studies have been published on QTL analysis of metal accumulation and hyperaccumulation in *T. caerulescens* and in *A. halleri*. A first QTL analysis involved the phenotyping of an F2 population generated from a cross between a high Zn-accumulating *T. caerulescens* ecotype, isolated in a non-metalliferous soil at Lellingen, and a relatively lower Zn-accumulating ecotype, from a calamine soil in La Calamine (Belgium). Two major QTLs were identified for increased root Zn accumulation (Assunção et al., 2006). A second publication reported QTLs for both root and shoot Cd and Zn accumulation by analysing the segregation of a mapping population derived from a parent from a Pb/Cd/Zn-contaminated site near La Calamine, and a second parent selected from a site with similar soil characteristics near Ganges (France) (Deniau et al., 2006). Two QTLs for root Cd accumulation and two QTLs for root Zn accumulation were identified, as well as three shoot Zn accumulation QTLs and one QTL for shoot Cd accumulation.

A QTL mapping performed in *A. halleri* led to the identification of genes involved in Zn or Cd tolerance. By segregation analysis of one F2 progeny, derived from interspecific crosses between *A. halleri* and *A. lyrata*, it was evidenced that a single major gene determined Zn tolerance (Macnair et al., 1999). Three major Zn tolerance QTLs were found in the backcross progeny of the interspecific cross *A. halleri* × *A. lyrata* (Willems et al., 2007). In each one of these QTLs the allele from *A. halleri* increased the level of tolerance. As a whole these QTLs explain 42% of the total phenotypic variance for this trait. The QTLs have been located on chromosomes 3, 4 and 6. In the F2 of a similar interspecific cross, three QTLs for Zn accumulation were mapped on chromosomes 4, 6 and 7 instead (Filatov et al., 2007). A partial overlapping for some of these QTLs mapping could be hypothesized (Roosens et al., 2008b). As described in more detail below, syntenic relationships among *A. halleri* and *A. thaliana* allowed the mapping of specific genes associated with the QTL or within the QTL regions, and made possible to correlate candidate genes for metal transport to the quantitative loci.

The identification of QTL regions conferring metal hyperaccumulation or tolerance holds great promise for the identification of the genes and gene networks mainly responsible for the phenomenon. The dissection of a QTL into discrete Mendelian loci is difficult in species for which a dense physical map or complete sequencing are not available yet. The closer relationship of *A. halleri* with *A. thaliana* and the high synteny among the genetic maps of the two species allowed the colocalization of some candidate genes within QTL regions. An example is the genetic mapping of AhHMA4 (see below), a member of P-type ATPase family involved in the transport of transition metals, which is colocalized with the peak of the QTL involved in both Zn and Cd tolerance (Courbot et al., 2007; Willems et al., 2007). Data derived from transcriptomics and functional genetics analyses are helping to find more candidate genes.

## 3. Molecular biology of metal hyperaccumulation

Molecular studies to dissect the different genetic components of the metal hyperaccumulation trait have benefited from the comparison between either of the two hyperaccumulators *A. halleri* and *T. caerulescens* with the model plant *A. thaliana*. The comparison was possible because the genomes of the first two species have on average a 94% and 88.5% DNA identity in coding regions, respectively, with the genome of *A. thaliana* (Becher et al., 2004; Rigola et al., 2006). These studies have entailed the identification of a number of genes involved in the process of uptake, translocation and distribution of metals in the plants, and have helped to elucidate differences between hyperaccumulators and non-accumulators (Table 2). The following section discusses single genes implicated in specific processes. Nomenclature of genes in the different species is sometimes confusing, and Table 2 reports for each of them the gene name(s) and the unique identifier of the Arabidopsis Genome Initiative (AGI; www.arabidopsis.org) locus, corresponding to that gene in the genome of *A. thaliana*.

### 3.1. Active transport across membranes

Metal bioavailability and mobility in the rhizosphere is an important aspect of the uptake from the soil into the roots. The chemical environment in the rhizosphere affects bioavailability; for example, the chemical form of nitrogen in the soil affects plant uptake of Cd and Zn in *T. caerulescens* (Xie et al., 2009). In the rhizosphere of hyperaccumulators, metal availability is reported to be higher than in non-hyperaccumulating plants (Puschenreiter et al., 2005). Root exudates such as organic acids and phytosiderophores, together with acidification by protons, play a role in the metal mobilization from the soil to the roots (Marschner, 1995). In this context a specific role of rhizosphere microbe community has been suggested to influence both the metal uptake and the plant

**Table 2**
Nomenclature of the genes selected as good candidates in determining the phenotype of hyperaccumulators in *Arabidopsis thaliana*, *Arabidopsis halleri* and *Thlaspi caerulescens*.

| AGI locus gene | Description | *A. thaliana* | *A. halleri* | *T. caerulescens*[a] |
|---|---|---|---|---|
| At1g10970 | Member of Zrt- and Irt-related protein (ZIP) family | AtZIP4 | | ZNT1/TcZIP4 |
| At2g46800 | Member of the zinc transporter (ZAT) and cation diffusion facilitator (CDF) families | MTP1a/ZAT, MTP1b | AhCDF1-3/AhMTP1 | TcZTP1/ MTP1 |
| At4g30120 | Encodes a protein similar to Zn-ATPase, a P1B-type ATPases transport zinc | HMA3 | AhHMA3 | TcHMA3 |
| At2g19110 | Encodes a protein with similarity to Zn ATPase | HMA4 | AhHMA4 | TcHMA4 |
| At4g19690 | Fe(II) transport protein (IRT1) | AtIRT1 | AhIRT1 | TcIRT1, TcIRT1-1G, TcIRT1-2P |
| At1g60960 | Member of Fe(II) transporter isolog family | AtIRT3 | AhIRT3 | TcZNT2-LC TcZNT2-G |
| At1g07600 | Metallothionein, binds to and detoxifies excess copper and other metals, limiting oxidative damage | AtMT1a | | TcMT1 |
| At3g09390 | Metallothionein, binds to and detoxifies excess copper and other metals, limiting oxidative damage | AtMT2a | | |
| At5g02380 | Cysteine-rich protein with copper-binding activity | AtMT2b | | |
| At3g15353 | Metallothionein, binds to and detoxifies excess copper and other metals, limiting oxidative damage | AtMT3 | | |
| At5g04950 | Nicotianamine synthase 1 | NAS1 | | TcNAS1 |
| At5g56080 | Nicotianamine synthase 2 | NAS2 | | TcNAS2 |
| At1g09240 | Nicotianamine synthase 3 | NAS3 | AhNAS3 | TcNAS3 |
| At1g56430 | Nicotianamine synthase 4 | NAS4 | | TcNAS4 |
| At5g24380 | Closest Arabidopsis homolog of *Zea mays* metal-phytosiderophore/metal-nicotianamine transporter ZmYS1 | AtYSL2 | | |
| At5g53550 | Oligopeptide transporter | AtYSL3 | | TcYSL3 |
| At1g65730 | *Arabidopsis thaliana* metal-nicotianamine transporter | AtYSL7 | | TcYSL7 |
| At3g27020 | *Arabidopsis thaliana* metal-nicotianamine transporter YSL6 | AtYSL6 | AhYSL6 | |
| At1g47240 | Member of the NRAMP2 gene family of metal ion transporters | AtNRAMP2 | | |
| At2g23150 | Encodes a member of the Nramp metal transporter family | AtNRAMP3 | | TcNRAMP3 |
| At5g67330 | Encodes a member of the Nramp metal transporter family | AtNRAMP4 | AhNRAMP4 | TcNRAMP4 |
| At3g22460 | Cysteine synthase, putative/O-acetylserine (thiol)-lyase | OAS-TL | | |
| At5g10180 | Encodes a low-affinity sulfate transporter | SULTR2.1 | | |
| At4g14680 | ATP sulphurylase | APS3 | | |

