Research review

Thlaspi caerulescens, an attractive model species to study heavy metal hyperaccumulation in plants

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Summary

Studying heavy metal hyperaccumulation is becoming more and more interesting for ecological, evolutionary, nutritional, and environmental reasons. One model species, especially in the era of high throughput genomics, transcriptomics, proteomics and metabolomics technologies, would be very advantageous. Although there are several hyperaccumulator species known, there is no single model species yet. The Zn, Cd and Ni hyperaccumulator species *Thlaspi caerulescens* has been studied to a great extent, especially for Zn and Cd hyperaccumulation and tolerance. Its physiological, morphological and genetic characteristics, and its close relationship to *Arabidopsis thaliana*, the general plant reference species, make it an excellent candidate to be the plant heavy metal hyperaccumulation model species.

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Heavy metal hyperaccumulator plants

Already back in 1865 the first reference to heavy metal hyperaccumulation in plants was made when *Thlaspi calaminare* (now *Thlaspi caerulescens*) growing on Zn-rich soils near the German-Belgium border, was reported to contain 17% of Zn in its ash (Risse in Sachs, 1865). Nevertheless, the study of plant heavy metal hyperaccumulation is relatively recent. It was the discovery in 1948 by Minguzzi and Vergnano of extreme Ni accumulation in *Alyssum bertolonii* from serpentine hills in Italy, reaching 10 000 µg Ni g^{-1} (1%) of plant dry weight (d. wt) (Minguzzi & Vergnano, 1948), which marks the beginning of an increasing interest in this subject.

Brooks *et al.* (1977) first coined the term 'hyperaccumulator' to define plants with Ni concentrations higher than 1000 µg g^{-1}

d. wt (0.1%). This value was not chosen arbitrarily. Ni is a plant micronutrient and is found in the vegetative organs of most plants in the range of 1–10 µg g^{-1} d. wt. Toxicity occurs at concentrations higher than 10–50 µg g^{-1} d. wt (Marschner, 1995). Initial surveys revealed very few plants containing between 300 and 1000 µg Ni g^{-1} d. wt (Brooks *et al.*, 1977) suggesting a clear distinction between nonaccumulators and hyperaccumulators, with the 1000 µg g^{-1} threshold as a good criterion for defining Ni-hyperaccumulation. However, after finding plant species with Ni concentrations in the range of 100–3000 µg g^{-1} upon analysing additional serpentine floras, Reeves (1992) suggested that 'ultimately the concept of hyper-accumulation as a distinct form of serpentine behaviour may be most useful if it can be linked to a small range of well defined uptake mechanisms or metabolic processes'. In the

absence of such proven mechanisms the Ni hyperaccumulator definition was specified as 'a plant in which a Ni concentration of at least 1000 µg g^{-1} has been recorded in the dry matter of any above-ground tissue in at least one specimen growing in its natural habitat' (Reeves, 1992).

Threshold values are also provided to define hyperaccumulation of several other trace elements. For Zn, another plant micronutrient, this value is 10 000 µg g^{-1} d. wt (1%) (Baker & Brooks, 1989). Most plants have Zn concentrations between 30 and 100 µg g^{-1} d. wt Concentrations above 300 µg g^{-1} are considered toxic (Marschner, 1995). Cd is not an essential or beneficial element for plants, although they generally exhibit measurable Cd concentrations, particularly in roots, but also in leaves, most probably as a result of inadvertent uptake and translocation. A foliar concentration above 100 µg g^{-1} d. wt (0.01%) is considered exceptional and is used as a threshold value for Cd hyperaccumulation (Baker *et al.*, 2000).

An extensive analytical survey on many plant species has been performed in the past decades, resulting in an ever-increasing list of known metal hyperaccumulators. Approximately 400 species have now been reported, mainly occurring on metal-rich soils in both tropical and temperate zones and belonging to a wide range of unrelated families. The metal hyperaccumulation characteristic is not common in terrestrial higher plants. The identified metal hyperaccumulators account for less than 0.2% of all angiosperms (Baker *et al.*, 2000). The list encompasses hyperaccumulators of Ni, Zn, Cd, Pb, Cu, As, Co and Mn (Baker & Brooks, 1989; Brooks, 1994; Baker *et al.*, 2000; Ma *et al.*, 2001). The majority are Ni hyperaccumulators (> 317 species; Baker *et al.*, 2000). Although these species are scattered geographically and belong to different genera and families, the genera *Alyssum* and *Thlaspi* from the Brassicaceae family are reported to be the ones that contain the highest number of Ni hyperaccumulators. Extreme Ni hyperaccumulators were also found in the genera *Phyllanthus* (Euphorbiaceae), *Homalium* (Flacourtiaceae), *Geissois* (Cunoniaceae) and *Hybanthus* (Violaceae) (Baker & Brooks, 1989; Reeves, 1992).