[a] G: Ganges accession; P: Prayon accession; LC: La Calamine accession.

growth (Whiting et al., 2001; Wenzel et al., 2003). Soil bacteria can affect the metal mobility and availability in many ways, including lowering the pH, producing compounds such as antibiotics and antifungals, organic acids, hormones (e.g. indoleacetic acid) and metal-chelating agents, enhancing plant biomass increase at root level (Wenzel et al., 2003; Xiong et al., 2008). The rhizosphere of hyperaccumulators is rich in metal-resistant bacteria (Whiting et al., 2001; Abou-Shanab et al., 2003). Rhizosphere microorganisms significantly increase the uptake of Cd, Zn, and Pb in *Sedum alfredii* Hance, and enhance root elongation (Xiong et al., 2008). In *T. caerulescens*, root proliferation is enhanced in metal-enriched soil patches (Whiting et al., 2000), but not all populations behave in this way (Dechamps et al., 2008). Root exudates from *T. caerulescens* however do not increase the mobility of metals (Cd and Zn) in the rhizosphere in a significant way as compared to non-accumulating plants (Zhao et al., 2001). Root environment for hyperaccumulator and non-accumulator plant species seems to have different effects on metal bioavailability and root uptake, but more studies are needed to ascertain this.

Enhanced metal uptake from the roots and translocation to the shoots in hyperaccumulators seems to be generated by specific members of metal transporters. A number of different plant micronutrient/metal transporter families have been identified. These classes include: (i) plasma membrane transporters involved in uptake; (ii) tonoplast transporters for uptake; or for (iii) remobilization from the vacuole; (iv) transporters for xylem loading; and (v) endomembrane transporters. Some of these families, such as the ZIP (ZRT/IRT like protein), CDF (cation diffusion facilitator), Nramp (natural resistance and macrophage protein) and HMA (heavy metal ATPase) have been identified firstly in *A. thaliana*.

### 3.1.1. Transporters involved in uptake into cells

*TcZNT1* is a member of the ZIP family of transport proteins, identified in *T. caerulescens* (Pence et al., 2000) based on homology to the high-affinity Zn uptake transporter in yeast, ZRT1, and the *A. thaliana* Fe transporter, IRT1/ZIP4 (At1g10970). TcZNT1 is a putative plasma membrane localized transporter. Restoration of function in complementation assays using a yeast *zrt1 zrt2* mutant (zhy3), which was not able to grow on low Zn medium, indicates that TcZNT1 acts both as a high- and as a low-affinity Zn transporter (Pence et al., 2000). In *T. arvense* and *A. thaliana*, the gene is expressed at very low levels in roots and shoots grown on sufficient and high Zn; but in condition of Zn deficiency the expression increases, presumably to facilitate Zn uptake through the synthesis of more transporters (Grotz et al., 1998). In *T. caerulescens*, expression of TcZNT1 is very high in both Zn-deficient and Zn-sufficient plants, while at very high Zn levels there is a downregulation of its expression. However, even at very high Zn levels, TcZNT1 expression is higher than that of its homologues in non-accumulator plants (Pence et al., 2000; Assunção et al., 2001).

The homologue of TcZNT1/AtZIP4 is also expressed in roots of *A. halleri* but not in *A. thaliana*, with a level of expression decreasing under conditions of Zn excess (Chiang et al., 2006; Talke et al., 2006; Weber et al., 2006). In hyperaccumulator plants the regulation of ZNT1/ZIP4 seems to respond to changes in plant Zn status in different ways as it happens in non-accumulator plants. Though in *A. thaliana* ZIP4 is the closest homologue to TcZNT1, sharing 90% DNA sequence identity as well as 81% homology at the amino acid level, it is involved in the transport of different metals. In fact, in *A. thaliana* (Wintz et al., 2003) AtZIP4 is induced both by Zn deficiency, and by Cu deficiency. In addition, AtZIP4 does not complement with the zrt1 zrt2 Zn uptake deficient yeast mutant as does TcZNT1 (Pence et al., 2000). Instead, it complements the ctr1 yeast mutant, which is defective in Cu uptake, restoring the growth of this yeast mutant under limited Cu conditions (Wintz et al., 2003). These evidences point out that AtZIP4 is involved in Cu uptake rather than in Zn/Cd uptake. The cell specific expression of these transporters suggests that they all have different roles in different plant species. The

expression of the AtZIP4 promoter::β-glucuronidase (GUS) construct in *A. thaliana* indicates that AtZIP4 expression is localized to the stele, thus suggesting a role in trans-root metal transport and not in metal uptake from the soil as previously suggested (Milner and Kochian, 2008). Using a quantitative in situ hybridization technique in *T. caerulescens*, it has been found that TcZNT1 is preferentially expressed in leaf mesophyll, bundle sheath and guard cells (Küpper et al., 2007), hence it can be argued that it plays a role in normal leaf Zn nutrition and not in metal hyperaccumulation. These transgenic approaches coupled with complementation between yeast and *A. thaliana* have thus proved that the differences in behaviour are ascribed to a different function, rather than to a different regulation.

Recent genetic analyses with hyperaccumulator plants failed to show a precise correlation between this gene and the metal accumulation trait. In a QTL analysis of two accessions of *T. caerulescens*, which differed in their degree of Zn accumulation, Assunção et al. (2006) did not find any QTL linked with this gene. The same authors speculate that this gene does not show a segregation in interaccession crosses because it is expressed at comparable levels in different accessions (Assunção et al., 2001; Deniau et al., 2006). A similar result was found for the *A. halleri* homologue, which, in a F2 cross with *A. petraea*, mapped in a region of chromosome 1, which does not contain any QTL for Zn accumulation (Filatov et al., 2007).