The list of Zn hyperaccumulators is much shorter than for Ni hyperaccumulators, with *c.* 15 species identified (Baker *et al.*, 1992; Brooks, 1994). They are mainly found growing on calamine soils, contaminated with Pb, Zn and Cd due to mining activities or metal smelting, although for some species like *T. caerulescens* and *Arabidopsis halleri* (formerly *Cardaminopsis halleri*), nonmetalicollous accessions have also been reported (Meerts & Van Isacker, 1997; Bert *et al.*, 2000; Schat *et al.*, 2000). Zn hyperaccumulation appears to be limited to the genera *Thlaspi* (11 species) and *Arabidopsis* (1 species) (Brassicaceae) and just a few species in other families (Baker & Brooks, 1989). Hyperaccumulation of Cd was only reported for the Zn hyperaccumulators *T. caerulescens* and *A. halleri* (Brown *et al.*, 1995; Küpper *et al.*, 2000; Lombi *et al.*, 2000) and the Ni hyperaccumulator *Thlaspi goesingense* (Lombi *et al.*, 2000).

In the past decade or so, the phenomenon of heavy metal hyperaccumulation has attracted considerable scientific attention for several different reasons. Metal hyperaccumulation probably evolved as an adaptive trait, offering a new niche to plants, maybe as an extension of the metal tolerance trait found in many more species. The current view is that the trait itself evolved independently in various genera. Boyd & Martens (1992) summarized five hypothetical selective forces to explain metal hyperaccumulation. One of these has emerged to be most plausible. It states that metal hyperaccumulation is a way to reduce damage by herbivory and parasitism (Boyd & Martens, 1992) and is supported by circumstantial experimental evidence (Boyd & Martens, 1994; Martens & Boyd, 1994; Pollard & Baker, 1997; Jhee *et al.*, 1999; Davis & Boyd, 2000; Ghaderian *et al.*, 2000; Davis *et al.*, 2001). These studies indicate that, e.g. Ni hyperaccumulation functions as a general defence, being effective against a broad range of organisms like bacteria, fungi and insects, and that Zn hyperaccumulation can at least protect against insect herbivory.

Next to these interesting ecological and evolutionary issues that can be studied with heavy metal hyperaccumulators, there are two other reasons why these species currently receive increasing attention. This interest is also fuelled by the prospect of exploiting the heavy metal hyperaccumulator species, or their genes, for phytoextraction and phytomining purposes (Lasat, 2002). Metal contamination of soil is considered to be more and more a threat to human health, and conventional cleaning methods are either very costly or simply practically impossible to perform in view of the large area of contamination. A better understanding of the physiology and molecular genetics of metal hyperaccumulator might lead to the desired breakthrough towards commercially attractive phytoremediation applications.

Recently, another application aspect arose in the possibility of using the Zn hyperaccumulation traits to improve the nutritional value of food for people living largely on a vegetative diet, which is normally relatively poor in iron and zinc (Grusak, 2002). When considering that about 1.5 billion people worldwide suffer from zinc-deficiency, improving the zinc content in food could clearly aid in solving a problem.

In all, these increasing interests helped the establishment of metal hyperaccumulation in plants as a growing and exciting field of research. Still there are many issues unresolved and many questions to answer. For instance, why do hyperaccumulators accumulate metals to concentrations that are toxic to most organisms? Which are the selective factors that provoked the evolution of the trait? What physiological mechanisms are employed to maintain the high intracellular metal levels? Are these high levels driven by altered uptake and transport mechanisms? Which genes are involved, are they novel and species-specific, or modified versions of genes commonly used by other, nonaccumulator species, but expressed or regulated at different levels?

In this review we want to advocate the use of *T. caerulescens* as a very convenient model plant species to study many aspects of this exciting research field and to finally provide answers for the unresolved issues.

Thlaspi caerulescens, a Ni, Zn, Cd hyperaccumulator

T. caerulescens J. & C. Presl is best known as a Zn hyper-accumulator, although it also hyperaccumulates Cd and Ni. Accumulation of 30 000 µg Zn g^{-1} d. wt (Brown *et al.*, 1995), 14 000 µg Cd g^{-1} d. wt (Lombi *et al.*, 2000) and 4700 µg Ni g^{-1} d. wt (Schat *et al.*, 2000) has been reported in healthy plants growing either in contaminated soil or in metal-rich nutrient solutions. The species belongs to the Brassicaceae or Cruciferae family, which is well represented among the reported hyperaccumulator plants. Also within the *Thlaspi* genus, there are many species known to hyperaccumulate metals, most often Ni (Reeves, 1988). Perhaps the best-studied species next to *T. caerulescens* are *T. goesingense*, originating from Central Europe (Salt, 2001) and *T. montanum*, native to the eastern USA (Boyd & Martens, 1998).

The taxonomy of the genus *Thlaspi* and also the species *T. caerulescens* is quite complex (Koch & Mummenhoff, 2001). Initially several subspecies were distinguished, largely based on differences in geographical distribution and metal preference. Recent isozyme analyses of several metallophytic and nonmetallophytic accessions could not confirm this sub-division in subspecies (Koch *et al.*, 1998). The species is found at scattered sites throughout Europe (Tutin *et al.*, 1993), from Spain, France and the UK up to Poland in the east. It has been introduced and is now naturalized in Scandinavia and Finland. Although the species appears to have a preference for metal-containing calamine and serpentine soils, it can also grow on nonmetalliferous soils and as such is classified as a facultative metallophyte. The species is biennial or perennial, generally flowering in spring. Greenhouse-grown plants normally require an extensive vernalization period of at least 1 month to induce flowering. Upon flowering, plants produce long racemes with many flowers, eventually bearing heart-shaped winged fruits (silicula), each containing 8 seeds maximum. Seed dormancy is either absent or broken after a few weeks of dry storage.

Many accessions are known (Koch *et al.*, 1998), but only few are frequently used for physiological and genetic studies of metal uptake, transport and tolerance. The 'Prayon' accession originates from the north-east of Belgium (Ramaut, 1964), not far away from the site where 'La Calamine' was collected (Schat *et al.*, 2000). Both originate from Zn/Cd/Pb-rich calamine soil. The 'Ganges' accession originates from Zn/Cd-rich soil from the south of France (Lombi *et al.*, 2000). Accession 'Monte Prinzera' was collected from Ni-containing serpentine soil in the north of Italy (Schat *et al.*, 2000) and 'Lellingen' is an accession collected in Luxembourg, from a nonmetalliferous site (Meerts & Van Isacker, 1997).

Metal hyperaccumulation in *T. caerulescens*

Like any other hyperaccumulator, *T. caerulescens* exhibits enhanced metal uptake, as well as enhanced metal translocation to the shoots (Lasat *et al.*, 1996; Shen *et al.*, 1997; Schat *et al.*, 2000). The requirement for zinc is that strong that plants, especially the Zn-tolerant accessions, show Zn-deficiency symptoms at 10 times the internal Zn concentration normally observed for Zn deficiency in nonaccumulator species (Shen *et al.*, 1997, A. Assunção & H. Schat, unpublished). The physiological mechanisms underlying the strongly enhanced metal uptake and root-to-shoot translocation in hyperaccumulators are not yet known. There are a number of possibilities associated with different sequential processes, i.e. mobilization in the rhizosphere, metallophilic root proliferation, uptake, chelation and compartmentalization in the roots, xylem loading, chelation in the xylem, xylem unloading, and chelation and compartmentalization in the leaf cells.

Mobilization from the soil

An enhanced capacity to mobilise metals from the rhizosphere has been suggested to play a role in the metal hyperaccumulation phenomenon. The mobilization of Zn from nonlabile pools through rhizosphere acidification (Knight *et al.*, 1997; McGrath *et al.*, 1997; Luo *et al.*, 2000) and/or release of root exudates (Zhao *et al.*, 2001) was not found to be more efficient for *T. caerulescens* than found for nonhyperaccumulators (Whiting *et al.*, 2001a, 2001b).

Metallophilic root foraging

T. caerulescens has been observed to exhibit preferential root proliferation in Zn- and Cd-enriched soil patches (Schwartz *et al.*, 1999; Whiting *et al.*, 2000). However, this zincophilic root foraging trait is not an absolute requirement for Zn hyperaccumulation. When comparing plants from two accessions with different degrees of Zn accumulation and tolerance, Haines (2002) found that the accession with the highest Zn accumulation efficiency, derived from a slightly Zn-polluted soil, did not exhibit the Zn foraging trait, whereas the calamine accession did. Whiting *et al.* (2000) found Cd foraging in a Cd hyperaccumulating population, but could not observe the phenomenon in a population with low Cd accumulation. In both of the above cited studies, it was the most tolerant population out of the two, i.e. Prayon (Zn) and Ganges (Cd), respectively, that showed the foraging response. Root metal foraging could therefore be associated with high levels of tolerance to the metal in question, rather than with high metal accumulation efficiency.

Uptake

Lasat *et al.* (1996) started a detailed physiological character-
ization of Zn uptake in hydroponically grown seedlings of
T. caerulescens (accession Prayon) and *T. arvense* (a nonhyper-
accumulator congener). Using radiotracer flux techniques,
they recorded a concentration-dependent Zn^{2+} influx into the
root. In both species, there was a saturable component
following Michaelis-Menten kinetics. While the K_m values
appeared similar for both species, the V_{max} was much higher
in *T. caerulescens* than in *T. arvense*, suggesting a higher
expression of functionally very similar Zn transporters in
T. caerulescens compared to *T. arvense* roots (Lasat & Kochian,
2000). Time-course analysis of Zn accumulation in roots and
shoots supported this finding. At the beginning of the
experiment *T. caerulescens* accumulated two times more Zn in
roots than *T. arvense* did, despite a much higher rate of Zn
translocation to the shoot (Lasat *et al.*, 1996).