The metal transporter *IRT1* (At4g19690), plays a role in metal homeostasis in *T. caerulescens*. TcIRT1 is a close homologue of AtIRT1 with 90.2% identity at amino acid level. AtIRT1 is an Fe-regulated transporter cloned by complementation of the yeast double mutant fet3 fet4 lacking the high and low-affinity Fe uptake systems (Eide et al., 1996). AtIRTI is expressed in roots, where it is induced by Fe deficiency, and shows an altered regulation pattern in plant lines bearing mutations that affect the Fe uptake system (Eide et al., 1996). Thus, the physiological role of IRT1 seems to fall among the mechanisms of Fe uptake from the rhizosphere across the plasma membrane, in the root epidermal cell layer. Localization of IRT1 in transgenic plants (carrying its promoter fused to GUS reporter gene) through colorimetric analysis and in situ hybridization, showed that IRT1 is expressed in the external cell layers of the root, specifically in response to Fe starvation (Vert et al., 2002). A role of IRT1 in the transport of Cd and Zn has been also evidenced (Eide et al., 1996; Connolly et al., 2002). In particular, the observation that transgenic plants for the constructs 35S::IRT1, 35S being the promoter of the Cauliflower Mosaic Virus 35S RNA, accumulate levels of Cd and Zn higher than the wild-type plants, suggests that IRT1 is involved also in the uptake of these metals (Connolly et al., 2002). A homologue to IRT1 has been cloned in *T. caerulescens* (Lombi et al., 2002), where this gene is highly expressed upon Fe deficiency in the Cd hyperaccumulating accession Ganges, but it is expressed at lower levels in the Cd-excluding accession Prayon (Lombi et al., 2002). The expression of IRT1-G found in Gange is enhanced by Cd exposure when the plants received sufficient Fe supply (Plaza et al., 2007). In the *T. caerulescens* accession La Calamine, a Cd-tolerant non-hyperaccumulator plant (van de Mortel et al., 2006, 2008), in condition of Fe deficiency or in response to Cd supplement, a low expression the Fe homeostasis genes IRT1, IRT2 and FRO2 was found (Assunção et al., 2003). Consequently, a role of ITR1 in Cd accumulation has been taken into consideration. Recently, two copies of IRT1 were identified in *T. caerulescens*, and both the Gange and Prayon ecotypes harbour a full-length and a truncated version of the gene in their genomes. The Gange ecotype expresses only the full-length version of TcIRT1, whereas the Prayon ecotype expresses the truncated variant (Plaza et al., 2007). Both the full-length and truncated versions of TcIRT1 are able to complement the fet3fet4 yeast double mutant (Plaza et al., 2007). Through microarray experiments, relevant levels of expression of IRT1 have been found in *A. thaliana* but not in *A. halleri* (Becher et al., 2004).

### 3.1.2. Vacuolar sequestration

Vacuolar sequestration is considered an important aspect in plant metal homeostasis, and in plant detoxification from heavy metals (Martinoia et al., 2007). *AtNramp3* (At2g23150) is a metal ion transporter of the vacuolar membrane, involved in Fe and Cd uptake; it belongs to a membrane metal transporters family of broad specificity. It can complement deficiencies in Mn and Fe transport in yeast (Thomine et al., 2000) and has been found overexpressed in *A. halleri* (Weber et al., 2004; Chiang et al., 2006; Filatov et al., 2006; Talke et al., 2006) and in *T. caerulescens*, at all Zn concentrations (van de Mortel et al., 2006). AtNramp3 expression is also induced by Cd in *A. thaliana* (Herbette et al., 2006). It has been reported to be associated with a QTL for Zn tolerance in *A. halleri* (Roosens et al., 2008a) and with a QTL for Zn accumulation (Filatov et al., 2007). *A. thaliana* overexpressing AtNramp3, controlled by the CaMV 35S promoter, evidenced Cd hypersensitivity and increased Fe accumulation, whereas the accumulation of Cd was not affected (Thomine et al., 2000). Recently, a *T. caerulescens* NRAMP3 has been isolated (Wei et al., 2009; Oomen et al., 2009); its expression in roots is induced by Fe starvation and by Cd and Ni. Overexpression of TcNRAMP3 in yeast increased Cd content, enhancing Ni resistance and reducing Ni accumulation. Overexpression of TcNRAMP3 in tobacco resulted in roots light Cd sensitivity, but did not cause Ni resistance. These results suggest a role of TcNRAMP3 in metal cations homeostasis rather than in metals accumulation (Wei et al., 2009). Overexpression of TcNRAMP3 in *A. thaliana* confirmed tonoplast localization, and the ability to rescue the Cd and Zn hypersensitivity in mutants defective in AtNRAMP3 (Oomen et al., 2009). Recent evidence suggests that the difference between hyperaccumulators and non-accumulators should lie in the higher level of expression, rather than on functional differences of the proteins (Oomen et al., 2009).

Other transporters of the same class such as AtNramp4 (At5g67330), AtNramp5 (At4g18790), AtNramp2 (At1g47240), have been isolated and were induced in *A. thaliana* and in *A. halleri* by Zn or Cd treatments (Herbette et al., 2006; van de Mortel et al., 2006; Becher et al., 2004; Chiang et al., 2006).

The metal transporter *ZAT/ZTP1/MTP1* has been identified in *T. caerulescens* bearing sequence homology to the *A. thaliana* transporter AtMTP1 (At2g46800). The gene belongs to the CDF (cation diffusion facilitators) family of cation transporters so far 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 sequence homology with TgMTP1, isolated in the Ni hyperaccumulator, *Thlaspi goesingense* (Kim et al., 2004). Contrary to previous reports indicating a plasma membrane localization (Kim et al., 2004), TgMTP1 localizes to the vacuolar membrane (Gustin et al., 2009), like TcZTP1 and AtMTP1. These two homologues could have different functions, as suggested by the phenotype observed in transgenic plants: TgMTP1 conferred tolerance to different metals (Persans et al., 2001; Gustin et al., 2009) whereas AtMTP1 conferred tolerance to and increased Zn accumulation (van der Zaal et al., 1999). T-DNA inactivation mutants of *A. thaliana*, which do not express AtMTP1, are more sensitive to Zn (Kobae et al., 2004). AtMTP1 level of expression is higher in hyperaccumulator species (Becher et al., 2004; Chiang et al., 2006; van de Mortel et al., 2006). Three unlinked copies of MTP1 have been found in the genome of *A. halleri*, two of which cosegregate with the Zn tolerance trait in a backcross progeny (*A. halleri* × *A. lyrata*). Individuals harbouring both genes from the *A. halleri* parents have higher tolerance to Zn, while individuals carrying only one gene

show intermediate levels of tolerance (Dräger et al., 2004). The non-tolerant *Arabidopsis* species *A. thaliana* and *A. lyrata* possess only one copy of MTP1. Two copies of this gene have been associated with the QTLs Zntol-2 and Zntol-3 for Zn tolerance (Willems et al., 2007).