Further progress was made when Lasat *et al.* (2000) and
Pence *et al.* (2000) described the isolation of the *ZNT1* Zn
transporter gene from *T. caerulescens* accession Prayon by
functional complementation of the yeast *zhy3* mutant, which
is defective in Zn uptake (Zhao & Eide, 1996). *ZNT1* is a
member of the *ZIP* family of metal transporter genes (Grotz
et al., 1998), with members in the fungal, plant and animal
kingdoms (Eng *et al.*, 1998; Guerinot, 2000; Mäser *et al.*,
2001). Homology to the *Arabidopsis thaliana ZIP4* gene was
used by Assunção *et al.* (2001) to clone the slightly longer
ZNT1 gene from accession La Calamine, as well as an apparent
paralogue, the *ZNT2* gene. In nonaccumulator species like
A. thaliana or *T. arvense*, the orthologues of *ZNT1/ZNT2* are
mainly expressed in roots, but only under Zn deficiency con-
ditions. At normal or elevated Zn supply their transcription
is strongly downregulated (Grotz *et al.*, 1998; Pence *et al.*,
2000; Assunção *et al.*, 2001). By contrast, in *T. caerulescens*
both *ZNT1* and *ZNT2* are highly expressed in roots and at
a lower level in shoots, not only under conditions of Zn defi-
ciency, but also at normal or elevated Zn supply (Pence *et al.*,
2000; Assunção *et al.*, 2001). ZNT1-mediated Zn uptake in
yeast showed a saturable component (Pence *et al.*, 2000) with
a K_m value very similar to the one found for *T. caerulescens*
(Lasat *et al.*, 1996). Furthermore, the V_{max} of Zn influx in
roots of *T. caerulescens* grown under different Zn concentra-
tions correlated very well with the root *ZNT1* transcript levels.
Also, the K_m values were very similar at all the Zn exposure
levels tested (Pence *et al.*, 2000). While the ZNT1 and ZNT2
transporters may account for the majority of Zn uptake in
roots, *T. caerulescens*, like *A. thaliana*, will contain many more
genes potentially encoding Zn transporters (Mäser *et al.*,
2001). As long as these have not been studied, a role for them
in Zn uptake cannot be excluded.

Still, an increased expression of *ZNT1* and *ZNT2* may be
one of the evolutionary changes on the way from nonaccumu-
lator to hyperaccumulator. Why these genes are apparently

overexpressed is still unknown. It could be that a Zn-responsive
element in the *ZNT1* promoter has been altered, altering
the Zn-imposed transcriptional downregulation. However,
since two genes (*ZNT1* and *ZNT2*) are overexpressed, an
alteration in the Zn receptor and signal transduction machinery
appears more plausible (Lasat *et al.*, 2000; Pence *et al.*, 2000).
Recently a gene encoding a basic helix-loop-helix (bHLH)
transcription factor involved in the regulation of iron status
was identified in tomato. Mutation of this gene leads to much
lower expression of the main root iron transporter (Ling *et al.*,
2002). One can envision that a similar transcription factor in
T. caerulescens is involved in the regulation of zinc status.
Modification of one transcription factor often changes the
expression of several genes, as could be the case for the *ZNT1*
and *ZNT2* paralogues. Alternatively, if the Zn sequestration
machinery is much more efficient in *T. caerulescens* than in
nonaccumulator species (perhaps operating constitutively
rather than exclusively under Zn excess), this can create a state
of 'physiological Zn deficiency'. In that case, the cellular sensing
machinery does not sense Zn at appropriate levels, even though
the Zn supply rates and total cellular Zn concentrations would
be adequate or even toxic for nonhyperaccumulator plants.

Although Cd and Zn are chemically quite similar, the
mechanism of Cd uptake might be different from Zn uptake
in *T. caerulescens*. Lombi *et al.* (2001) and Zhao *et al.* (2002)
established the kinetic parameters of Cd and Zn influx into
the roots in plants from two calamine *T. caerulescens* accessions,
Prayon and Ganges, with different Cd accumulation capacities
(much higher in Ganges). The nonsaturable component of
the Cd influx was the same in both accessions. However, the
V_{max} of the saturable component was about five times higher
in Ganges than in Prayon, while the maximum saturable Zn
influx rates were about equal. Cd uptake was significantly
suppressed in the presence of equimolar concentrations of Zn
and Mn in Prayon, suggesting that Zn transporters largely
mediate Cd uptake in Prayon. However, as this treatment did
not affect Cd uptake in Ganges, it prompted the authors to
postulate the existence of a transporter with high selectivity to
Cd, as compared to Zn and Mn at least, which would be
much higher expressed in Ganges than in Prayon.

Root-to-shoot transport

The enhanced translocation of metals from the roots to the
leaves, which is characteristic for metal hyperaccumulators,
typically results in high metal concentrations in the xylem
fluid and in shoot-to-root metal concentration ratios higher
than 1 (Krämer *et al.*, 1996; Lasat *et al.*, 1996; Shen *et al.*,
1997; Lasat *et al.*, 1998; Schat *et al.*, 2000). Lasat *et al.* (1998)
found indications of a faster Zn efflux out of the vacuoles of
root cells of *T. caerulescens* (Prayon) compared with *T. arvense*,
when determining Zn compartmentalization by radiotracer
efflux analysis. Additionally, after 96 h of exposure, Zn
accumulation in roots was higher in *T. arvense* than in

T. caerulescens (Lasat *et al.*, 1996). This could indicate a difference in root tonoplast transport characteristics, with a smaller influx and a larger efflux of root vacuoles from *T. caerulescens* when compared to *T. arvense* (Lasat & Kochian, 2000). As a result, *T. caerulescens* should have a higher amount of Zn readily available for loading into the xylem than *T. arvense* (Lasat *et al.*, 1998). When immersing separated leaf sections in radiolabelled Zn solutions, Zn accumulation was higher in *T. caerulescens* than in *T. arvense*, though only at the relatively high external Zn concentration of 1 mM (Lasat *et al.*, 1998). Together, this suggests that both enhanced xylem loading and enhanced uptake into leaf cells could play a role in Zn hyperaccumulation in *T. caerulescens* (Lasat & Kochian, 2000).

Metal tolerance in *T. caerulescens*

Accumulation of large amounts of metals requires the presence of high-capacity detoxification mechanisms. Metal chelators generally play an important role in metal detoxification (Ernst *et al.*, 1992) and in long-distance metal transport via the xylem, irrespective of metal-hyperaccumulation (Senden *et al.*, 1995; Krämer *et al.*, 1996; Pich & Scholz, 1996). The principal classes of metal chelators in plants are organic acids, amino acids, phytochelatins and metallothioneins (Clemens *et al.*, 2002).

Organic acids

In general, hyperaccumulators exhibit high concentrations of several organic acids, usually citrate and/or malate (Brooks *et al.*, 1974; Pelosi *et al.*, 1976). In *T. caerulescens* leaves, malate and citrate levels are constitutive, but in roots citrate levels are induced upon metal exposure (Shen *et al.*, 1997). In all the Brassicaceae hyperaccumulators investigated thus far, including *T. caerulescens*, only malate is present at concentrations sufficient to account for the chelation of at least a major part of the foliar Zn content (Pelosi *et al.*, 1976; Tolrà *et al.*, 1996; Shen *et al.*, 1997; Sarret *et al.*, 2002). The exact role of organic acids in the hyperaccumulation trait still remains elusive. Using X-ray absorption spectroscopy, Sarret *et al.* (2002) found the majority of foliar Zn to be complexed by malate in *A. halleri*. However, Salt *et al.* (1999), applying the same methodology to *T. caerulescens*, concluded that *c.* 40% of the foliar Zn was bound to citrate, and did not find any evidence of a Zn-malate complex in this species. In roots on the other hand, Sarret *et al.* (2002) found Zn-malate, Zn-citrate and particularly Zn-phosphate in *A. halleri*, whereas Salt *et al.* (1999) exclusively found Zn-histidine and cell wall-bound Zn in *T. caerulescens*. These observations are remarkable since *A. halleri* and *T. caerulescens* exhibit an almost equal malate to citrate molar ratio and very similar foliar malate and citrate concentrations and root organic acid concentrations (Tolrà *et al.*, 1996; Shen *et al.*, 1997; Sarret

et al., 2002). In general, organic acids are supposed to be localized in the vacuole, suggesting that the Zn-citrate and Zn-malate complexes specifically represent vacuolar Zn. According to Salt *et al.* (1999), cytoplasmic Zn would predominantly occur as Zn-histidine in *T. caerulescens*, which raises the question of why this complex was not detected in *A. halleri* roots (Sarret *et al.*, 2002). The stability constants of organic acid complexes with Zn are probably too low to account for an effective detoxification through Zn binding as such. Moreover, a nonmetallicolous *T. ochroleucum* accession has been found to exhibit constitutive malate and citrate levels comparable to those in *T. caerulescens* (e.g. Shen *et al.*, 1997). This accession was neither metal-hyperaccumulating, nor particularly metal-tolerant, showing that organic acid accumulation as such, is not sufficient to account for either of these traits (Verkleij & Schat, 1990). It has been suggested that citrate or malate would serve as metal complexing agents during xylem transport. However, model calculations as well as direct measurements suggested that Zn, Ni and Cd are largely present as free hydrated cations in the xylem (Krämer *et al.*, 1996; Salt *et al.*, 1999).