We can conclude that the MTP1 genes from *A. halleri*, *T. caerulescens* and *T. goesingense* are involved in the influx of Zn in the vacuole, increasing Zn sequestration. Recent evidence suggests that expression of MTP1 induces a systemic response to Zn deficiency, leading to increased Zn uptake and accumulation (Gustin et al., 2009).

#### 3.1.3. Xylem loading

The gene *HMA4* was first identified in *T. caerulescens* by yeast complementation and screening of recombinant yeast strains for increased Cd tolerance (Bernard et al., 2004; Papoyan and Kochian, 2004). HMA4 is a member of the P-type ATPase superfamily, more specifically, of the P1B subfamily of ATPases, which are supposed to be involved in heavy metals transport. TcHMA4 was found expressed primarily in roots, induced both by Zn deficiency and Zn excess, as well as in response to exceeding Cd (Papoyan and Kochian, 2004). The *A. thaliana* homologue of TcHMA4 (At2g19110) has been characterized in detail; it is expressed primarily in the root stele, where it is probably involved in loading Zn into the xylem for transport to the shoots (Hussain et al., 2004; Verret et al., 2004; Sinclair et al., 2007). In shoots of transgenic *A. thaliana* plants overexpressing AtHMA4, accumulation of Zn and Cd is increased, suggesting a role of HMA4 in loading metals into the xylem (Verret et al., 2004). When both AtHMA4 and its close relative, AtHMA2, are knocked out in *A. thaliana*, reduced Zn accumulation in the shoot ensues (Hussain et al., 2004). Efficient translocation of metals from the root to the shoot is a hallmark of metal hyperaccumulators, thus it has been suggested that TcHMA4 plays a crucial role in heavy metal transport from root to shoot (Papoyan and Kochian, 2004). To support this hypothesis TcHMA4 was found overexpressed in *A. halleri* (Chiang et al., 2006; Talke et al., 2006). Complementation studies with Zn and Cd hypersensitive strains of yeast revealed that both AtHMA4 and AhHMA4 proteins restored the mutants to a wild-type phenotype (Talke et al., 2006). Other evidences confirm that HMA4 is a strong candidate in determining the Cd hyperaccumulator phenotype: results from a cross between *A. halleri* and *A. lyrata* showed how a major Cd tolerance QTL colocalized with AtHMA4 (Courbot et al., 2007). The strongest evidence of a pivotal role of HMA4 in metal accumulation comes from a work of Hanikenne et al. (2008): by using RNA interference to downregulate HMA4 expression in *A. halleri*, they showed that Zn hyperaccumulation and full tolerance to Cd and Zn depend on HMA4. In addition, the increased expression of a series of Zn deficiency responsive genes (ZIP4 and IRT3) followed an increase in HMA4 activity, further enhancing metal accumulation. At least three copies of HMA4, sharing 99% identity, are present in the *A. halleri* genome and the three promoter sequences regulating their expression are more efficient than the one regulating the same gene in *A. thaliana*. This can explain the higher expression level of these genes in the hyperaccumulator plants. *A. thaliana* plants expressing a genomic clone of the HMA4 gene promoter and coding sequence showed enhancement of metal influx, demonstrating that HMA4 suffices in modifying heavy metal accumulation. In *A. thaliana* HMA4 has been shown also as responsible for translocation of Cd from roots to shoots (Wong and Cobbett, 2009).

Another member of this class, *AtHMA3* (At4g30120), is the most overexpressed gene in shoots of *A. halleri*, both in control and in high Zn conditions (Becher et al., 2004). It is also induced by Zn deficiency in *A. thaliana* (van de Mortel et al., 2006). Overall, it is thought to be involved in loading Zn into the xylem.

### 3.2. Metal ligands

Another aspect of hyperaccumulation rests on the ability of the plant to sequester and/or bind metals to molecules or structures to limit their otherwise negative effects. Vacuolar transporters, as previously described, partly fulfil this role, contributing to the partitioning of metals in the vacuole (Martinoia et al., 2007).

The most frequently cited metal protective proteins are metallothioneins. *Metallothioneins* are cysteine-rich, low-molecular-weight, metal-binding proteins that can form mercaptide bonds with various metals. These proteins have been implicated in metal homeostasis primarily in mammals (Cobbett and Goldsbrough, 2002). Plant metallothioneins are grouped into four subfamilies, MT1, MT2, MT3, MT4, with different expression in plant tissues during development and possibly fulfilling different functions (Cobbett and Goldsbrough, 2002). Even if at present no correlations between the levels of MT proteins and the metal concentrations in the plants have been established, some differences, at least in the expression levels, between hyperaccumulators and non-accumulators have been found. In non-accumulator plants, like *A. thaliana* and poplar, MT1a and MT1b are expressed at high levels in roots during exposure to Cd, Cu and Zn (Garcìa-Hernàndez et al., 1988; Zhou and Goldsbrough, 1994; Kohler et al., 2004), while in *T. caerulescens* the levels of MT1 mRNA were found in leaves constitutively higher than in roots; the levels increased with exposure to Cu (Roosens et al., 2005). *A. halleri* and *T. caerulescens* have a constitutively high expression of MT2 (Roosens et al., 2005; Chiang et al., 2006; van de Mortel et al., 2006) like in the Cu-tolerant plants *Silene paradoxa* Lapeyr. Hist. Pl. and *Silene vulgaris* (Moench) Garcke (van Hoof et al., 2001; Mengoni et al., 2003). The overexpression of MT2b is associated with Cu tolerance, but this gene is not a major player in the process (Schat et al., 1996). Overexpression of AtMT2b in tobacco led to increased arsenic translocation from root to shoot (Grispen et al., 2009). The expression of MT3 genes increases during leaf ageing and upon exposure to Cu in non-accumulator plants (Guo et al., 2003; Kohler et al., 2004; Roosens et al., 2004). In *T. caerulescens*, MT3 expression is induced by Cd exposure, but functional studies in yeast demonstrate that TcMT3 confers much greater levels of tolerance to Cu than to Cd. The increase in yeast Cu tolerance in a Cu sensitive mutant background was stronger with TcMT3 as compared with AtMT3 (Roosens et al., 2004). It has been postulated that TcMT3 plays its role allowing the maintenance of a normal Cu homeostasis under conditions of high Cd and Zn in the cytoplasm. This suggestion is reinforced at molecular level by the discovery of a smaller cavity adapted for Cd chelation in the MT3 protein of *A. thaliana* as compared with the analogous protein from *T. caerulescens* (Roosens et al., 2004, 2005). MT4 is highly expressed in seeds of *A. thaliana* (Guo et al., 2003). It plays a role in metal homeostasis during seed development and seed germination rather than in metal decontamination. Transgenic plants expressing metallothionein genes usually result in an increase in metal tolerance (see as example Zimeri et al., 2005; Zhigang et al., 2006): an increased Cd accumulation in roots and leaves was reported in transgenic tobacco expressing the MThis gene from *S. vulgaris* (Gorinova et al., 2007). In a novel approach, the expression of MT1 from *Brassica rapa* L. has been ectopically targeted to chloroplasts of *A. thaliana* (Kim et al., 2007). The transgenic plants showed an increase in tolerance to Cd and to oxidative stress. These observations are complementary to observations in *A. thaliana* mutants lacking MT1a and MT2b (Guo et al., 2008); the double mutant had normal Cu tolerance but Cu accumulation in roots was reduced of 30% by the lack of MT1a. A triple mutant combined with impaired phytochelatin synthesis showed reduced tolerance to Cu and Cd. It can be concluded that metallothioneins play some role in metal homeostasis, but not in metal accumulation, even if they can contribute to the phenotype