Amino acids

Although the amino acid histidine has been suggested to play a role in Zn homeostasis in *T. caerulescens* roots (Salt *et al.*, 1999, see earlier section), this amino acid is more often found in hyperaccumulation of Ni, especially in the Ni hyperaccumulating Brassicaceae *Alyssum lesbiacum* (Krämer *et al.*, 1996). In a recent study, Kerkeb & Krämer (2003) showed that the xylem loading of Ni is coupled to that of histidine. The formation of a Ni-His complex is enhanced under conditions of a high endogenous-free histidine pool in the roots, such as that typically found in Ni hyperaccumulating *Alyssum*. The accumulation of Ni in the root, however, was found to be independent of the root-internal histidine pool, suggesting that histidine functions to prevent the trapping of Ni in the root cell vacuoles, possibly through complexation in the cytoplasm. Ni-induced histidine accumulation has also been observed in Ni hyperaccumulating *T. caerulescens* (J. A. C. Smith, unpublished), suggesting that the same mechanism might operate in this species too. In view of the relatively high stability of Zn-histidine at cytoplasmic pH (Salt *et al.*, 1999), it is conceivable that histidine might play a similar role in Zn hyperaccumulation. However, Zn-induced histidine accumulation in the xylem is lacking both in Zn and Ni hyperaccumulating *T. caerulescens*. Zn also did not induce histidine xylem loading in Ni hyperaccumulating *Alyssum*, suggesting that Zn-translocation in Zn hyperaccumulators does not depend on the root histidine pool (Krämer *et al.*, 1996, J. A. C. Smith, unpublished). It remains possible that other amino acid chelators with a demonstrated role in root to shoot metal transport in nonhyperaccumulators, such as nicotianamine (Stephan *et al.*, 1996), play a decisive role.

Phytochelatins

The role of phytochelatins (PCs) in metal tolerance of *T. caerulescens* has been studied extensively. Schat *et al.* (2002) found Cd-imposed PC accumulation in roots and, to a lower degree, in leaves of three *T. caerulescens* accessions with contrasting metal-specificities for tolerance and accumulation. However, the rates of PC accumulation, compared at similar Cd accumulation rates, were inversely related to the Cd tolerance levels of the accessions. The accession with normal Cd sensitivity exhibited PC-thiol to Cd molar ratios comparable to those found in nonmetallophytes and it showed significantly increased Cd sensitivity upon treatment with the γ-glutamylcysteine synthetase inhibitor, buthioninesulfoximine (BSO), suggesting that PC-synthesis was essential for Cd detoxification in this accession. The Cd-hypertolerant accession showed much lower PC-SH to Cd molar ratios, comparable with those reported for Cd hypertolerant nonhyperaccumulator plants, and did not respond to the BSO treatment. This suggests that PC-dependent, constitutive Cd tolerance exists in *T. caerulescens*, though only in nonCd-hypertolerant accessions. In the Cd-hypertolerant accession there is a decreased dependency on PC-based sequestration (Schat *et al.*, 2002). In agreement with this, Ebbs *et al.* (2002) found no involvement of PCs in Cd tolerance in *T. caerulescens* accession Prayon, which also has a high level of Cd tolerance.

Metallothioneins

A possible role for metallothioneins (MTs) in metal accumulation or metal tolerance in *T. caerulescens* or any other hyperaccumulator species, has not been explored thus far. In general MT genes are very abundant in plant tissues, sometimes reaching close to 3% of all transcripts (Cobbett & Goldsbrough, 2002). Also, in *T. caerulescens* MTs appear to be quite abundant. In a set of around 700 *T. caerulescens* ESTs, a type 1 MT-like gene was represented by 10 clones, and a type 2 MT-like gene was represented by 2 clones (D. Rigola & M. Aarts, unpublished). There are currently no clues to assume a possible role of these MTs in Zn / Cd / Ni hyperaccumulation. Rather than a function in Cd detoxification, as found for many animal MTs, plant MTs appear to be more involved in Cu homeostasis (Cobbett & Goldsbrough, 2002).

Sequestration

Metal sequestration in the vacuole is one of the ways to remove excess metal ions from the cytosol. In *T. caerulescens* leaves, Zn is sequestered mainly in the vacuoles of epidermal cells (Vázquez *et al.*, 1994; Küpper *et al.*, 1999; Frey *et al.*, 2000). However, the mechanisms of Zn transport across the tonoplast have not yet been identified. Several candidates are available. CDFs (Cation Diffusion Facilitators) form a family

of metal transporters occurring in bacteria, fungi, plants and animals. So far, they appear to be involved in metal efflux from the cytosol (Mäser *et al.*, 2001). The *Arabidopsis* ZAT1 protein is a plant member of the CDF family, which has been shown to confer increased Zn tolerance and Zn accumulation in roots upon ectopic overexpression, suggesting a role in the plant-internal compartmentation of Zn (Van der Zaal *et al.*, 1999). A number of homologous genes have been identified within the *Arabidopsis* genome, although these have not been functionally characterized thus far (Mäser *et al.*, 2001). Persans *et al.* (2001) characterized *TgMTP1t1* and *TgMTP1t2*, two CDF cDNAs from the Ni hyperaccumulator, *Thlaspi goesingense*. These clones are supposed to originate from two mRNA splicing variants derived from one single-copy genomic sequence (*TgMTP1*). When overexpressed, both proteins complemented the metal sensitivity of two yeast mutants, *cot1* and *zrc1*, which lacked a functional vacuolar CDF metal transporter. Since the metal-specificity patterns were different for each protein, they appear to play a distinct role in the vacuolar sequestration of metals in plants (Persans *et al.*, 2001). In *T. caerulescens* the *ZTP1* CDF has been identified but alternative splicing was not noticed (Assunção *et al.*, 2001). This gene is constitutively expressed with higher expression in leaves compared to roots and higher expression in *T. caerulescens* compared to *T. arvense*. Further characterization of the *T. caerulescens ZTP1* gene could elucidate its role in metal hyperaccumulation and tolerance in this species and provide insight into the Zn sequestration mechanism.