of metallicolous accessions enhancing tolerance (Hassinen et al., 2009).

Studies have focused on the role in metal accumulation of the non-protein amino acid, *nicotianamine* (NA); these studies were conducted in *T. caerulescens* and *A. halleri*, but also in *A. thaliana* and *Zea mays* L. NA is a small molecular weight chelator, enzymatically synthesised through a pathway involving four NA synthase genes (NAS1–4). The same have been shown involved in long distance Fe, Cu and Zn transport (Stephan and Scholz, 1993; Wintz et al., 2003). NA proved relevant in Fe unloading from vascular tissues to leaves and floral tissues, in complexing Fe, Zn, Cu in the phloem, Zn and Cu in the xylem (Wintz et al., 2003; Haydon and Cobbett, 2007).

*T. caerulescens* ecotypes which also hyperaccumulate Ni possess a TcNAS1 gene which cloned in yeast conferred high levels of Ni tolerance (Mari et al., 2006). *In planta*, TcNAS1 is expressed in shoots only and induction initiated 6 h after Ni treatment. After this 6-h exposure to Ni, high levels of NA were found in roots, and NA–Ni complexes became detectable in the xylem sap. In response to Ni, NA seems to be translocated to the roots where it chelates the absorbed Ni thus facilitating its transport to the shoot. Further evidences that TcNAS1 can increase Ni tolerance came from transgenic tobacco and *A. thaliana* overexpressing TcNAS1 (Pianelli et al., 2005): in both plants it was observed a significant increase in Ni tolerance and Ni accumulation within the shoot. Transcriptomic studies with microarrays showed a high expression of NAS3 but not of NAS1 or NAS2 in *A. halleri* compared to *A. thaliana* shoots, both at high and low Zn (Becher et al., 2004; Talke et al., 2006). When AhNAS3 was expressed in the yeast Zn hypersensitive zrc1 cot1 double mutant, the yeast cells recovered the capacity to grow at elevated Zn concentrations (Becher et al., 2004). Overexpression of NAS4 (At1g56430) was also reported in *A. halleri* (Talke et al., 2006). Also nicotinamide and nicotinic acid can increase metal tolerance, though in a more indirect way (Berglund, 1994; Semane et al., 2007). Metal sensitive clones of *Salix viminalis* supplemented with different nicotinamide doses prior metal treatments with Cd, Zn or Cu manifested an increase in metal tolerance correlated with an increase in glutathione (GSH), which protects the cell from oxidative stress caused by ROS (reactive oxygen species). ROS are in fact one of the first chemical consequences of the presence of excess free metal ions (Ohlsson et al., 2008). The increase in GSH as a straight consequence to the supplement of nicotinamide remains to be elucidated.

In the Graminaceae, a series of enzymatic reactions produce, starting from NA, the mucigenic acid (MA), which is as efficient as NA in chelating and transporting primarily Fe, in the form $Fe^{3+}$, as well as Zn, Cu, and Ni (Curie and Briat, 2003; Roberts et al., 2004).

In addition to higher expression of NAS genes, some Yellow Stripe Like (*YSL*) genes are also induced under Zn deficiency in *A. thaliana* (van de Mortel et al., 2006). The first member of the YSL protein family was identified as the putative Fe-phytosiderophore uptake transporter in maize roots, and other YSL members were hypothesized to be involved in the transport of NA–metal and MA–metal complexes (Curie et al., 2001; Suzuki et al., 2006; Waters et al., 2006). Three members of the YSL family have been characterized in *T. caerulescens*: TcYSL3, TcYSL5 and TcYSL7, with sequence homology to *A. thaliana* YSLs (Gendre et al., 2007). All three are highly expressed in *T. caerulescens* more than their *A. thaliana* orthologues. TcYSL3 and TcYSL7 were found to be expressed in the root stele, becoming associated with the xylem. Functional analysis in yeast demonstrated that TcYSL3 is able to transport both Ni–NA and Fe–NA complexes. Based on these and other findings, it can be hypothesized that TcYSL3 is involved in long distance Ni translocation in *T. caerulescens*.

*Histidine* (His) is considered important as a free amino acid involved in hyperaccumulation (Haydon and Cobbett, 2007). It forms stable complexes with Ni, Zn and Cd and it is present at high concentrations in roots of hyperaccumulators; in the hyperaccumulator *Alyssum lesbiacum* the concentration of His is increased by Ni treatment, but the genes of the biosynthetic pathway are not induced (Persans et al., 1999; Ingle et al., 2005). In *A. lesbiacum* there is an enhanced expression of ATP-phosphoribosyltransferase, the first enzyme of the His biosynthetic pathway, in comparison with the non-accumulator *Alyssum montanum*; the same gene overexpressed in transgenic *A. thaliana* plants increases tolerance but not accumulation (Ingle et al., 2005). Hyperaccumulators show a larger pool of His available for chelation in roots. A similar picture emerges from recent results obtained with *T. caerulescens*, showing high His concentrations in roots, and suggesting that Ni-His complex formation in the cytoplasm of root cells decreases the sequestration of Ni in vacuoles. This could explain the higher rate of Ni loading in the xylem sap, as compared with the non-accumulator *T. arvense* (Richau et al., 2009). Until now, transcriptomics and proteomics have not found instances of constitutively high expression of His biosynthetic genes in *Thlaspi* and *A. halleri*.

### 3.3. Sulphate assimilation

The gene O-acetylserine(thiol)lyase (*OAS-TL*, At3g22460) was identified as overexpressed in *A. halleri* (Weber et al., 2004, 2006). The gene belongs to the cysteine synthetic pathway, producing an amino acid which is a fundamental constituent of all metal chelators: metallothioneins, glutathione, phytochelatins, and nicotianamine (Bonner et al., 2005; Eapen and D'Souza, 2005). The same sequence was also found overexpressed in shoots of *A. halleri* in control conditions (Becher et al., 2004; Talke et al., 2006), but this was not confirmed (Chiang et al., 2006). Tobacco plants with altered levels of this protein in the cytosol and/or chloroplasts showed enhanced tolerance to Cd, Se and Ni, but not to Pb or Cu (Kawashima et al., 2004). In particular, the plants expressing the protein both in the cytosol and in the chloroplasts had a higher Cd tolerance, and enhanced levels of Cys and GSH. The same plants also accumulated more Cd.

Genes involved in sulphate assimilation were found to be differentially expressed in *T. caerulescens* in response to Cd exposure or Zn deficiency, such as SULTR2;1 (At5g10180) (van de Mortel et al., 2008). In *A. thaliana* this gene is induced in response to short term Cd exposure (Herbette et al., 2006); it is specifically expressed in the central cylinder (xylem parenchyma and pericycle) of roots and in the vascular tissues (phloem parenchyma) of leaves (Takahashi et al., 1997) suggesting a role in enhancing the sulphate translocation capacity. Overexpression of SULTR2;1 in condition of Cd exposure could simply imply its role in the adaptive process required to ensure an adequate supply in sulphur-containing compounds in stress conditions. *A. thaliana* with an antisense suppression of SULTR2;1 exhibited a reduced translocation of sulphate to seeds and a decrease in thiols such as Cys and GSH (Awazuhara et al., 2005). Sulphate translocation is therefore essential in providing S atoms for synthesis of ligands.

Also the sulphate adenyltransferase or ATP sulphurylase *APS3* (At4g14680), the first enzyme involved in sulphate assimilation, is expressed at high levels in *T. caerulescens* and *A. halleri* in conditions of Zn deficiency and/or Cd exposure (Weber et al., 2006; van de Mortel et al., 2008). Although overexpression of the *A. thaliana* APS1 gene in *Brassica juncea* confers increased Se accumulation and tolerance (Pilon-Smits et al., 1999), no evidence that APS3 can have a similar role has been provided. Both enzymes have a plastidic localization (Murillo and Leustek, 1995) and therefore their functions can be similar.

### 3.4. Lignin biosynthesis

When discussing the mechanisms for metal sequestration and chelation, it is to be remembered that plant cell walls constitute a

vast extension of material which can bind and effectively sequester metal ions. Several genes involved in root lignin biosynthesis, few others implicated in suberin biosynthesis, and some involved in wax synthesis were identified as overexpressed in *T. caerulescens* compared to *A. thaliana* when exposed to Zn (van de Mortel et al., 2006, 2008). This finding correlated with the increased lignification of the endodermal cell layer and the observation of two endodermal cell layers in roots of *T. caerulescens*. A more strongly developed endoderm in the hyperaccumulator could function to minimize the remobilization of metals accumulated in the stele during the trans-root processes resulting in metal loading into the xylem. The genes found differently expressed in *A. thaliana* in respect to *T. caerulescens* under Zn or Zn/Cd treatments have been at times related to lignification processes even if in some cases they are either involved in wax synthesis or in general phenylpropanoids metabolism: flavonoid, flavones, anthocyans, and other pigments (Whetten and Sederoff, 1995; Hamberger and Hahlbrock, 2004). Lignification starts with the synthesis of monolignols from the common precursor L-phenylalanine, through a branched, and if need be channelling, pathway, which is also species specific, tissue specific and different during the stages of plant development. Polymerized monolignols create lignins whose deposition occurs on the cell wall of specific cell types (Whetten and Sederoff, 1995; Rogers and Campbell, 2004). In *A. thaliana*, "biosynthetic mutants" (*brown midrim, mb*) and "regulatory mutants" (*brevipedicellus, bp*) helped to identify the genes encoding for the isoenzymes families involved in lignin deposition, their internal regulation mechanisms, related to the genes promoter sequence, and their response to environmental and developmental cues (Venglat et al., 2002; Rogers and Campbell, 2004). Deposition of suberin, a lipophylic polyester, and cellulose, constituted by glucans microfibrils, present in the cell wall with lignin has not been found in response to metal stress neither in *A. thaliana* nor in *T. caerulescens* by microarray experiments (van de Mortel et al., 2006, 2008). The genes, univocally responsible for lignification, which have been found active during metal response in *T. caerulescens* can be summarised thus: genes coding for members of the (i) 4-coumarate-CoA ligase family, 4CLL (At1g20490, At1g20500, At5g38120); (ii) caffeoyl-CoA 3-O-methyltransferase family, CCOMTL (only At1g67980); (iii) cinnamyl-alcohol dehydrogenase family, CAD (only At1g72680) (Whetten and Sederoff, 1995; Rogers and Campbell, 2004; van de Mortel et al., 2006, 2008; Friedmann et al., 2007; Rowland et al., 2007).

Hyperaccumulators show an increased expression in key genes involved in production of ligands for metals, including sulphur compounds and plant cell wall constituents. Induction of low molecular weight ligands, such as phytochelatins and metallothioneins, looks likely to be a short-term response, similar in concept to a stress response with the purpose of limiting the immediate toxic effects of metals. In hyperaccumulators, it is likely that long-term responses can be more sustainable and effective: sequestration in plant cell walls should fulfil this role. Higher focus on genes and enzymes involved in plant cell wall synthesis and maintenance should be necessary. In particular, cellulose and other polysaccharides have received comparatively less attention than lignin, but it is to be expected that their role could be as important, at least, due to their ability in forming stable bonds with metals because of their highly ordered structure (Marmiroli et al., 2005).