T. caerulescens as a model species for heavy metal hyperaccumulation and tolerance: prospects and limitations

In spite of an increasing amount of research, many of the basic physiological, biochemical and molecular genetic mechanisms underlying the metal (hyper)accumulation and tolerance traits in plants are not yet known. Currently most progress has been made in the general model species *Arabidopsis thaliana*, especially in identifying genes controlling metal homeostasis traits such as metal uptake, chelation and sequestration. However, a full understanding, let alone manipulation of plant metal (hyper)accumulation, is still a long way ahead (Clemens *et al.*, 2002). So, is there a need for an additional plant model species to study the way plants deal with heavy metals? We think there certainly is, for two reasons. The first is that the adaptive traits associated with plant heavy metal *hyper*accumulation and tolerance are simply not present in any of the current model species. The second, and equally important, reason is that a full understanding of these traits can increase tremendously when studied with the full set of high-throughput genomics, transcriptomics, proteomics and metabolomics tools that are rapidly coming available.

The research on metal hyperaccumulation in general and that of Zn, Ni and Cd in *T. caerulescens* in particular, gave strong clues that the mechanisms used by naturally evolved metal hyperaccumulators to cope with increased metal exposure are quite different from the mechanisms used by nonaccumulators. It would therefore not be wise to design metal hyperaccumulation strategies based on the low level metal uptake and accumulation mechanisms found in nonaccumulator species, especially for biotechnological applications. It is more attractive to use the long-lasted evolution and perfection of adaptive traits that lead to the fine-tuned metal hyperaccumulation mechanism found in adapted species. As far as has been examined, the hyperaccumulator species appear to use similar genes as found in nonaccumulator species, but perhaps due to changes in the metal response signalling and regulation mechanisms, the expression of these genes has changed. For instance, even in the absence of zinc, the root zinc transporter genes appear to be expressed at a much lower level in nonaccumulator species than in *T. caerulescens* (Pence *et al.*, 2000; Assunção *et al.*, 2001). In addition, Cd hypertolerance in *T. caerulescens* and in other metallophytes, appears to be independent of the phytochelatin-based Cd-detoxification mechanism, which is well-established in nonmetallophytic species (Schat *et al.*, 2002). These examples indicate that metal hyperaccumulation and tolerance are clearly adaptive traits worth studying and not the result of an exaggerated stress response.

When there is the need for a heavy metal hyperaccumulator model species, what are the criteria it should comply with? Based on analyses of plant materials collected from field hyperaccumulation has been reported for Ni, Zn, Cd, Pb, Cu, As, Co and Mn (Baker & Brooks, 1989; Brooks, 1994; Baker *et al.*, 2000; Ma *et al.*, 2001). However, there are reasons to doubt the validity of some of these reports. For example, Cu and Pb hyperaccumulation appeared not to be reproducible under controlled laboratory conditions (Kohl *et al.*, 1997, M. R. Macnair, personal communication), suggesting that high foliar concentrations of these metals in field-collected samples might result from aerial contamination rather than from enhanced uptake via the roots. Preferably the model species can hyperaccumulate and tolerate as many of these metal species as possible (Baker *et al.*, 1994). It should also be easy to grow, preferably in relatively high density in a greenhouse or climate chamber, and preferably also in a hydroponics growing system, to carefully control different metal concentrations and combinations. When trying to identify the genes contributing to the mechanism, the ability to perform genetic analyses is very advantageous. In order to do this efficiently the model species is diploid; preferably has a relatively short life cycle of several months at the most; can produce many seeds; is predominantly self-fertilizing or can be forced to self-fertilization; and can be easily outcrossed. Since it makes little sense to do genetics if there is no genetic variation, it should be relatively easy to collect or obtain several accessions

or mutants with different phenotypes for a range of properties contributing to the heavy metal hyperaccumulation and tolerance trait. In the current era of '-omics' technologies, it is extremely useful that high-throughput technologies can be applied to obtain genomic, transcriptomic, proteomic or metabolomic data, and that these data can be reliably compared to similar data collected for other species. In plants there are at the moment two well established reference species, *Arabidopsis thaliana* and *Oryza sativa*, respectively, belonging to the dicot family of Brassicaceae and the monocot family of Gramineae, for both of which the complete genome sequences are largely known. There are also two emerging model species, *Lycopersicon esculentum* and *Medicago truncatula* belonging to the dicot families of Solanaceae and Leguminosae, respectively, for which recently many EST sequences have been produced (Journet *et al.*, 2002; Van der Hoeven *et al.*, 2002). Since metal hyperaccumulator species are highly represented in the Brassicaceae family, *Arabidopsis thaliana* appears to be the most attractive reference species.