## 4. Metal adaptation, costs and trade-offs

Costs associated with adaptation to metals can be considered in terms of energy and resources allocation. The presence of elevated concentrations of metals in the soil increases maintenance costs because the organism needs to spend energy to counter potentially toxic effects. Active physiological processes, such as those described in this paper, require ATP and highly energetic compounds to operate in processes such as active transport, sequestration, delocalization and concentration. The metabolic cost to perform these tasks can be substantial and the increase in the energy expenditure, required to constantly maintain these functions, is a pre-requisite of a broad plastic response. However, trade-off can arise when individuals spend more than others to maintain functions because they display specific mechanisms like resistance, which are not plastic. This can increase survival in stressful condition, such as excessive metals in the soil, but at the same time leaves less energy for reproduction, growth and other processes (Audet and Charest, 2008). The emergence of these specialised allocation patterns associated with resistance/tolerance behaviour leads necessarily to the development of a trade-off between maintenance and reproduction, and subsequently to a trade-off between resistance/tolerance and fitness. Cellular mechanisms devoted to sequestration of metals in organelles or organs and to protection of cell structures through ligands concur to this trade-off (Pierce et al., 2005). In productive habitats these tolerant/resistant organisms which exhibit adaptive morphological changes (stress-tolerators or ruderals, *sensu* Grime, 1979) are overgrown by competitors. In the presence of high soil concentrations of metals, a more conservative use of resources permits survival and a competitive advantage, notwithstanding the constant stress (Kazakou et al., 2008). Wiley et al. (2005) have shown increased caesium accumulation in plants belonging to the stress-tolerant and ruderal growth strategies. However, the foregoing speculations do not completely explain the hyperaccumulator phenotype which is associated to a trait of resistance/tolerance, even though genetically independent, but at the same time does not fit with the idea of trade-off based only on resources allocation. In fact the general natural mechanism underlying the hyperaccumulator phenotype fits more with the idea of resources conservation rather than allocation.

Many hyperaccumulator species have a decreased growth rate, limited energy expenditure and decreased metabolic rate. These plants could be at a disadvantage when resources are abundant, but find themselves at ease in disturbed habitats because, for example, the high concentration of metals in their organs deters animals to graze upon them (Pollard and Baker, 1997; Jiang et al., 2005). A recent meta-analysis of the literature showed that herbivores vary in their preference or aversion to high metal concentration in plant tissues; therefore, the advantage conferred by hyperaccumulation would depend on the type of herbivores feeding on the particular plant species (Vesk and Reichman, 2009). On the other hand, some insects feed on hyperaccumulating plants and accumulate metal in their tissues; in turn, they may exploit this as a defense against predators (Boyd, 2009). This contradiction helps to explain why many hyperaccumulators grow in complex assemblages of plant species that include both hyperaccumulators and non-hyperaccumulating competitors. Spatial environmental variability may explain the apparent juxtaposition of both strategies alongside each other. Allelopathy may be an additional aspect of hyperaccumulation behaviour; hyperaccumulators cause a local increase in metal soil concentration (phytoenrichment, through leaf fall), thereby decreasing the fitness of neighbouring non-tolerant species (Morris et al., 2009).

Ruderal species tend to be representative of disturbed environments, short-lived, fast-growing and rapidly go to seed when confronted with environmental stress. Stress-tolerators grow slowly and delay reproduction in unfavourable conditions. Neither strategy appears to explain or typify all metal hyperaccumulator plants. In fact it has been shown that for some hyperaccumulators the presence of the metal at high concentration in the soil is essential for a normal growth (Küpper et al., 2001). When analysing the existence of trade-offs between traits and environments, it has to be considered that the physiological performance needs to be associated with fitness but under a range of natural conditions.

Resistance/tolerance represent the physiological performance in a small range: the presence of the metal. The hyperaccumulator phenotypes could represent the physiological response on a broader range. The extension of the trade-off to animals feeding or to other environmental cues can be an example of this "extended" fitness. Are hyperaccumulator plants more or less "specialised" than the tolerant/resistant plants? From the data reported in this review, the hyperaccumulators share common mechanisms with tolerant plants, but show peculiar mechanisms as well which make them more generalist examples of trade-off between trait and environment. This could result in phenotypic plasticity half way between the specialised tolerant and the generalist non-tolerant species.

## 5. From present to future research

Fig. 1 reports some critical mechanisms which have been advocated for explaining the difference between hyperaccumulators and non-accumulators, listed according to relevance. At the basis of the graph the main mechanisms, described with examples taken from the literature on *T. caerulescens* and *A. halleri* in this paper, are listed: (i) the existence of gene variants and copy number variation, (ii) specific regulation responses, (iii) subcellular or organ localization of proteins, (iv) sequestration of ions in organelles or tissues, (v) activation of enzymes, (vi) regulation of protein modification and stability. The top of the graph lists potential mechanisms which have not yet been described as relevant for hyperaccumulation: (i) codon usage bias leading to different protein structure, (ii) RNA splicing and alternative splicing, and (iii) interfering RNA. Hopefully in the near future these mechanisms will be explored also in hyperaccumulators, and the prediction is that future research interests could focus on these aspects.

It is interesting to note that the two plant species *T. caerulescens* and *A. halleri* have evolved different mechanisms to control hyperaccumulation. One example: the triplication of MTP1 and HMA4 in *A. halleri* which has no equivalent in *T. caerulescens*. It sounds reasonable to say that the regulation of critical genes in hyperaccumulators differs in two basic features: homologues genes can be overexpressed in hyperaccumulators in control conditions, and/or they can respond differently to metals normal presence or scarcity.

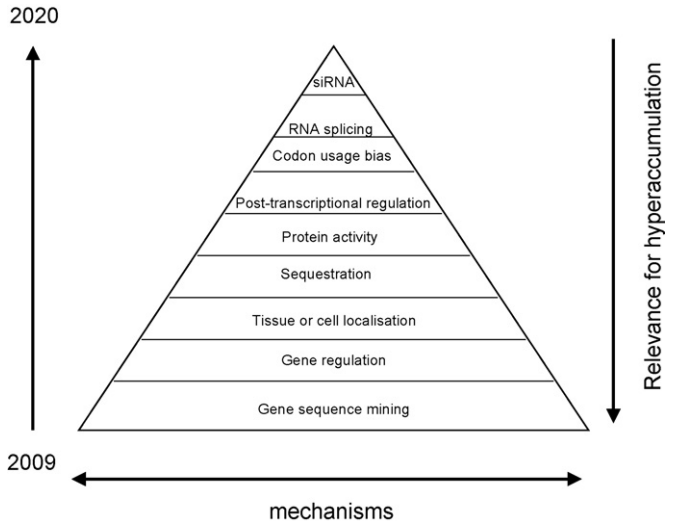


**Fig. 1.** Genetic mechanisms which can account for the differences between hyperaccumulators and non-accumulators. Mechanisms are listed from bottom to top according to decreasing relevance (arrow on the right, see text for comments). The arrow on the left indicates the prediction for future research interest in coming years moving from the current topics of interest to those yet unexplored.

Many of the isolated genes abide this description. In some instances, the genes lead to a protein with different tissue-specificity or cell-specificity, leading to the conclusion that its function in the hyperaccumulator is different from the non-accumulator. Finally, gene dosage plays an important role.

The phenomenon of constitutive over-expression of a large set of genes seems to be a common process in the adaptation of plants to extreme environments. Gene duplication could be a system to get a higher expression of a specific gene, when the phenomenon of gene silencing was not affected. This is the case of *A. halleri* HMA4 found in 3 copies, in which modification of cis-regulatory elements contributes to the high expression level of each copy (Hanikenne et al., 2008).

A gene present in a higher number of copies in the hyperaccumulator, could lead to its enhanced expression. Differences in expression, cellular localization, and function, should be correlated in an overarching frame to sequence diversity and polymorphisms at gene level, and to differences in translational and post-translational modification of proteins. Investigations on this latter subject are still rare in the literature. Recently sequence divergence between *A. thaliana* and *A. halleri* in the gene HMA4 were studied (Hanikenne et al., 2008) and it was found that divergence in the 5′ regions was the highest. Experiments using transgenic organisms confirmed that the three promoters from *A. halleri* were as effective as the CaMV 35S promoter, and more effective than the promoter of the *A. thaliana* gene AtHMA4.

There is also a nexus between the regulatory genes controlling the cascade expression of structural genes and the response to metals in different plants. Comparison of the root transcriptomic profile of *T. caerulescens* vs. that of *A. thaliana* in the presence of different Zn and Cd concentrations pinpointed that a number of genes encoding for transcription factors and regulators are differentially expressed (van de Mortel et al., 2006, 2008).

Although a number of different metal transporters and enzymes have been implicated in metal hyperaccumulation, the protein activity and their substrate affinity are still poorly understood. A deeper insight into the relationship between structure and function, presence and localization of the corresponding protein is required, as evidenced also by Dickinson et al. (2009) and by Verbruggen et al. (2009).

Most of the changes in gene expression obtained by comparing hyperaccumulators vs. non-accumulators provide only correlations on a possible functional role, not a clear demonstration of the existence of any connections between genotype and phenotype. A bottom–up approach requires the isolation and characterization of mutants with the desired phenotype followed by (i) isolation of the gene involved and (ii) comparison of the proteomic profiles of the mutant and the wild-type eventually. *A. thaliana* mutants resistant to caesium were isolated and the phenotype characterized by evaluating the intracellular localization of Cs and other ions with μ-SRXF (synchrotron radiation induced X-ray micro-fluorescence) and μ-XANES (micro X-ray absorption near edge spectroscopy) techniques (Marmiroli et al., 2009). That a single gene impairs Cs uptake and translocation, and K and Ca homeostasis and plant biomass production was demonstrated in this way.

A number of approaches to exploit the genomic of contrasting accessions and/or species are also utilised because they are facilitated by the synteny and homology between a target and a model species (i.e. *T. caerulescens* and *A. thaliana*). However, it is quite unlikely that the mechanisms identified in just two plant genuses of the Brassicaceae family can be of general significance for a plethora of other hyperaccumulator plants, which are as taxonomically distant as ferns with angiosperms or gymnosperms. Like in animal evolution any phenotypic homology might derive from the convergence of non-homologous mechanisms rather than from synonymous mechanisms.

The broad extent of genetic variability in metal hyperaccumulation between ecotypes from different regions of the world needs to be exploited more efficiently to further forward the understanding the hyperaccumulation trait. The integration of population genetics, ecology, molecular evolution and phylogenetic studies could help to determine the molecular basis of metals adaptation through hyperaccumulation (Purugganan and Gibson, 2003).

During a cooperation financed partly by COST Action 859 between University of Parma and Wageningen University, we studied the role played by genetic variation in the ecological adaptation of *Thlaspi* species (Andrea Pirondini, 2007, PhD thesis, University of Parma). A natural Ni adapted population of *T. caerulescens* grown on the Ni-rich serpentine hill of Monte Prinzera (MP) in Italy was compared with the other *T. caerulescens* populations collected on metalliferous and non-metalliferous soils and with other *Thlaspi* species in the rest of Europe. DNA sequences were determined and analysed for different genes involved in metal homeostasis. Phylogenetic analyses revealed that the MP population resembled more the Zn and Cd accumulator accessions from Austria than other *T. caerulescens* accessions from non-metalliferous soils. A higher level of sequence variation was found in the metal responsive genes, compared to genes not involved in the metal response. This evidence suggests that a continuous selective pressure exercised by the metal concentrations in the soil could have played a role in the evolution of the phenotype because it contributed to the ecological adaptation to the metalliferous environment. Moreover, selection tests conducted in *T. caerulescens* populations for the different stress related genes previously analysed showed that the most significant variation concerned the ZNT2 metal responsive gene. The *in silico* analysis of the amino acid sequences and the reconstruction of hydrophobicity profile in ZNT2 and in the orthologous At5g36250 indicates that some of the sequence variations found in these two genes could have had an effect on the proteins function. The sequence analyses of candidate genes for metals hyperaccumulation substantiate the evidence that during evolution, when environmental stimuli or stressors (in this case the metal composition of the soil) could have oriented the genetic variation, genetic variability steered towards a condition favouring eventually a new trade-off between the plants and the environments. Besnard et al. (2009) in a recent paper dealing with *T. caerulescens* population genetics in western Switzerland also described TcZNT2 as a candidate gene marker which showed a positive correlation with metal status of the *Thlaspi* populations: its genetic differentiation increased according to differences in the Zn status of soils. ZNT2 is a Zn transporter similar to TcZNT1, previously described in this paper (Table 2). Structural models and finally crystallographic analyses of purified protein should provide *in vivo* evidences of the possible distinctive role of specific transporters in different *T. caerulescens* accessions. Few examples were reported in literature about protein structures in hyperaccumulators: for instance, the structural model of the TcMT3 metallothionein protein revealed that in *A. thaliana* the cavity for metal chelation is smaller than in *T. caerulescens*. The *Thlaspi* isoform was functionally more effective than the *Arabidopsis* isoform in conferring tolerance to Cu in transgenic yeast (Roosens et al., 2004). Similar experiments could clarify the relevance of structural modifications due to genetic variability in hyperaccumulator accessions.

Explaining the mechanisms, degree and the role of the biological variability in the context of environmental adaptation is a prerequisite to harness and exploit the potential of hyperaccumulating plants in phytoremediation (see also Dickinson et al., 2009). Advancement of knowledge will also contribute towards defining the criteria for the design of the ideal plant prototype for metals remediation in specific environments.

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