Taking all these criteria into account, two putative model species emerge: *Arabidopsis halleri* and *Thlaspi caerulescens*, both belonging to the Brassicaceae family. It is hard to make a choice between them. *A. halleri* ($2n = 16$) is a European perennial Zn/Cd hyperaccumulator. Together with *Arabidopsis lyrata* it is probably one of the closest relatives of *Arabidopsis thaliana* (Mitchell-Olds, 2001), the best characterized general plant model species. On average it has 90–95% DNA identity in coding regions compared with *A. thaliana*, allowing relatively easy and reliable DNA cross-hybridization for genomic and transcriptomic applications. The species is easy to grow in high density in a climate chamber or greenhouse and hydroponics culture has been well established (Sarret *et al.*, 2002). *A. halleri* plants can produce many seeds, but since plants are generally self-incompatible this requires the maintenance of the plants in small mixed populations or through labour-intensive manual cross-fertilization. There are several accessions known, but the genetic variation for Zn/Cd hyperaccumulation and tolerance is generally less than for *T. caerulescens* (Bert *et al.*, 2002). To overcome this problem, *A. halleri* has been crossed and back-crossed with the closely related nonaccumulator congener *A. lyrata* to produce segregating back-cross populations (Macnair *et al.*, 1999). However, the rate of genetic recombination between the two parental chromosomes still needs to be established.

As a comparison, *T. caerulescens* ($2n = 14$) is less related to *A. thaliana* than *A. halleri*, with only 88% DNA identity in coding regions (D. Rigola & M. G. M. Aarts, unpublished). Still, this level of identity is enough to perform reliable heterologous cDNA microarray hybridizations using labelled *T. caerulescens* cDNAs on *A. thaliana* arrays (J. van de Mortel & M. G. M. Aarts, unpublished). Despite this small disadvantage of *T. caerulescens* there are two major advantages. First of all, *T. caerulescens* is fully self-compatible and also easily outcrossed. More important, however, is the high level of apparently uncorrelated variation in the degrees and metal-

specificities of metal accumulation, root-to-shoot transport and tolerance traits between different *T. caerulescens* accessions (Meerts & Van Isacker, 1997; Escarré *et al.*, 2000; Lombi *et al.*, 2000; Schat *et al.*, 2000). This intraspecific variability permits a genetic study of these traits in segregating F_2 or F_3 populations derived from interaccession crosses. This approach has already been used on a small scale, yielding new genotypes with contrasting and novel phenotypes. As is often the case when considering polygenic quantitative traits, the progeny of a cross contains individuals with more extreme phenotypes than any of the parents (A. Assunção & H. Schat, unpublished). Besides the generation of new genotypes with extreme phenotypes, which can be used for comparative transcriptome, proteome or metabolome studies, these populations will be used for Quantitative Trait Loci mapping (Alonso-Blanco & Koornneef, 2000), eventually expected to lead to the identification of the relevant genetic loci contributing to the Zn, Ni or Cd hyperaccumulation or tolerance traits.

What is needed to fully develop *T. caerulescens* as a useful model species? Next to a continued physiological interest in the species, more species-specific '-omic' tools are required. Although the tools and databases developed for *A. thaliana* can often be used, it will be extremely useful to have additional *T. caerulescens* cDNA and genomic libraries, mutant collections, genetic markers and cDNA, protein and metabolome data. Some cDNA libraries are already available (Lasat *et al.*, 2000; Pence *et al.*, 2000; Assunção *et al.*, 2001) and EST data production is in progress (D. Rigola & M. G. M. Aarts, unpublished), as is the development of an efficient genetic transformation method enabling large-scale insertional mutagenesis or functional gene analysis (D. Salt *et al.*, unpublished). Perhaps the only limitations hampering the widespread use of *T. caerulescens* as the heavy metal hyperaccumulation and tolerance model species are the difficulty to grow large numbers of healthy plants and the relatively long vernalization period needed to induce flowering. Maybe because their natural defence is based on metal accumulation, plants grown in normal potting soil in greenhouses appear to be highly sensitive to various pests, like fungi (damping-off, powdery mildew) and insects (thrips, aphids, black flies). Sensitivity to damping-off disease was also reported for Ni-hyperaccumulating species (Ghaderian *et al.*, 2000). To induce flowering, plants with a rosette-size of around 10 cm diameter need to be transferred to cold conditions (< 7°C) for at least 4–6 weeks. However, with an increasing experience in growing large populations, these problems will undoubtedly be solved. A solution to the long vernalization requirement may be to first screen mutagenized populations of attractive accessions for early flowering mutants and convert these to 'laboratory strains'.

Finally, with one model species, *T. caerulescens*, supplemented with the continuing research on *A. thaliana* and *A. halleri*, it should be possible to make considerable progress in the understanding of Zn, Cd and Ni hyperaccumulation, tolerance and homeostasis in plants. Although the group of

hyperaccumulator species reported up to now contains many unrelated species and accumulators of several different metals, we anticipate that much of the knowledge on this subject will apply to more or maybe all hyperaccumulators, although of course metal specificities and species specificities will exist.

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